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NEURONAL LOCALIZATION OF COPPER-ZINC SUPEROXIDE DISMUTASE PROTEIN AND mRNA WITHIN THE HUMAN HIPPOCAMPUS FROM CONTROL AND ALZHEIMER'S DISEASE BRAINS

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The distribution of cells containing copper-zinc superoxide dismutase (CuZn SOD) protein and mRNA was studied in hippocampi from normal humans and patients with Alzheimer's disease (AD) by using immunohistochemistry and *in situ* hybridization. Using antisera against native and denatured CuZn SOD protein, we have determined that immunostaining was intense in pyramidal neurons of the cornu ammonis, in granule cells of the dentate gyrus and very weak in other cells. In the hippocampus of an Alzheimer's patient, successive immunostaining of the same tissue section by antiCuZn SOD and antipaired helical filaments antisera show that both normal and degenerating cells were labeled by the antiCuZn SOD antiserum. Thus, large pyramidal neurons which are susceptible to degenerative processes in AD have the property to contain high amount of CuZn SOD protein.

In situ hybridization was performed on paraformaldehyde-fixed hippocampus sections of normal human brains and AD brains with a 35S labeled DNA probe homologous to human CuZn SOD mRNA. Our results show that CuZn SOD transcripts are present at high abundance in pyramidal neurons of the CA1-CA4 fields, subiculum, and in granule cells of the dentate gyrus. This cellular distribution is similar to that obtained with the antiCuZn SOD antiserum. This might indicate that biochemical pathways leading to superoxide radicals generation are specially active in these neurons, requiring an active transcription of CuZn-SOD gene.

KEY WORDS: CuZn superoxide dismutase, oxygen free radicals, human hippocampus, Alzheimer's disease.

INTRODUCTION

The hippocampal formation is particularly vulnerable to the pathological mechanisms that operate in Alzheimer's disease (AD), adult Down's syndrome (DS) and to a lesser extent in normal human aging. All the associated neurodegenerative events, including cell death, senile plaques (SP), neurofibrillary tangles (NFT), and granulovacuolar degeneration are expressed preferentially in the hippocampus during the disease process.[1-3] The nature of the biochemical events which lead to the loss of

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specific hippocampal neurons is not understood, but altered activity of cellular antioxidant systems have been implicated in the neuronal cell loss that is associated with aging[4-6] and with degenerative diseases of the central nervous system like DS[7-9] and/or AD.[10-12] Part of the system of antioxidant defenses is the copper-zinc superoxide dismutase (CuZn SOD; EC 1. 15. 1. 1.) which catalyses the dismutation of superoxide anions ($O_2^{\cdot -}$) into oxygen and hydrogen peroxide (H_2O_2)[13] and presumably protects oxidizable compounds like catecholamines and subcellular zones such as membranes against the toxic effects of superoxide anions. $O_2^{\cdot -}$ are highly reactive radicals formed by the one-electron reduction of oxygen and by autoxidizing catecholamines,[14] and may be particularly important in the genesis of cell damage. Moreover, the harm may arise from the activation of H_2O_2 (iron-catalysed Haber-Weiss or Fenton reactions), with the generation of the highly reactive species: hydroxyl radicals ($OH^{\cdot}$) or singlet oxygen $^1(O_2)$[15] leading to armfull oxydations of cell components.[10,16,17] CuZn SOD has been found widely distributed in all regions of human brain examined[11,18] with approximately the same specific activity, however a study at the cellular level has not yet been carried out.

To further investigate the free-radical hypothesis of neuronal death[19] and to know more about the CuZn SOD content of the different cell types of the human hippocampus, the CuZn SOD protein and mRNA was studied by immonohistochemistry and by in situ hybridization, within the various neuronal subpopulations of the human hippocampus.

MATERIALS AND METHODS

Immunohistochemistry

Samples of tissue from 3 control and 3 AD patient brains were obtained within 5–10 h of death as described by Delacourte and Defossez.[20] Controls, deceased from heart attack, were 50, 80 and 85 years old. AD patients were 70, 75 and 77 years old at the time of death. Cachexia complicated by lung infection was, in all cases, the cause of death.

Human CuZn SOD was purified from red blood cells according to Hartz and Deutsch.[21] The purity of our preparation was checked by electrophoresis in denaturing conditions. Three different antisera were raised against CuZn SOD: one against the native CuZn SOD preparation; two against denatured CuZn SOD. Denaturation of the protein was carried out in two ways: by heat-treatment alone (5 min) in potassium phosphate buffer 0.05 M-NaCl 0.1 M, pH 7.0 or by heat-treatment in 2% sodium dodecyl sulfate (SDS) followed by dialysis against water lyophilization. Monospecificity of the antisera was checked by immunoblotting after electrophoresis in denaturing conditions. The anti-PHF was prepared as published by Persuy *et al.*[22]

Pieces of various cortical areas, and hippocampus were fixed in Carnoy's solution (ethanol, chloroform, acetic acid: 6/3/1(v/v) for 24 h before embedding in paraffin. The blocks were cut in 5-μm thick sections. Immunoperoxidase reactions were performed by using an indirect method according to Persuy *et al.*[22]

The anti-PHF and anti-CuZn SOD antisera were used at 1/1000 and 1/100 dilutions respectively and the conjugated sheep anti-rabbit immunoglobulin at 1/50 (Pasteur Production, Paris). Peroxidase reaction was carried out in a 100 ml solution containing 0.1 M Tris buffer, pH 7.6, 4-chloro-1-naphthol, 20 mg and hydrogen peroxide 0.001% (w/w). Controls fro the anti-CuZn SOD immunoreaction was performed with

the antiserum previously immunoabsorbed with the purified CuZn SOD protein and gave no labeling of cells. The elution technique[23] was used in order to compare the successive immunolabeling of two antibodies on the same tissue section. The first immunolabeling was performed with anti-PHF or anti-CuZn SOD antiserum. Microphotographs were then taken. After the destaining of chloro-naphthol with acetone and the complete elution of bound antibodies with an aqueous solution containing $KMnO_4$ (0.25%) (w/v) and H_2SO_4 (0.005%), the second immunolabelling was performed with antiCuZn SOD antibodies or anti-PHF antibody. Microphographs of the same area were taken and compared with the previous ones.

In situ Hybridization Histochemistry

Brain tissues from 5 control subjects (mean age 80 ± 2 years) with no known neurological or neuropsychiatric disorders were obtained between 2 and 6 hr postmortem (mean 3.6 ± 0.8 hr). Within 2 hours after autopsy, the brains were sliced in coronal sections. Tissues blocks containing the mid rostro-caudal level of the hippocampus were dissected and fixed by 3 days immersion at 4° C in 4% (wt/vol) paraformaldehyde as previously described.[24] Tissue sections (15 μm) were cut in a cryostat, mounted on gelatine-coated slides and stored at − 70° C until use.

The construction and characterization of the human recombinant CuZn-SOD cDNA in plasmid pBR 322 have been described.[25] The 620-base-pair insert containing the complete coding and 3′-untranslated sequences was purified by electroelution after agarose gel electrophoresis and labeled by a random priming technique[26] using 35_S-substituted deoxycytidine 5′-[α-thio]-triphosphate (Amersham) to specific activities of 2–4 × 10^8 dpm/μg.

Experiments on slide-mounted sections were carried out as described by Berod *et al.*[27] for *in situ* hybridization. Autoradiograms of tissue sections were generated by apposition of ^{35}S-labeled sections to Hyperfilm (Amersham). Films were developed after 3–6 days, and the sections were dipped in NTB-2 emulsion (Kodak), exposed at 4° C for 20 days and then developed and stained with cresyl violet.

Control experiments were performed by incubation of sections with RNase A (50 μg/ml in 0.1 M sodium phosphate, pH 7.4 for 30 min at 37° C and dehydrated prior to hybridization) or by hybridization of sections with a ^{35}S-labeled human tyrosine hydroxylase cDNA probe.[28]

RESULTS

Immunostaining with our anti-CuZn SOD antibodies, carried out on normal aged human brains (Figure 1b) was compared to the general staining of nervous cells revealed by hemalun-erythrosin (Figure 1a). It was observed that the cell bodies of pyramidal cells were intensely labeled whereas other cells were very weakly marked except for the hippocampal granule cells which were moderately but significantly labeled. Results were identical with anti-native and anti-denatured CuZn SOD antisera. This immunostaining pattern indicates that the CuZn-SOD protein content is higher in pyramidal neurons than in other cell types. This was observed in the two brain areas that we studied: the hippocampus and the associative cortex (data not shown). At high magnification, the staining was apparently homogenous excluding

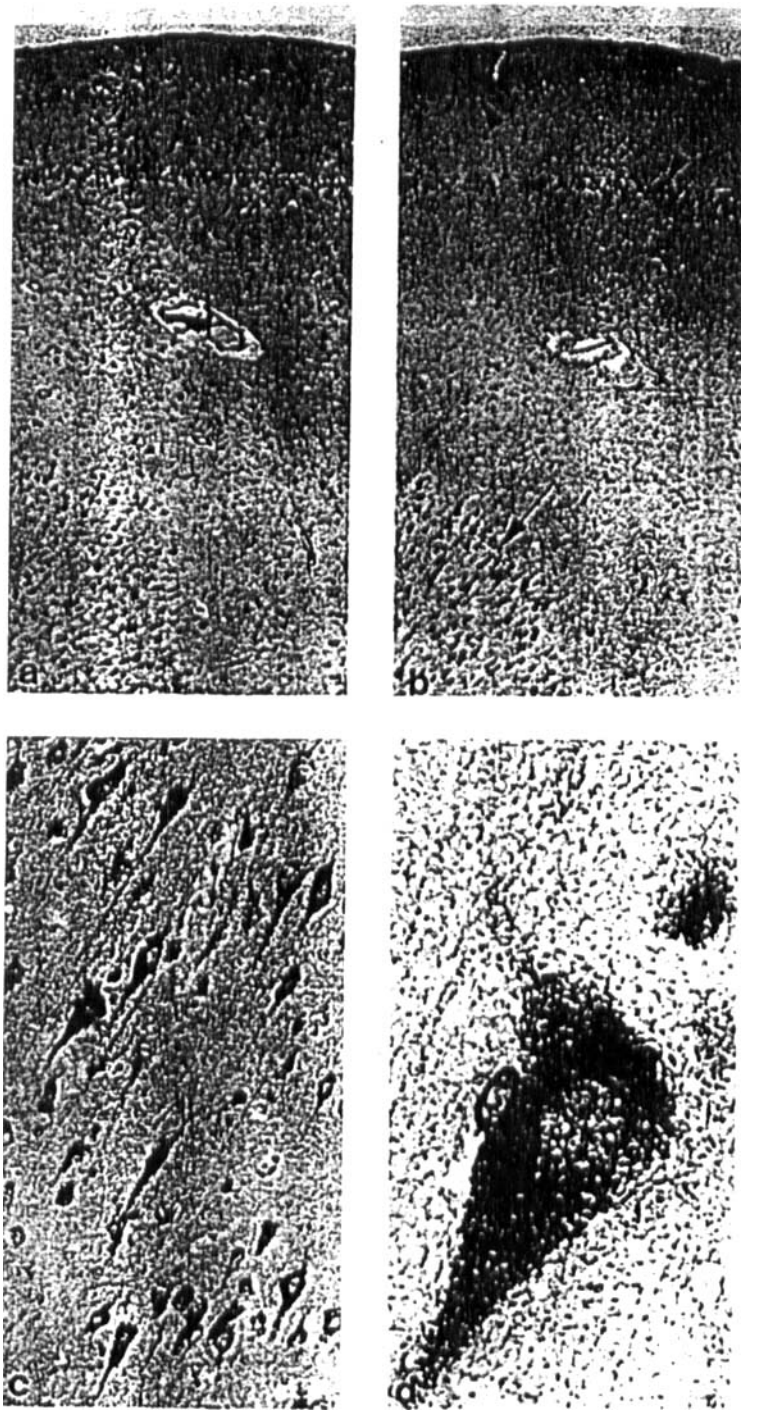

FIGURE 1 a, b: Adjacent histological sections of hippocampus from a normal aged patient. Field CA4 (X80).
a: hemalun erythrosin staining. b: anti-CuZn SOD immunostaining. Note the important labeling of pyramidal cells by the immunoserum (arrow), the moderate labeling of the hippocampal granule cells (arrow heads) and the weak labeling of other cells. c,d: observation of the immunolabeling at a higher magnification (c, × 400; d, × 2400). The labeling is confined to the perikaryon and the proximal dentrites.

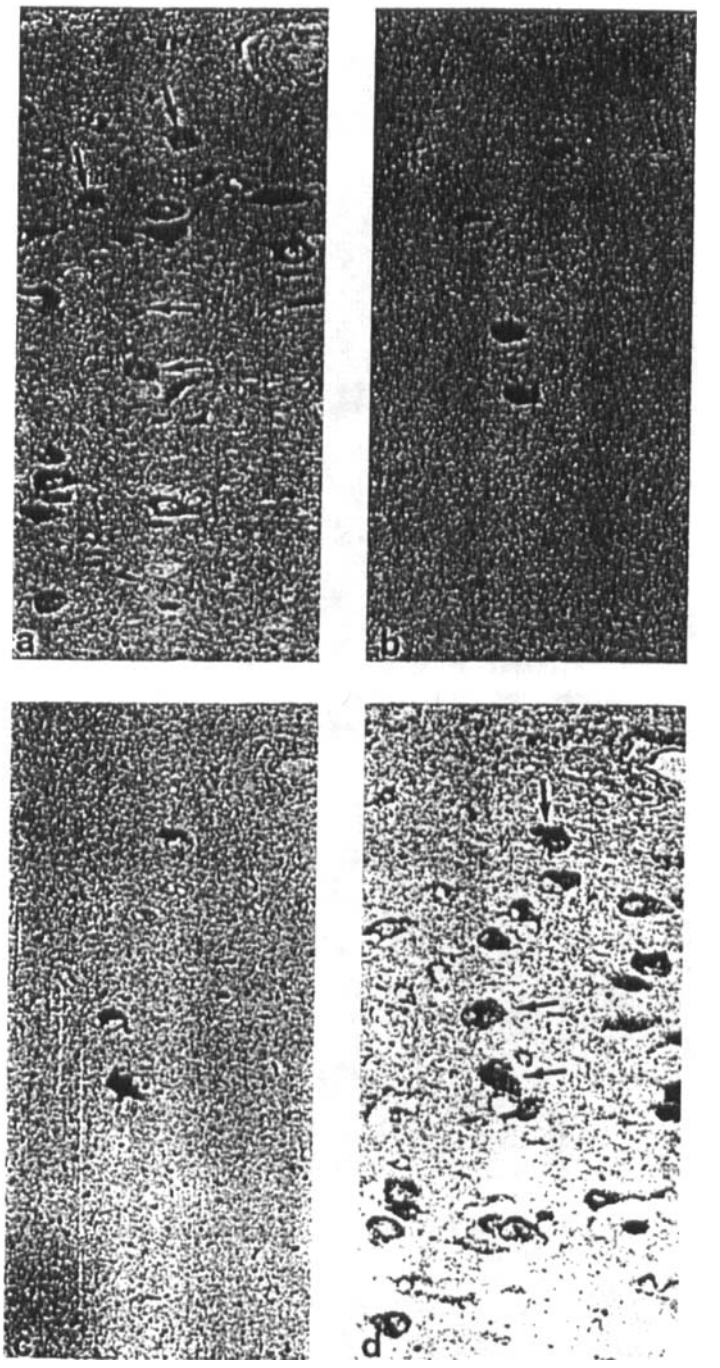

FIGURE 2 a, b: Two photographs of the same section of AD hippocampus. a: the distribution of neurons immunolabeled with the anti-CuZn SOD antiserum. b: the degenerating neurons immunolabeled with the anti-PHF antiserum, after elution of the first antiserum (arrows in a). c, d: two photographs of the adjacent sections. c: labelling with anti-PHF. d: elution and restaining with the anti-CuZn SOD antiserum. Note that degenerating neurons (arrows) are among the population of the CuZn SOD immunostained neurons. × 400.

AUTORADIOGRAPHIC LOCALIZATION OF CuZn SUPEROXIDE DISMUTASE mRNA IN HUMAN HIPPOCAMPUS BY IN SITU HYBRIDIZATION

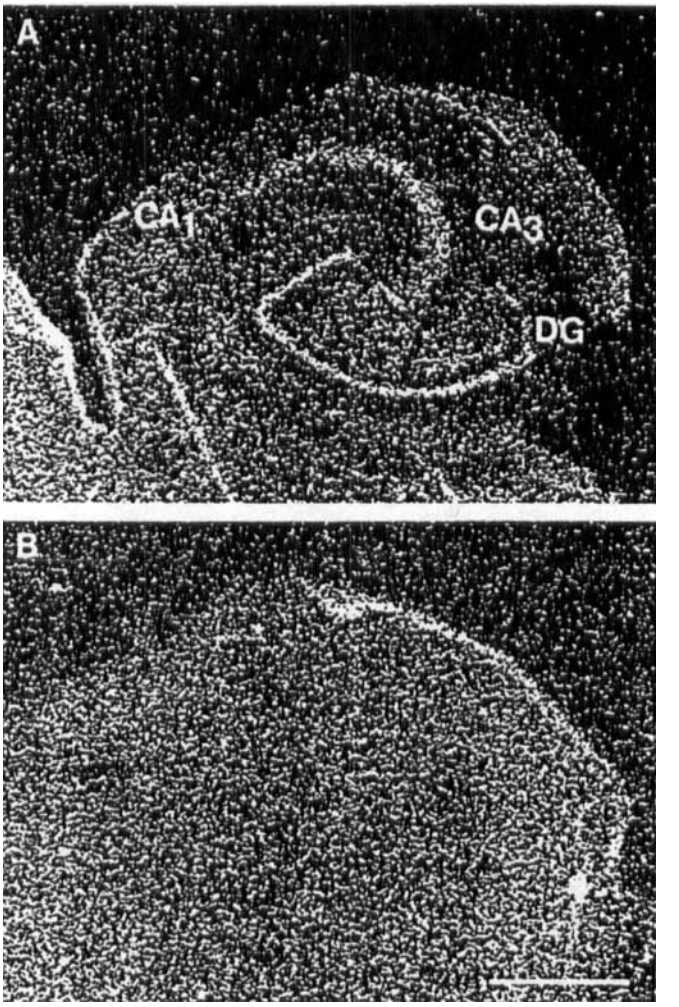

FIGURE 3 Film autoradiograms of adjacent sagittal sections of a human hippocampus hybridized with a human ^{35}S-labeled CuZn SOD cDNA probe (A) or with a human ^{35}S-labeled tyrosine hydroxylase cDNA probe (B). Coronal tissue section from mid-rostrocaudal levels of the hippocampal formation were exposed in direct contact with x-ray film for 2 days. - An intense labeling is observed with the CuZn SOD cDNA probe over the pyramidal cell layers of the Ammon's horn (CA), the CA1 to CA4 fields, the subiculum, and in the granule cell layer of the dentate gyrus. - The specificty of the hybridization pattern is demonstrated by the absence of labeling with a radioactive cDNA unrelated to the hippocampus such as the tyrosine hydroxylase cDNA probe (B). Magnification: Bar indicates 2 mm.

CELLULAR LOCALIZATION OF CuZn SUPEROXIDE DISMUTASE mRNA IN HUMAN HIPPOCAMPUS BY IN SITU HYBRIDIZATION

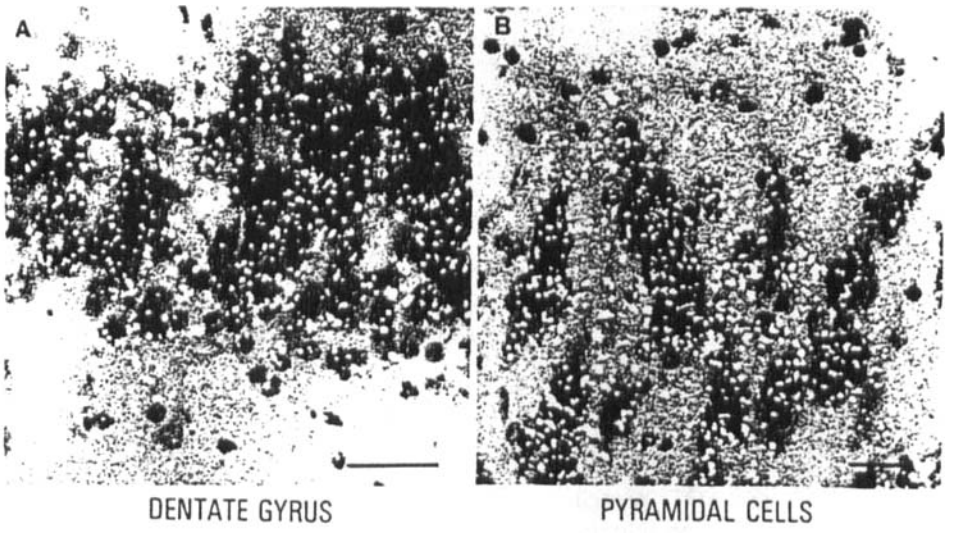

FIGURE 4 Photomicrograph of granule cell layer from the dentate gyrus (A) and pyramidal cells of the CA1 region of Ammon's horn (B) after hybridization with ^{35}S-labeled CuZn SOD cDNA probe. Cresyl violet stain of emulsion-coated sections viewed using a combined brightfield and darkfield illumination. Note the accumulation of grains over the clustered granule cells (A) and over the pyramidal neurons (B). Exposure time 21 days. Magnification: Bar indicates 20 μm.

any obvious sublocalization of CuZn SOD to the membrane or the nucleus of these cells (Figure 1c,d).

In Alzheimer's brains, cell distribution of the CuZn-SOD immunostaining was similar to that observed in control brains. In one of these brains, successive immunostainings were performed with anti-CuZn SOD and anti-PHF antibodies or vice-versa (Figure 2). Among the population of pyramidal cells that were stained with the anti-CuZn SOD antiserum, several neurons were affected by the neurofibrillary degeneration as shown by the anti-PHF labeling. In pyramidal cells which did not contain any detectable PHF material as well as in other cells, CuZn SOD immunostaining was similar to that observed in normal brain.

Autoradiograms of the hippocampal sections hybridized with the ^{35}S-labeled CuZn SOD probe showed a characteristic, heterogeneous distribution of grains corresponding to different regions of the hippocampus. A heavy density of grains was observed over the dentate gyrus, in the CA fields of the Ammon's horn (CA) and in the subiculum (Figure 3A) with a background corresponding to white matter areas. The same qualitative distribution of grains was observed in the five different hippocampi examined and results were reproductible between different tissue sections of the same hippocampus.

The specificity of the hybridization reaction is evidenced by control experiments: first, only background grain density was observed when sections were pretreated with RNase before hybridization (data not shown) and second, sections treated with a radioactive cDNA unrelated to the hippocampus such as the human tyrosine hyd-

roxylase (TH) cDNA probe did not exhibit the characteristic pattern of labeling previously described with the CuZn SOD cDNA probe (Figure 3B). Microscopic examination of emulsion-coated sections showed a heavy neuronal labeling appearing as an accumulation of silver grains over cell bodies. Two types of hippocampal cells were preferentially labeled: the granule cells of the dentate gyrus (Figure 4A), the pyramidal cells of CA1 (Figure 4B), CA2, CA3 and CA4 fields and the pyramidal cells in the subiculum. In the other layers of the hippocampal formation, no specific labeling of cells could be detected. The grains overlaid cell somata and appeared densely packed over the pyramidal cells (Figure 4B) and more sparse over the clusters of cells of the dentate gyrus (Figure 4A). In control experiments with the human TH cDNA probe, emulsion-coated sections did not exhibit positively hybridized pyramidal or granule cells, as could be expected from anatomical data.[29] The characteristic cellular distribution of CuZn SOD mRNA within the human hippocampus was superimposable to the disposition of the CuZn SOD protein previously evidenced by immunohistochemical methods.

DISCUSSION

Although the CuZn SOD protein is an unbiquitous enzyme as suggested by the distribution of the enzyme activity in the human brain,[18] a higher level of CuZn SOD mRNA and protein in subsets of hippocampal neurons i.e. the pyramidal and granule cells is now demonstrated. This might suggest that the biochemical pathways leading to the generation of $O_2^{\cdot -}$ radicals are particularly active in these neurons, thus requiring a high CuZn SOD content to facilitate the removal of the toxic radicals. Alternatively, the high cellular CuZn SOD activity might contribute to the neurodegenerative process itself. This hypothesis is motivated by several observations. The gene coding for CuZn SOD protein in human is located on chromosome 21 and in Down's patients, increased CuZn SOD activity in various tissues[9] including brain[8] reflects a gene dosage effect associated with a corresponding increase in CuZn SOD mRNA.[25] It is noteworthy that patients with Down's syndrome develop an accelerated aging and brain histopathological changes are reminiscent to that of Alzheimer's disease.[1] Moreover, CuZn SOD activity was significantly elevated in familial Alzheimer's fibroblasts.[30] Recent publications further support this hypothesis. Mouse cells expressing the transfected human CuZn SOD gene showed an induction of glutathione peroxidase activity, an enzyme which catalyses the reduction of H_2O_2.[31] In addition, PC12 cells overexpressing the human CuZn SOD gene have impaired neurotransmitter uptake resulting from modifications of the membrane properties of chromaffin granules, likely secondary to lipid peroxidation.[32]

The elevated abundance of CuZn SOD mRNA and protein in the pyramidal neurons of the human hippocampus suggest that they represent a subset of neurons with distinct characteristics. This may account for their preferential vulnerability in aging or in neurodegenerative diseases such as Alzheimer's disease. To further consider this concept, it would be worthwhile investigating other enzymatic steps involved in cellular antioxidant defense system i.e. catalase and glutathione peroxidase. Moreover, quantitative analysis by *in situ* hybridization are in progress to evaluate the level of the transcription of CuZn SOD gene in the different populations of hippocampal neurons in control human brains as well as in brains from patients deceased with AD.

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References

1. M.J. Ball and K. Nutall (1980) Neurofibrillary tangles, granulovacuolar degeneration, and neuron loss in Down's Syndrome: quantative comparison with Alzheimer's dementia. *Annals of Neurology*, **7**, 462–465.
2. M.J. Ball, V.T. Hachinski and A. Fox (1985) A new definition of Alzheimer's disease: a hippocampal dementia. *Lancet*, **I**, 14–16.
3. I.T. Lott (1982) Down's syndrome, aging and Alzheimer's disease: a clinical review. *Annals of the New York Academy of Science*, **396**, 15–27.
4. D. Harman (1981) The aging process. *Proceedings of the National Academy of Sciences USA*, **78**, 7124–7128.
5. E.W. Kellog and I. Fridovich (1976) Superoxide dismutase in the rat and mouse as a function of age and longevity. *Journal of Gerontology*, **31**, 405–408.
6. Y. Mizuno and K. Ohta (1986) Regional distribution of thiobarbituric acid-reactive products, activities of enzymes regulating the metabolism of oxygen free radicals, and some of the related enzymes in adult and aged rat brains. *Journal of Neurochemistry*, **46**, 1344–1352.
7. K.G. Anneren and C.J. Epstein (1987) Lipid peroxidation and superoxide dismutase-1 and glutathione peroxidase activities in trisomy 16 fetal mice and human trisomy 21 fibroblasts. *Pediatric Research*, **21**, 88–92.
8. B.W.L. Brooksbank and R. Balazs (1984) Superoxide dismutase, glutathione peroxidase and lipoperoxidation in Down's Syndrome fetal brain. *Development in Brain Research*, **16**, 37–44.
9. P.M. Sinet (1982) Metabolism of oxygen derivatives in Down's Syndrome. *Annals in New York Academy of Science*, **396**, 83–94.
10. B. Halliwell and M.C. Gutteridge (1985) Oxygen radicals and the nervous system. *Trends in Neuroscience*, **8**, 22–26.
11. S.L. Marklund, R. Adolfsson, C.G. Gottfries and B. Windblad (1985) Superoxide dismutase isoenzymes in normal brains and in brains from patients with dementia of Alzheimer's type. *Neurology Science Journal*, **67**, 319–325.
12. R.N. Martins, C.G. Harper, G.B. Stokes and C.L. Masters (1986) Increased glucose 6-phosphate dehydrogenase activity in Alzheimer's disease may reflect oxidative stress. *Journal of Neurochemistry*, **46**, 1042–104.
13. I. Fridovich (1974) Superoxide dismutases. *Advances in Enzymology*, **41**, 35–97.
14. G. Cohen and Heikkila (1974) The generation of hydrogen peroxide, superoxide radical and hydroxyl radical by 6-hydroxydopamine and related cytotoxic agents. *Journal of Biological Chemistry*, **249**, 2447–2452.
15. A. Puppo and B. Halliwell (1988) Formation of hydroxyl radicals from hydrogen peroxide in the presence of iron. *Biochemistry Journal*, **249**, 185–190.
16. J.W. Hartz and H.F. Deutsch (1972) Subunit structure of human superoxide dismutase. *Journal of Biological Chemistry*, **247**, 7043–7050.
22. P. Persuy, A. Defossez, A. Delacourte, G. Tramu, B. Bouchez and G. Arnott (1985) Anti-PHF antibodies: an immunohistochemical marker of the lesions of the Alzheimer's disease. *Virchows Archives A.*, **407**, 13–23.
23. G.P. Ramu A. Pillez and J. Leonardelli (1978) An efficient method of antibody elution for the successive or simultaneous localization of two antigens by immunohistochemistry. *Journal of Histochemistry and Cytochemistry*, **26**, 322–324.
24. A. Graybiel, E.C. Hirsch and Y. Agid (1987) Differences in tyrosine hydroxylase-like immunoreactivity characterize the mesostriatal innervation of striosomes and extrastriosomal matrix at maturity. *Proceedings of the National Academy of Sciences USA.*, **84**, 303–307.
25. L. Sherman, N. Dafni, J. Leman-Hurwitz and Y. Groner (1983) Nucleotide sequence and expression of human chromosome 21 – encoded superoxide dismutase mRNA. *Proceedings of the National Academy of Sciences USA*, **80**, 5465–5469.

26. A.P. Feinberg and B. Volgestein (1983) A technique for radiolabeling restriction endonuclease fragments to high specific activity. *Analytical Biochemistry*, **132**, 6–13.
27. A. Berod, N. Faucon-Biguet, S. Dumas, B. Bloch and J. Mallet (1987) Modulation of tyrosine hydoxylase gene expression in the central nervous system visualized by in situ hubridization. *Proceedings in the National Academy of Sciences USA.*, **84**, 1699–1703.
28. B. Grima, A. Lamouroux, C. Boni, J.F. Julien, F. Javoy-Agid and J. Mallet (1987) A single human gene encoding multiple tyrosine hydroxylases with different predicted functional characteristics. *Nature*, **326**, 707–711.
29. I. Walass (1983) The hippocampus, chemical neuroanatomy. In P.C. Emson (Ed), Raven Press, New York, 337–359.
30. F.P. Zemlan, O.J. Thienhaus and H.B. Bosmann (1989) Superoxide dismutase activity in Alzheimer's disease: possible mechanism for paired helical filament formation. *Brain Research*, **476**, 160–162.
31. I. Ceballos, J. M. Delabar, A. Nicole, R.E. Lynch, R.A. Hallewel, P. Kamoun and P.M. Sinet (1988) Expression of transfected human CuZn superoxide dismutase gene in mouse L cells and NS20Y neuroblastoma cells induces enhancement of glutathione peroxidase activity. *Biochimica et Biophysica Acta*, **949**, 58–64.
32. O. Elroy-Stein and Y. Groner (1988) Impaired neurotransmitter uptake in PC12 cells overexpressing human CuZn superoxide dismutase: implication for gene dosage effects in Down's Syndrome. *Cell*, **52**, 259–267.

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