



Cytochrome Oxidase in Alzheimer's Disease: Biochemical, Histochemical, and Immunohistochemical Analyses of the Visual and other Systems

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Defects in oxidative metabolism have been implicated in Alzheimer's disease (AD). The present study evaluated the level of cytochrome oxidase (C.O.), an indicator of neuronal oxidative capacity, in various brain regions of post-mortem AD and control patients. We found a statistically significant reduction in C.O. levels in all cortical areas examined, including the primary and secondary visual cortices. In addition, all layers of the dorsal lateral geniculate nucleus and sublaminae of the primary visual cortex in AD cases examined suffered a reduction in their relative C.O. activity and protein amount. Our results suggest a generalized suppression of oxidative metabolism throughout the cortex, as well as in a major subcortical visual center in AD. Such hypometabolism may form the basis for not only deficits in higher cortical functions, but also a variety of visual dysfunctions known to occur in AD. © 1997 Elsevier Science Ltd

Alzheimer's disease Cytochrome oxidase Cortex LGN Mitochondria

INTRODUCTION

The documentation of a severe form of presenile dementia by Alois Alzheimer in 1907 (Alzheimer, 1907) heralded a new age of intense investigations into the cause, manifestations, and prognosis of the disease bearing the founder's name. Much is known about the existence and chemical nature of amyloid plaques and neurofibrillary tangles in affected brain regions. The genes for the amyloid precursor proteins have been cloned and sequenced (reviewed by Muller-Hill & Beyreuther, 1989; Selkoe, 1991). A number of neurotransmitters and neuromodulators, including acetylcholine, somatostatin and glutamate have been found to be deficient in Alzheimer's disease (AD) (Wurtman, 1992;

Epelbaum *et al.*, 1993). The involvement of tau proteins and apolipoprotein E has been reported (Han *et al.*, 1994). In recent years, however, attention has been turned to possible defects in energy metabolism in AD, as shown by positron emission tomography (PET) and single photon emission computed tomography (SPECT) in AD (Grady *et al.*, 1993; Cutler, 1986; Horwitz *et al.*, 1987; Johnson *et al.*, 1987; McGeer *et al.*, 1990; Kessler *et al.*, 1991; Nyback *et al.*, 1991; Blass & Gibson, 1991; Rapoport, 1991; Beal, 1992).

The major source of energy supply for neurons is ATP, which is generated mainly from oxidative metabolism in the mitochondria. Recent studies have implicated defects in mitochondrial oxidative phosphorylation as one of the key factors contributing to normal and premature aging. Dysfunction of the mitochondrial electron transport chain proteins has been associated with the pathophysiology of AD (reviewed in Blass & Gibson, 1991) as well as in another neurodegenerative disease, Parkinson's disease (Parker *et al.*, 1989).

A reliable and sensitive indicator of a neuron's capacity for oxidative metabolism is cytochrome oxidase (C.O.). The level of this enzyme is heterogeneous among neurons, and is tightly regulated by neuronal activity (reviewed in Wong-Riley, 1989). C.O. activity has been shown to be reduced in platelets of AD patients (Parker *et al.*, 1990), indicating a generalized molecular defect in

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AD. The activity of this enzyme has been studied in various brain regions of AD cases, but reports vary as to the precise location that showed defective C.O. activity (Kish *et al.*, 1992; Reichmann *et al.*, 1993; Simonian & Hyman, 1993; Mutisya *et al.*, 1994; Parker *et al.*, 1994b).

One region in question is the primary visual cortex, which does not show significant reduction in its regional metabolic rate when studied with PET (Haxby & Rapoport, 1986; Heiss *et al.*, 1991; Rapoport, 1991). Yet, visual dysfunction has been reported in many AD patients (Uhlmann *et al.*, 1991; Bassi *et al.*, 1993; Hutton *et al.*, 1993; Levine *et al.*, 1993). In an effort to understand the metabolic involvement of the visual and other systems in AD, we undertook a study to examine the histopathological presence of senile plaques and levels of C.O. measured biochemically, histochemically, and immunohistochemically. In particular, we compared C.O. levels in the visual cortex, dorsal lateral geniculate nucleus, and other brain regions between AD and age-matched non-AD patients.

MATERIALS AND METHODS

Human brain samples

Tissue from Alzheimer's disease brains was provided by the Wisconsin Alzheimer's Disease Brain Tissue Bank. All patients and controls were prospectively enrolled in the autopsy program and followed clinically before death. Information regarding medical history, records, and mode of death were reviewed by a neurologist to determine the clinical diagnosis in the case of AD patients, and the absence of neurological illness in the case of controls. At autopsy, tissue samples were collected for C.O. determinations. The diagnosis of AD was confirmed histologically by the Khachaturian criteria (Khachaturian, 1985). Samples were taken from various brain regions of 87 AD and 58 non-AD patients over a period of 9 yr. From these, we excluded: patients that were below 50 yr of age; those with other types of dementia or neurological diseases such as Creutzfeldt-Jacob disease, Huntington's disease, multiple sclerosis, Parkinson's disease, Pick's disease, significant cerebrovascular diseases, and brain tumors; and patients with visual system disorders (including blindness, cataracts, retinopathy, and optic gliomas). Among the AD patients, brains from 64 of them were processed for C.O. histochemistry, 51 for C.O. biochemistry, and 7 for C.O. immunohistochemistry. The mean age of this group was 76 yr (range: 58–95 yr), and the mean weight of their brains without the dura was 1088 g (range: 710–1490 g). The mean duration of illness for AD patients was 9.5 yr (range: 3–22 yr). Among the control group with no known neurological diseases, 26 were processed for C.O. histochemistry, 18 for C.O. biochemistry, and 8 for C.O. immunohistochemistry. While this group was age-matched with the AD group, with the mean age being 73 yr (range: 53–94 yr), the mean weight of their brains without the dura, 1215 g (range: 950–1430 g), was significantly higher than that of the AD group

($P < 0.001$). The average length of post-mortem delay was 8 hr for AD cases and 11 hr for non-AD cases. Data from these two groups went through a second screening, from which tissue samples that did not meet with our standard of preservation were discarded. Tissue integrity was of paramount importance, as AD brains tended to be more fragile and the values would be biased against them if poorly preserved AD brains were included in the final analysis. To avoid bias, tissue screening was carried out without prior knowledge of the diagnosis and irrespective of the levels of C.O. staining. The qualitative descriptions were based on well preserved samples, a subset of which were randomly chosen and subjected to further quantitative and statistical analysis as described below.

Histopathology

Brain sections of 6 μm thickness were processed with hematoxylin–eosin, luxol fast blue, King, and ubiquitin stains. King stain, a modified Bielschowsky stain, was used to reveal neuritic plaques and neurofibrillary tangles in various regions of the brain, including primary visual cortex, primary motor cortex, prefrontal cortex, parietal cortex (supramarginal gyrus), superior temporal cortex, insular cortex, hippocampus, and nucleus basalis of Meynert. The mean number of senile plaque counts per microscopic field (1 mm^2 at 200 \times magnification) in each brain region was recorded for each case and analyzed.

Cytochrome oxidase biochemistry

Samples were taken from the primary visual, somatosensory, motor, and auditory cortices, from the secondary visual cortex, prefrontal cortex, parietal cortex, and temporal cortex (superior temporal gyrus), hippocampus, cerebellum, substantia nigra, and the optic tract. Care was taken in dissecting only the gray matter (except in the case of the optic tract), and to include as little of the white matter as possible. Biochemical assays were conducted as previously described (Hevner *et al.*, 1993), except that 3.5–7.5% homogenates were used (with 3.5% being the final choice).

Cytochrome oxidase histochemistry and immunohistochemistry

A variety of fixatives were tested over the years to achieve an optimal combination of tissue and enzyme preservations. A large number of samples that did not meet our standards were discarded. Samples from various brain regions were dissected and placed in the cold fixative. The volume of each sample was approximately 3 cm^3 . For C.O. histochemistry, the optimal fixative was found to be 2.5% paraformaldehyde and 1.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.35, and 4% sucrose for 3–4 hr (Wong-Riley *et al.*, 1993). For C.O. immunohistochemistry, glutaraldehyde was omitted, the concentration of paraformaldehyde was increased to 4%, and the length of fixation was increased to 6 hr (Wong-Riley *et al.*, 1993). After cryoprotection with increasing concentrations of sucrose (10–20–30%) in 0.1 M sodium phosphate buffer, pH 7.35, brain

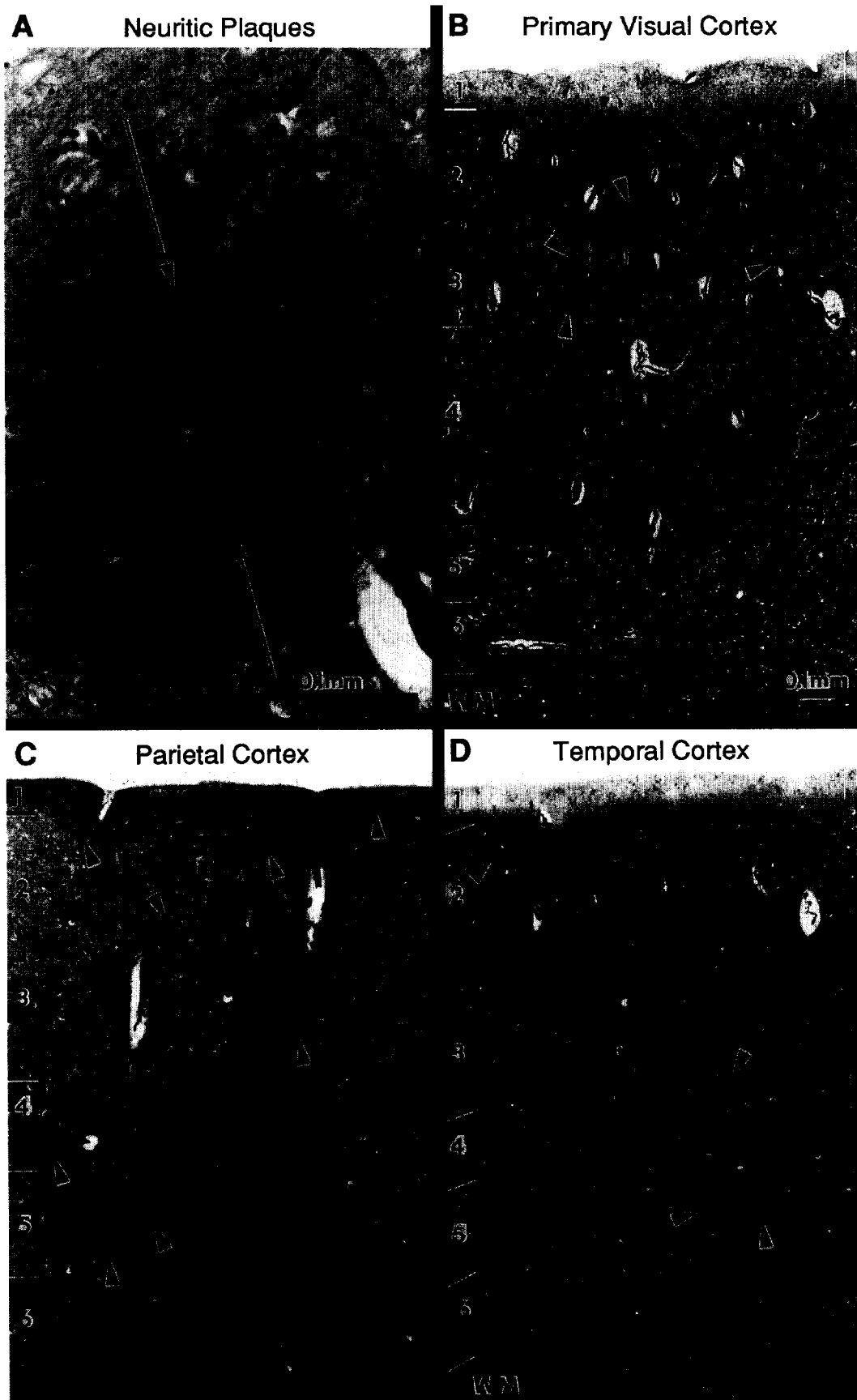


FIGURE 1. Sections of the cortex from AD cases stained with the modified Bielschowsky's (King's) stain. (A) Two classic neuritic senile plaques (arrows). The prevalence of neuritic plaques (arrowheads) is shown in cross-sections of the primary visual cortex (B), parietal cortex (C), and superior temporal cortex (D). Note that the parietal cortex has abundant plaques, but plaques are also present in the visual and other cortical areas. The layers are indicated on the left of each section. WM, white matter. (B)–(D) have the same magnification.

Senile Plaques in Alzheimer's Disease

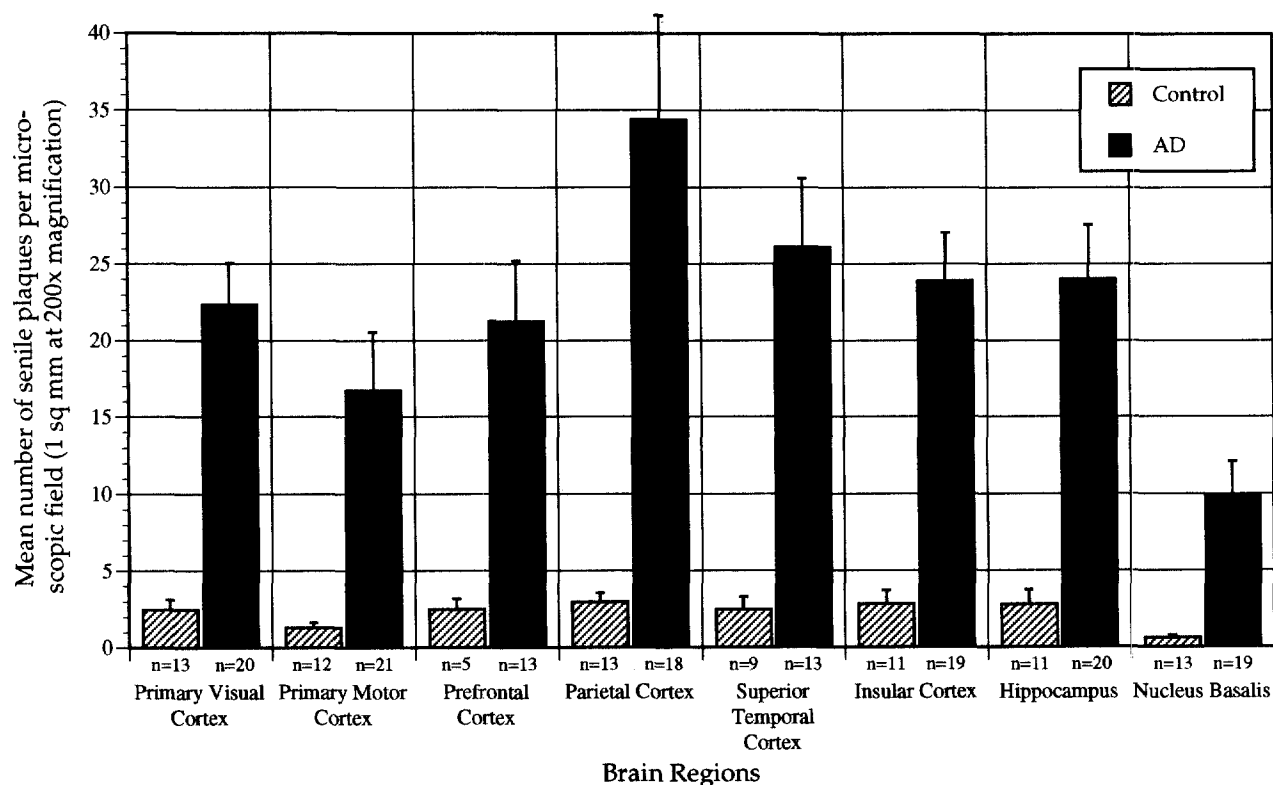


FIGURE 2. Graph of the mean numbers of senile plaques per microscopic field (1 mm^2 at $200\times$ magnification) in various brain regions of AD and control patients. All regions examined have significantly higher numbers than controls ($P < 0.0001$), confirming their diagnosis of AD according to Khachaturian criteria. The n for each region of each group was given.

samples were frozen in powdered dry ice for immediate or subsequent sectioning. Frozen sections at $40\text{--}50 \mu\text{m}$ thickness were processed for C.O. histochemistry (Wong-Riley, 1979) or C.O. immunohistochemistry against brain-specific C.O. (Hevner & Wong-Riley, 1989). Antibodies were used at dilutions of 1:1000 to 1:4000 and were detected by the indirect immunoperoxidase method. Controls included preimmune serum and the omission of primary antibodies.

Optical densitometry

Optical densitometric measurements were taken from various layers of histochemically and/or immunohistochemically reacted dorsal lateral geniculate nucleus (LGN), the primary visual cortex (area 17), parietal cortex, prefrontal cortex, hippocampus, and the entorhinal cortex of AD and control brains. Of the 64 AD and 26 control brains that were processed for C.O. histochemistry, we randomly selected 12 AD and 12 controls for optical densitometric measurements and statistical analyses for each of the brain regions described in the Results section (except for LGN, which had an n of 5 each). Of the seven AD and eight control brains that were processed for C.O. immunohistochemistry, optical density readings were taken from various brain regions of five AD and three controls that were randomly chosen.

The method for optical densitometry was as described previously (Wong-Riley *et al.*, 1993). All lighting conditions, measuring spot size, and magnifications were kept constant between AD and control samples for each brain region. At least 25 readings were taken for each laminae/sublaminae zone of each brain region in each case. The white matter of each section was used as the reference point and its value was subtracted from those of the gray matter in each section to achieve some uniformity in the level of baseline staining intensity. Statistical analysis was carried out using Student's t -test. A P value of 0.05 or less was considered significant.

RESULTS

Senile plaques

Twenty-one Alzheimer's disease and 13 control brains that were processed for the modified Bielschowsky stain were analyzed in detail. Neuritic plaques [Fig. 1(A)] reached Khachaturian criterion level for AD in many cortical areas [Fig. 1(B–D)], including the primary and secondary visual cortices. Figure 2 shows our quantitative analysis. The mean number (\pm SEM) of senile neuritic plaques per microscopic field (1 mm^2 at $200\times$ magnification) within the AD group was highest in the parietal cortex (supramarginal gyrus: 34.42 ± 6.73

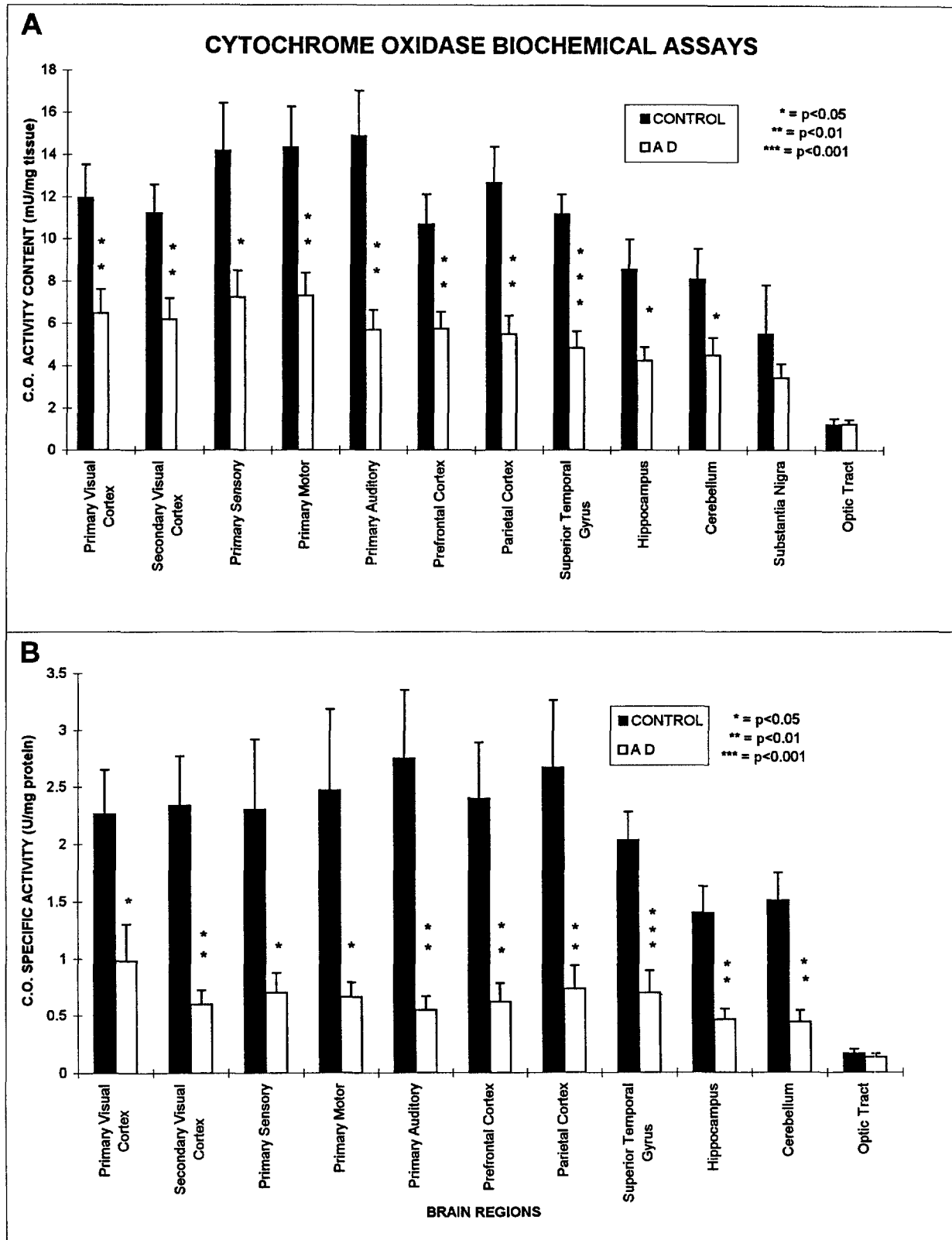


FIGURE 3. Cytochrome oxidase activity in various brain regions of post-mortem AD and control patients. (A) and (B) show C.O. activity content (by tissue wet weight) and C.O. specific activity, respectively, as determined by biochemical assays of tissue homogenates. Activity is significantly reduced (note P values) in all regions except substantia nigra and the optic tract. Specific activity was not done for substantia nigra because of limited samples.

plaques; $n = 18$), followed by the superior temporal cortex (26.12 ± 4.48 ; $n = 13$), hippocampus (24.05 ± 3.52 ; $n = 20$), insular cortex (23.91 ± 3.15 ; $n = 19$), visual cortex (22.35 ± 2.72 ; $n = 20$), prefrontal cortex (21.27 ± 3.96 ; $n = 13$), primary motor cortex (16.74 ± 3.83 ; $n = 21$), and lowest in the nucleus basalis of Meynert (9.97 ± 2.12 ; $n = 19$). All of these AD brains were included in our C.O. biochemical studies described below. In age-matched control cases, mean plaque densities in all of these regions were less than three per microscopic field (Fig. 2). The difference between AD and control values was statistically significant for all brain regions ($P < 0.0001$).

Cytochrome oxidase biochemical assays

Of the 51 AD and 18 control brains that were processed for C.O. biochemistry, we eliminated those that were used initially for the testing of various parameters to achieve optimal results, those that had questionable tissue integrity, as well as those that had exceptionally long post-mortem delay. Selections were made without knowledge of the biochemical data. Figure 3 shows the quantitative analysis of the remaining 26 AD and 12 control cases. It is clear that all cortical regions had a significant decrease in C.O. activity, with the greatest reduction in the superior temporal gyrus (STG) ($P \leq 0.001$), followed by the parietal cortex, prefrontal cortex, secondary visual cortex, primary auditory cortex, hippocampus, and the cerebellum ($P < 0.01$). The primary visual, primary sensory, and primary motor cortices had less severe though significant reductions in their enzyme activity ($P < 0.05$). By comparison, the substantia nigra and the optic tract did not show statistically significant changes when compared to control cases.

Cytochrome oxidase histochemical and immunohistochemical analyses

Within both the AD and control groups, there were intra-group variations in the intensity of C.O. reaction product obtained histochemically or immunohistochemically. However, as a whole, the AD group had significantly lower levels of C.O. in all regions processed histochemically and immunohistochemically than those of the control group.

Dorsal lateral geniculate nucleus (LGN)

The distribution of cytochrome oxidase in the human dorsal lateral geniculate nucleus resembled that in the macaque monkey (Liu & Wong-Riley, 1990). The two magnocellular layers had the highest level of activity, followed by parvicellular lamina 6, while the other parvicellular layers (3, 4, and 5) had the lowest activity. In AD, there was no change in the pattern of C.O. staining, but the level of histochemically and immunohistochemically detectable reaction product was significantly reduced in all six layers [Fig. 4(A,B); Fig. 5(A,B); $P < 0.05-0.001$].

Primary visual cortex (area 17)

The pattern of C.O. histochemistry or immunohistochemistry in the striate cortex of controls was as described previously in normal human subjects (Wong-Riley *et al.*, 1993). C.O. levels were highest in layer IVC (with IVC β slightly darker than IVC α), moderate-to-high in supragranular puffs (blobs or patches) and layer VI, and lowest in interpuffs, layers V and IVB. Puffs were extremely sensitive to suboptimal tissue preservation, such as extended post-mortem delay, freezing artifact, and overfixation, and were difficult to discern in some of the samples. Only those with acceptable tissue preservation, irrespective of C.O. levels were used for our final analyses.

The striate cortices from AD brains showed distinct variations in their levels of C.O. reaction product, with some brains exhibiting values slightly lower than those of the controls, while others had much reduced values. However, as a whole, the AD group had a consistent and statistically significant reduction in the relative activity (shown by histochemistry) and relative amount (shown by immunohistochemistry) of C.O. in all laminae of area 17 examined (puffs, interpuffs, layers IVC α , IVC β , V, and VI) as compared to the control group [Fig. 4(C,D); Fig. 5(C,D); $P \leq 0.001$ for all histochemical analyses; $P < 0.05-0.01$ for immunohistochemical analyses].

Parietal cortex, prefrontal cortex, and superior temporal cortex

These association cortical areas shared very similar patterns of C.O. distribution, in that the supragranular layers 2 and 3 exhibited slightly higher levels of activity than the granular (layer 4) and infragranular layers 5 and 6. In addition, pyramidal neurons in both supra- and infra-granular layers tended to have higher levels of C.O. than the majority of small, non-pyramidal neurons.

In AD brains, levels of C.O. analyzed histochemically and immunohistochemically were significantly reduced in the supra-, infra-, and granular layers of all three associational cortical areas (Figs 6 and 7; $P < 0.001$ for all histochemical analyses; $P < 0.01-0.001$ for immunohistochemical analyses; data from temporal cortex not shown).

Hippocampal formation and entorhinal cortex

The laminar pattern of C.O. reactivity in the human hippocampus and dentate gyrus resembled those of macaque monkeys and other species described previously (Kageyama & Wong-Riley, 1982). Stratum moleculare of fields CA1-3 and the outer molecular layer of the dentate gyrus exhibited intense neuropil staining; neurons of CA3 and CA4 were also darkly reactive, while those of CA1 had moderate levels of enzyme. Stratum radiatum of the hippocampus and dentate granule cell layer had the lowest level of C.O. activity.

In AD, all CA fields (1, 3, and 4) and sublaminae examined (stratum radiatum of CA1, stratum moleculare of CA1, the dentate granule cell layer, and the outer molecular layer of the dentate gyrus) showed a significant

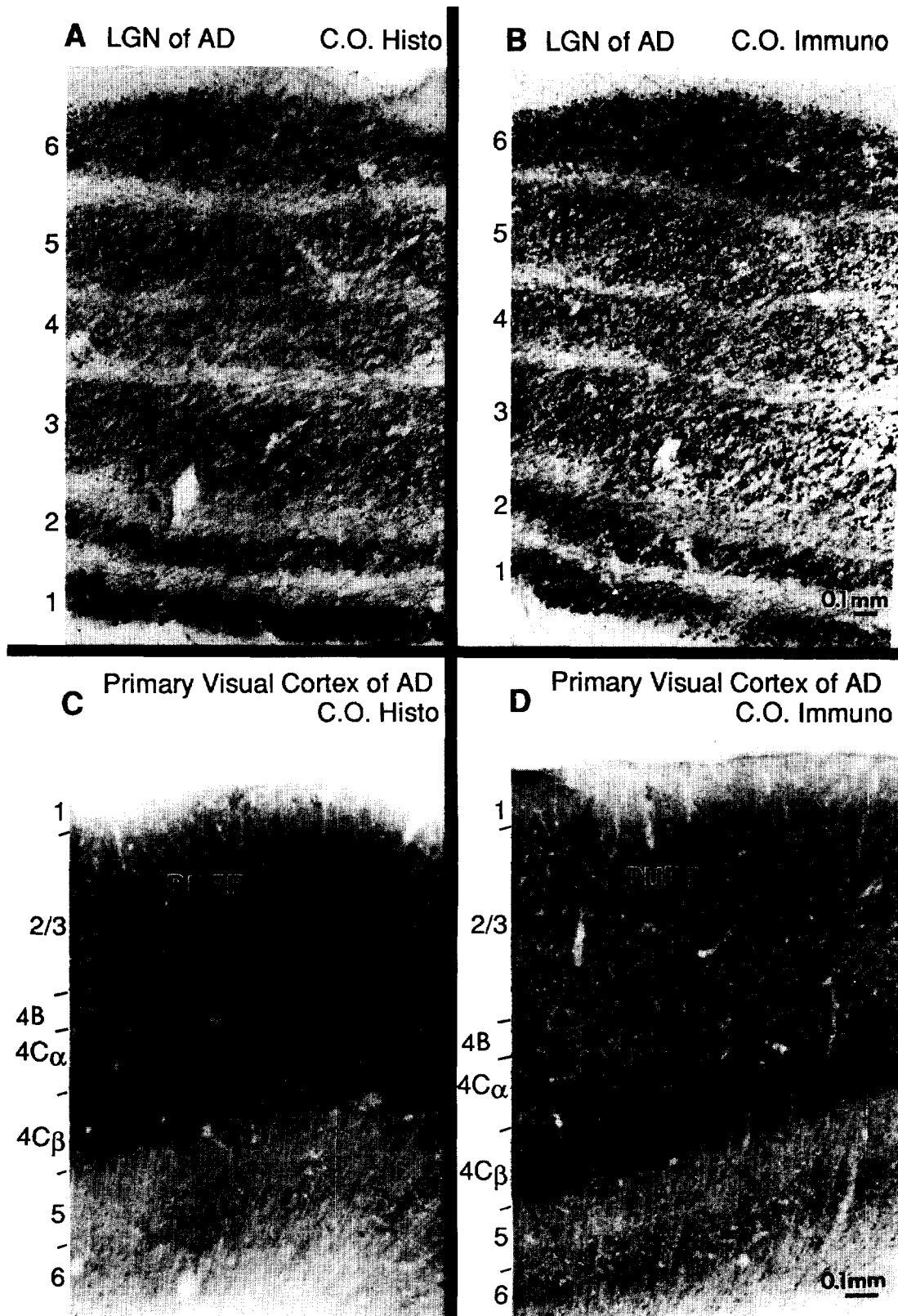


FIGURE 4. Coronal sections of the dorsal lateral geniculate nucleus (LGN) [(A) and (B)] and the primary visual cortex [(C) and (D)] reacted for C.O. histochemistry [(A) and (C)] and C.O. immunohistochemistry [(B) and (D)], respectively. The laminae and sublaminae of each region are listed on the left of each section. Puffs in the supragranular layers are shown by arrows in (C) and (D). These AD samples retained the same staining patterns as those in controls, but their levels of activity and immunoreactivity were significantly lower than those of controls (see Fig. 5). Since patterns of C.O. histochemistry and immunohistochemistry in various brain regions examined are similar between AD and control patients, only those from AD patients are shown. Control samples simply have higher levels of activity and immunoreactivity in most brain regions, as shown by optical densitometric readings in Figs 5, 7, and 9.

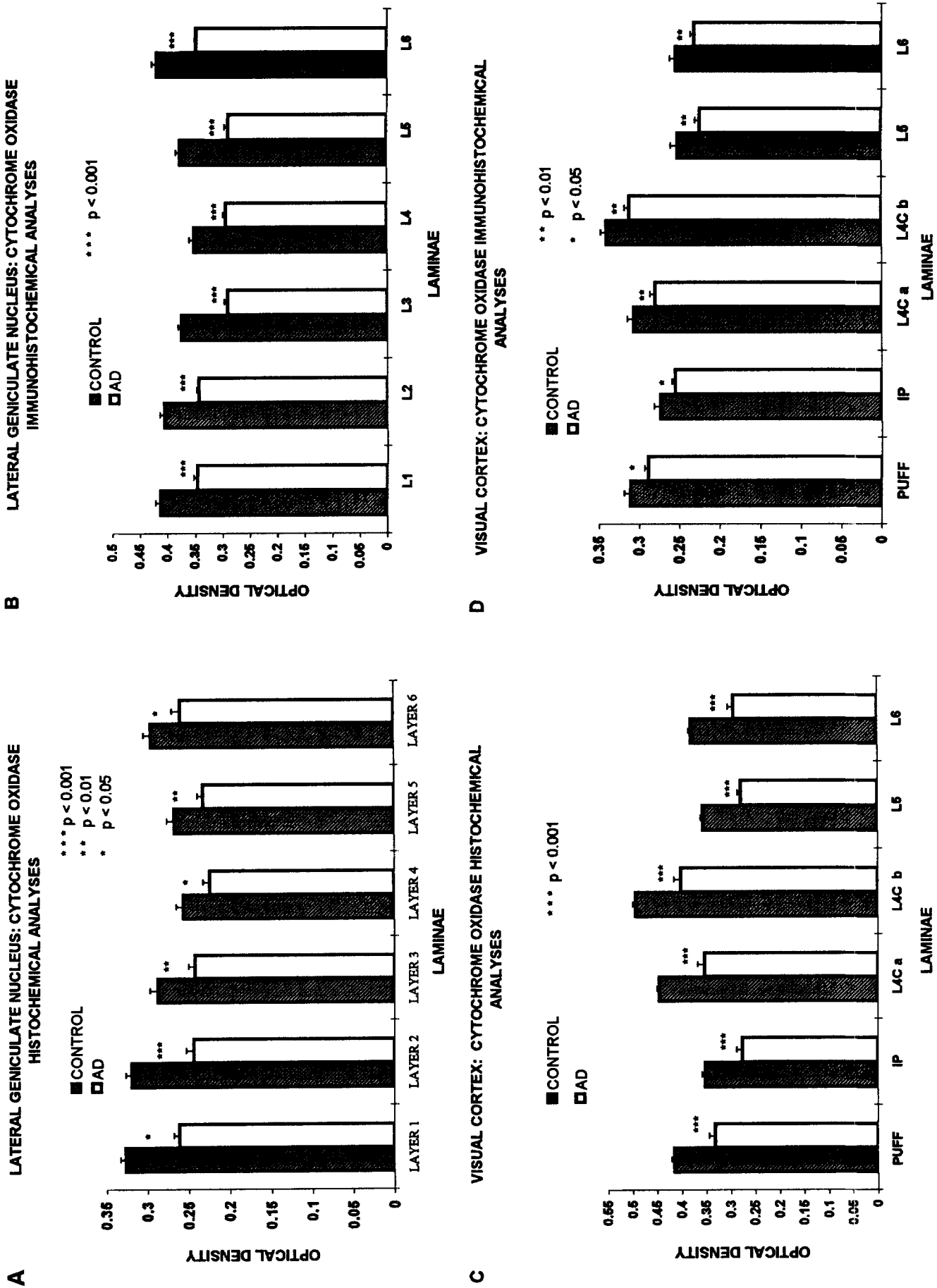


FIGURE 5. Optical densitometric measurements of reaction product of C.O. histochemistry [(A) and (C)] and C.O. immunohistochemistry [(B) and (D)] in the LGN [(A) and (B)] and primary visual cortex [(C) and (D)] of AD and control patients. A significant reduction in C.O. is seen in all laminae and sublaminae of AD samples examined (see *P* values). IP, interpufts; LACa, lamina 4C α ; LACb, lamina 4C β .

reduction in C.O. activity (Figs 8 and 9; $P \leq 0.001$). Reductions in C.O. immunoreactivity also reached statistical significance for CA1, 3, and 4 neurons, as well as for stratum moleculare of CA1 ($P \leq 0.001$).

The distribution of C.O. in the human entorhinal cortex has been described in detail previously and will not be repeated here (Hevner & Wong-Riley, 1992). We compared primarily field E_I (intermediate) between AD and controls, and found a reduction in C.O. activity both within layers II and III neuronal clusters forming C.O.-reactive islands and within the deep layers [Fig. 9(A); $P < 0.001$].

DISCUSSION

The present study documents that a key energy-generating mitochondrial enzyme, cytochrome oxidase, is significantly reduced in a number of brain regions, including the visual system of Alzheimer's disease patients. These findings confirmed and extended some of the recent reports linking defects in cytochrome oxidase and other oxidative stresses to AD. We shall examine some of these investigations below.

Defects in oxidative metabolism and AD

Several lines of evidence have pointed to abnormality in oxidative metabolism being involved in the pathogenesis of AD (reviewed in Blass & Gibson, 1991): (a) cultured cells exposed to a mitochondrial uncoupler CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) exhibited increased immunoreactivity against paired helical filaments and Alz-50, both of which are pathologic changes seen in AD (Blass *et al.*, 1990); (b) the synthesis and metabolism of neurotransmitters are highly sensitive to oxidative abnormality and severely impaired in AD (Gibson *et al.*, 1987); (c) cellular calcium homeostasis, being intimately linked to oxidative metabolism in the mitochondria, is abnormal in AD (Gibson *et al.*, 1987). Calcium is an important intracellular second messenger, and calcium/calmodulin-dependent protein kinase is known to phosphorylate tau proteins, the abnormal processing of which leads to the accumulation of paired helical filaments in AD (Baudier & Cole, 1987). Such correlations of oxidative stresses with AD have prompted Blass and Gibson (1991) to propose the "Mitochondrial hypothesis of Alzheimer's disease".

Consistent with the mitochondrial hypothesis is the finding of a high incidence of mitochondrial DNA (mtDNA) deletions in AD patients younger than 75 yr of age (Corral-Debrinski *et al.*, 1994). Point mutations of mitochondrial NADH dehydrogenase subunit 2 gene were also reported in AD (Lin *et al.*, 1992). Reichmann *et al.* (1993) searched for and failed to find deletions larger than 500 bp in parietal and entorhinal cortex of AD brains; however, they could not rule out the possibility of small-scale deletions or point mutations in AD. Mutation of mtDNA would be manifested as a non-Mendelian inheritance, and only about 10% of AD cases are Mendelian (Appel, 1981).

Mitochondrial DNA encodes 13 polypeptides that form

some but not all subunits of the four electron transport chain enzymes (complexes I, III, IV [C.O.], and V). A defect in mtDNA would be expected to result in deficiency of one or more of these enzymes. Reichmann's group (Reichmann *et al.*, 1993) did find a reduction in the activities of complexes II, III, and IV in AD brains, but Mutisya *et al.* (1994) claimed that complexes I and II/III were only mildly affected in the occipital but not the other cortical areas, indicating that C.O. might be selectively involved in AD.

Cytochrome oxidase and Alzheimer's disease

Parker's group first reported a significant reduction in cytochrome oxidase activity in platelets of AD patients (Parker *et al.*, 1990), suggesting strongly that the defect was a generalized phenomenon. This finding was subsequently challenged (Van Zuylen *et al.*, 1992) but was reconfirmed (Parker *et al.*, 1994a). Purified C.O. from AD brains showed an anomalous kinetic behavior compared to controls (Parker & Parks, 1995). C.O. activity was also found to be decreased biochemically in AD brains (Kish *et al.*, 1992; Reichmann *et al.*, 1993; Mutisya *et al.*, 1994; Parker *et al.*, 1994b), although the location and severity of deficits varied among these reports. Kish's group found a significant reduction in frontal (-26%) and temporal (-17%) cortices, but the other regions were either not affected (occipital cortex and putamen) or were nonsignificantly elevated (hippocampus). Reichmann and colleagues claimed a reduction in all four regions that they examined (temporal, parietal, and entorhinal cortices, and hippocampus), and Mutisya's group reported a 25-30% reduction in frontal, temporal, parietal, and occipital cortices. Thus far, only a single histochemical study has been reported on AD brains, and it concentrated solely on the hippocampus and dentate gyrus, both of which exhibited decreased C.O. activity (Simonian & Hyman, 1993), in contrast to Kish's biochemical findings (Kish *et al.*, 1992).

The present study represents a comprehensive analysis of C.O. by biochemical, histochemical, and immunohistochemical means, in multiple regions of a large group of AD cases over a period of several years. This approach allowed us to eliminate specimens that were suboptimal in structural and enzymatic preservations and to perform quantitative analyses on relatively well-preserved specimens. It is clear that all *cortical* regions examined exhibited a significant decrease in biochemical activity of C.O., with the greatest reduction in the superior temporal gyrus (STG) ($P \leq 0.001$), and definitely affecting the primary and secondary visual cortices ($P < 0.05-0.01$). By comparison, the substantia nigra and optic tract retained activities that were comparable to those of age-matched, non-AD cases with no known neurological diseases. The heterogeneous effect of AD will be discussed further below.

Histochemically and immunohistochemically, we were able to carry out the analyses to the laminar and sublaminar levels. All six layers of the dorsal lateral geniculate nucleus and all sublaminae of the primary

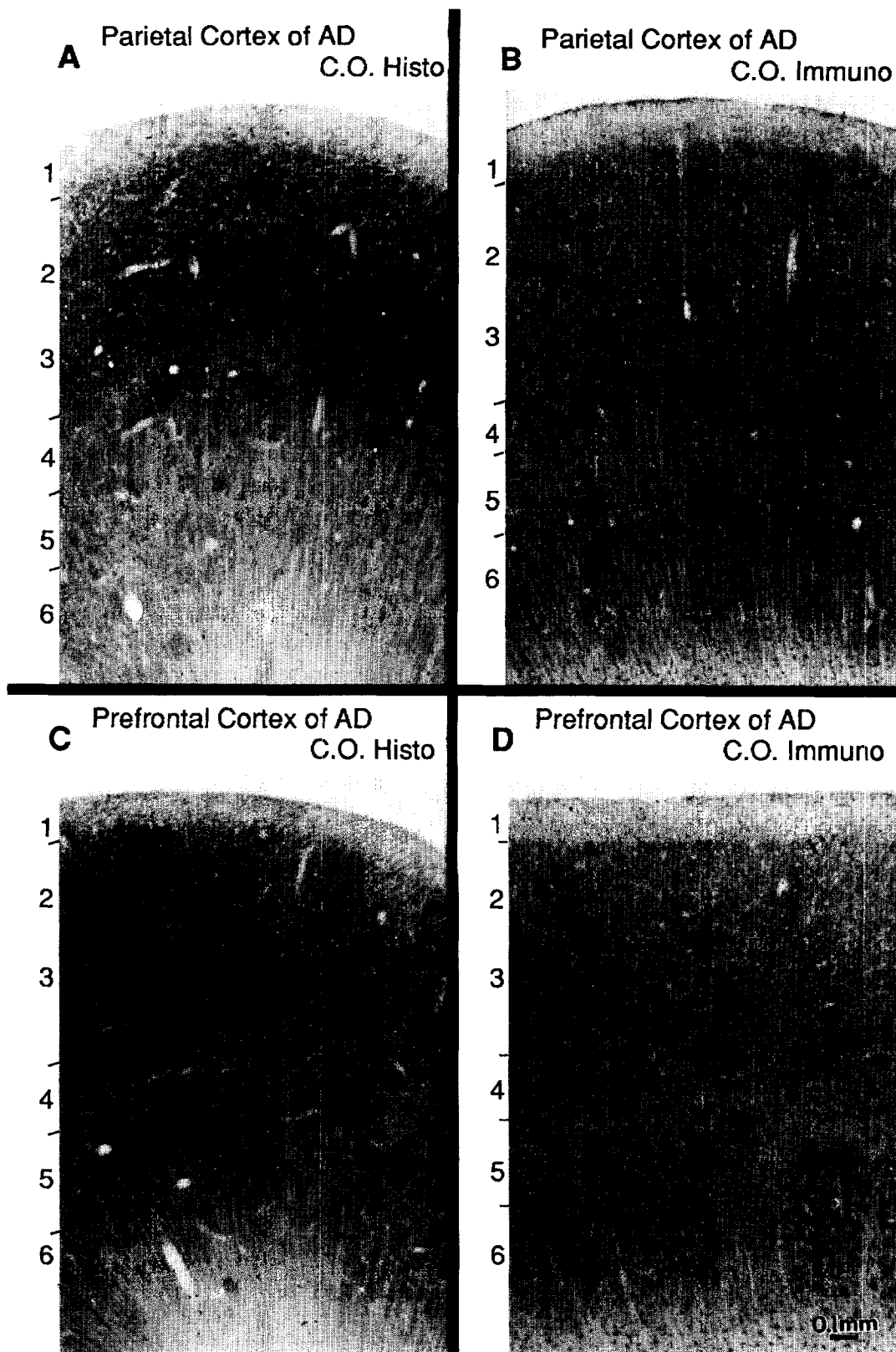


FIGURE 6. Sections perpendicular to the surface of parietal cortex [(A) and (B)] and prefrontal cortex [(C) and (D)] were reacted for C.O. histochemistry [(A) and (C)] and C.O. immunohistochemistry [(B) and (D)], respectively. The layers in each region are listed on the left of each section. These AD samples retained the same staining patterns as those in controls, but their levels of activity and immunoreactivity were significantly lower than those of controls (see Fig. 7). Since patterns of C.O. histochemistry and immunohistochemistry in various brain regions examined are similar between AD and control patients, only those from AD patients are shown. Control samples simply have higher levels of activity and immunoreactivity in most brain regions, as shown by optical densitometric readings in Figs 5, 7, and 9.

visual cortex examined exhibited statistically significant reductions in their C.O. activity ($P < 0.05-0.001$). Likewise, the supragranular, granular, and infragranular layers of prefrontal and parietal association cortices demonstrated a fall in relative enzymatic activity and relative protein amount ($P < 0.01-0.001$). Thus, our histochemical and immunohistochemical analyses confirmed our biochemical results and established that a reduction in C.O. activity was a generalized phenomenon in neocortical neurons and neocortical neuropil.

Within the hippocampal formation, our results are consistent with those of Simonian and Hyman (1993) in that all subfields and sublaminae examined exhibited a significant reduction in C.O. activity. In addition, neurons of CA1, 3, and 4 and the dentate molecular layer also had a loss in C.O. protein amount. These results are not surprising, since memory loss is one of the cardinal signs of AD. A corollary is our finding of a significant decrease in C.O. activity within neuronal clusters and deep layers of the entorhinal cortex, the major afferent source of the hippocampus. Again, this is consistent with known pathology in the entorhinal cortex of AD (Van Hoesen *et al.*, 1991).

Dysfunction of cytochrome oxidase would severely compromise several intra-mitochondrial functions: (a) electron transport, with the formation of oxygen free radicals known to induce mtDNA mutation and peroxidative membrane damage (Wallace, 1992); (b) ATP synthesis, affecting such ATP-dependent functions as the maintenance of a stable potential across the plasma membrane, as well as across the mitochondrial membrane; and (c) calcium sequestration, which affects intracellular calcium level and a host of calcium-dependent functions. In addition, disruption of cytochrome oxidase may be involved in lipid peroxidation found in the frontal cortex of AD patients (Volicer & Crino, 1990). Accumulation of these deficits with time could eventually lead to severe metabolic compromise and cell death.

At present, it is not known if C.O. deficiency is the cause or effect of AD. Either way, it is clear from the present and previous studies that this important enzyme is affected in many regions of the AD brain.

Regional differences

Our analyses of neuritic plaques are in general agreement with previous reports, which demonstrated that the prevalence of senile plaques increased from primary sensory areas to secondary and tertiary association areas (Pearson *et al.*, 1985; Rogers & Morrison, 1985). The distribution of abnormally phosphorylated tau proteins was uniformly high in the limbic, temporal, and parietal lobes of AD patients (Vermersch *et al.*, 1992), but tended to be more variable among patients for the occipital and frontal cortex. Among the AD cases for which we had performed C.O. biochemistry, neuritic plaque density was highest in the parietal cortex (supramarginal gyrus), followed by the superior temporal cortex. However, the densities were equally high in the

hippocampus, insular cortex, prefrontal cortex, and the primary visual cortex, indicating a close association between plaque deposits and reduced C.O. activity. Even the primary motor cortex and the nucleus basalis of Meynert had plaque densities significantly above those of control cases.

A reduction in C.O. activity is also consistent with PET findings that whole brain metabolic rate in AD is reduced, and that the reduction is related to the overall severity of dementia (Haxby & Rapoport, 1986). There appears to be a progression of hypometabolism from the parietotemporal association cortex (which are affected early in the disease and suffer the most severe regional metabolic disturbance) to the frontal lobe, but PET does not detect any deficits in primary sensory and motor cortical areas (Duara *et al.*, 1986; Jagust *et al.*, 1988; Heiss *et al.*, 1991; reviewed in Rapoport, 1991; Beal, 1992). In this regard, it is possible that C.O. biochemistry and histochemistry may have greater sensitivity and resolution than PET in revealing reduced oxidative metabolism in neurons. However, the latter has the distinct advantage of monitoring the living brain. Thus, the metabolic demands of different neuronal systems do differ and may form the basis for varying degrees of vulnerability in AD. Both neuropathologic (paired helical filaments, amyloid plaques) and imaging (CT, PET, and SPECT) studies suggest that brain regions of greatest vulnerability in AD include those particularly sensitive to oxidative impairments (Blass & Gibson, 1991). Discrepancies in the reported regions of metabolic disturbance may simply reflect the sensitivity of the measurement, the heterogeneous nature of the patient population, and the severity of the disease.

Visual system in AD

The present study showed unequivocally that, at least in our population of AD patients, both the dorsal lateral geniculate nucleus and the visual cortex exhibited significant reductions in C.O. activity and protein amount as compared to the control group. The primary visual cortex of these AD patients also had plaque density that was comparable to the affected prefrontal and insular cortices. Our findings are in agreement with those of Beach and McGeer (1992), who found a severe depletion of acetylcholinesterase fibers along with a significant aggregate of senile plaques and amyloid β -proteins (A β P) in the visual cortex of AD cases. However, they did not find a significant change in the density of synaptophysin there. A sizeable reduction (by $\sim 30\%$) in the mean neuronal density was also found in both areas 17 and 18, but the increase in glial density was significant only in area 17 (Leuba & Kraftsik, 1994).

While the common manifestations of AD include memory loss, impaired language ability, decreased praxis, impaired judgment, and learning, recent attention has been focused on visual dysfunction in AD (Cronin-Golomb *et al.*, 1991; Hof & Bouras, 1991; Mendola *et al.*, 1995; Pantel, 1995; Trick *et al.*, 1995). Frequently, topographic agnosia, visual agnosia, alexia without

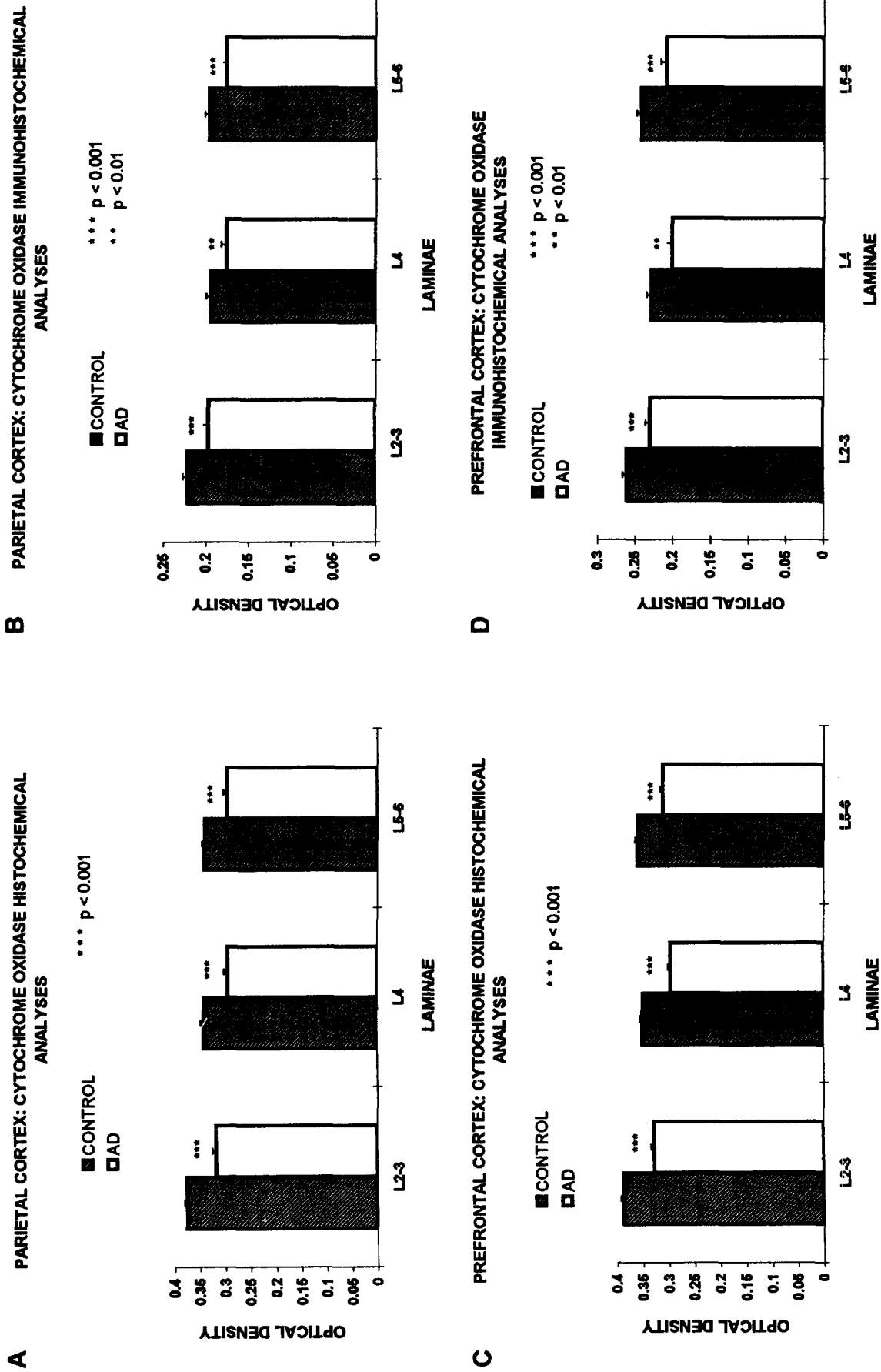


FIGURE 7. Optical densitometric measurements of reaction product of C.O. histochemistry [(A) and (C)] and C.O. immunohistochemistry [(B) and (D)] in the parietal cortex [(A) and (B)] and prefrontal cortex [(C) and (D)] of AD and control patients. A significant reduction in C.O. is seen in all layers of AD samples examined (see *P* values).

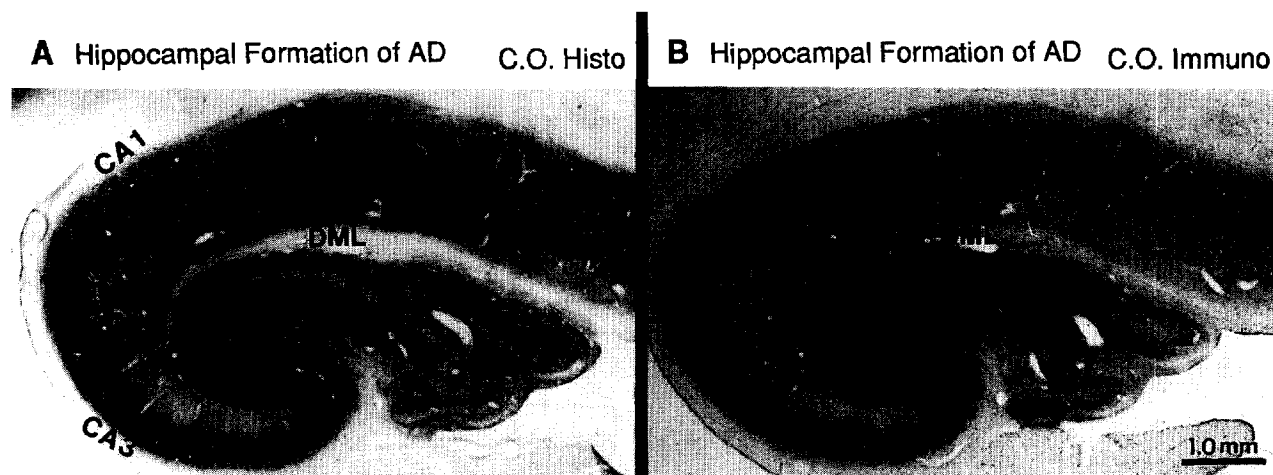


FIGURE 8. Coronal sections of the hippocampal formation reacted for C.O. histochemistry (A) and C.O. immunohistochemistry (B), respectively. Note that the granule cell layer (GCL) of the dentate gyrus is well demarcated by C.O. immunohistochemistry (B), but shows a low level of C.O. activity (A). The molecular layer of the dentate gyrus (DML) has relatively high levels of C.O. activity. The small, dark fold in the hippocampus in (A) is artifactual. These AD samples retained the same staining patterns as those in controls, but their levels of activity and immunoreactivity were significantly lower than those of controls (see Fig. 9). Since patterns of C.O. histochemistry and immunohistochemistry in various brain regions examined are similar between AD and control patients, only those from AD patients are shown. Control samples simply have higher levels of activity and immunoreactivity in most brain regions, as shown by optical densitometric readings in Figs 5, 7, and 9.

agraphia, and prosopagnosia are found during the clinical evaluation of patients with mild to moderate dementia of the AD type. Less commonly, the presenting symptoms of AD may consist of difficulty with seeing a picture as a whole. This very first symptom of AD is manifested in patients as an ability to describe individual components of a picture, but an inability to see them as parts in a larger context. This is consistent with a greater severity of C.O. deficits in the association cortical areas.

When compared with other age-matched controls, AD patients show specific deficits in contrast sensitivity (all but the lowest spatial frequency tested), while deficits in color vision were age-related and deficits in stereoacuity were related to other types of dementia (Bassi *et al.*, 1993; Hutton *et al.*, 1993; Levine *et al.*, 1993). AD patients had deficits in texture discrimination, blue-violet discrimination, and 4.72 deg/sec motion detection (Kurylo *et al.*, 1994). Selective degeneration of large ganglion cell axons was observed in the optic nerves of AD patients, suggesting an exclusive impairment of broad-band channel visual function; however, visual psychophysical tests indicate that broad-band visual capacities are not selectively impaired in AD (Kurylo *et al.*, 1994). Our analysis of the dorsal lateral geniculate nucleus also indicates that both magno- and parvocellular neurons were significantly affected in our population of AD patients. It has also been reported that AD patients were impaired at low rather than at high spatial frequencies, which contrasts with the normal aging pattern of high-frequency loss (Cronin-Golomb *et al.*, 1991). This would imply that regions subserving low spatial frequency processing in the primary visual cortex (cytochrome oxidase-rich puffs or blobs; reviewed in Wong-Riley, 1994) would be affected more than those for high spatial

frequency (interpuffs or interblobs) (Tootell *et al.*, 1988). Our data indicate that both puffs and interpuffs in our AD patients exhibited decreased cytochrome oxidase activity, and that there was a global reduction in oxidative capacity in both primary and secondary visual cortices.

The marked variability in C.O. levels among AD cases is not unexpected, as cases vary in their length and severity of illness. In the early stages of the disease, a bilateral increase in regional cerebral blood flow (rCBF) was actually observed in occipitotemporal extrastriate cortex during face matching, indicating a reliance on the extrastriate cortex to perform visuoperceptual tasks, similar to that seen in age-matched controls (Grady *et al.*, 1993). However, mild to moderately affected AD patients also showed an increased rCBF activation in regions of occipital and frontal cortex not seen in healthy controls, suggesting a need for increased attentional load due to a reduced cognitive capacity.

Future studies

The difficulty facing the pathogenesis of AD is that most, if not all, of the abnormalities reported are interrelated: altered processing of amyloid precursor protein, oxidative stress, oxygen free radicals, neurotransmitter abnormality, mtDNA mutation, hypometabolism, and neuronal degeneration. Defect in one would directly or ultimately lead to some or all of the other abnormalities. It seems clear, however, that cytochrome oxidase is compromised, perhaps more severely than the other enzymes of the electron transport chain, and that it affects certain brain regions more so than others.

Among the 13 subunits, mitochondrial-encoded C.O. 1 and 3 mRNAs were found to be significantly reduced (by 50–65%) in the temporal association, but not the primary

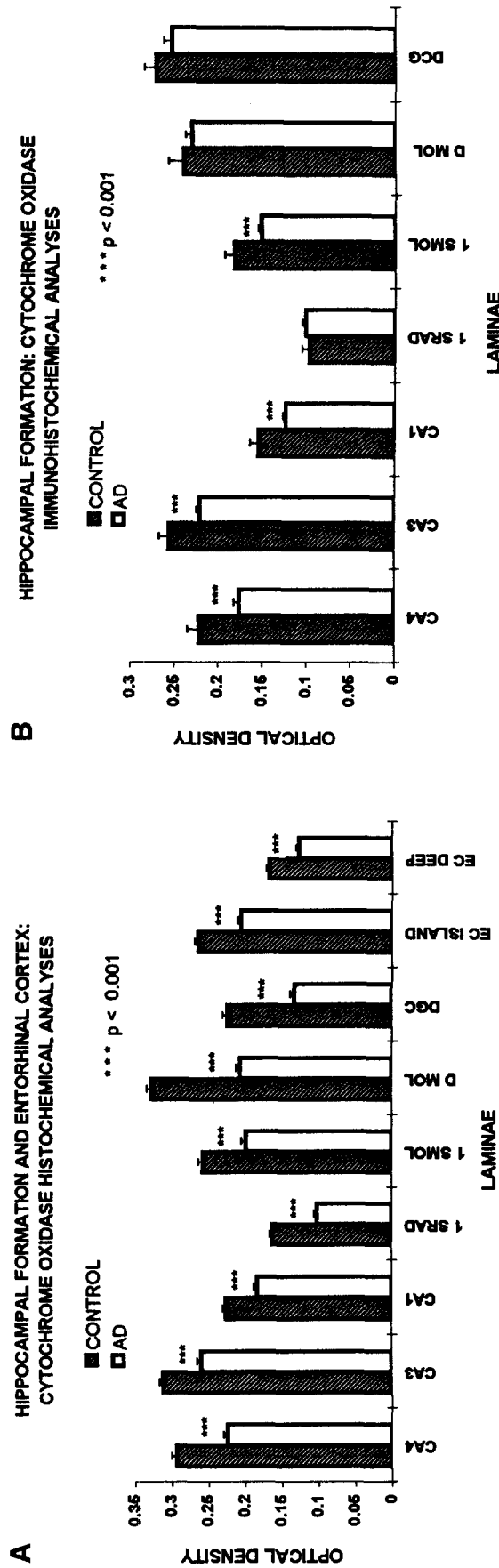


FIGURE 9. Optical densitometric measurements of reaction product of C.O. histochemistry (A) and C.O. immunohistochemistry (B) in the hippocampus and entorhinal cortex (EC) of AD and control patients. A significant reduction in C.O. activity is seen in all regions examined (A). Reduction in C.O. immunoreaction product reached statistical significance in CA1, 3, 4, and stratum moleculare of CA1 (1 SMOL), dentate molecular layer (DMOL) and dentate granule cell layer (DGC) of AD samples examined (B). The entorhinal cortex of AD was not processed for C.O. immunohistochemistry.

motor cortex of AD brains, suggesting the involvement of mtDNA in AD pathogenesis (Chandrasekaran *et al.*, 1994). Likewise, mitochondrial-encoded C.O. 2 was also reduced in the hippocampus of AD but, surprisingly, the nuclear-encoded C.O. 4 was reportedly not affected (Simonian & Hyman, 1994). These data suggest that reduced expression of C.O. genes in the mitochondria of AD brains may contribute to reduced oxidative metabolism in AD. However, mitochondrial-encoded C.O. subunit 3 gene was found to be overexpressed in temporal cortex of AD (Alberts *et al.*, 1992). The significance of such discrepancies is not clear at present. It is possible that the severity of the illness varies widely among AD patients, and that the disease manifests itself differently in disparate brain regions and to varying degrees.

The importance of oxidative stress in AD is undisputed. However, whether a genetic defect in oxidative enzymes is the cause or the effect of AD awaits further large-scale molecular studies.

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