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11. Mice were injected with morphine (10 mg/kg sc). After 30 min or 2 hours, wild-type mice were killed and blood was collected in vials containing sodium fluoride and potassium oxalate. Morphine concentrations in blood samples pooled from three mice per sample were 1500 ng/ml after 30 min and 83 ng/ml after 2 hours, as measured by mass spectroscopy analysis (Occupational Testing Division, LabCorp Inc., Research Triangle Park, NC). In similar experiments, βarr2-KO mice had a concentration of 93 ng/ml in the blood after 2 hours.
12. Naloxone (2.5 mg/kg sc), which immediately reverses the effects of opiates, was given 30 min after morphine (10 mg/kg). Naltrindole [P. S. Portoghese, M. Sultana, A. E. Takemori. *J. Med. Chem.* **88**, 1547 (1990)] was given 20 min before morphine, and nor-binaltorphimine [A. E. Takemori, B. Y. Ho, J. S. Naeseth, P. S. Portoghese, *J. Pharmacol. 'Exp. Ther.* **246**, 255 (1988)] was given 1 hour before morphine [H. W. Matthes *et al.*, *J. Neurosci.* **18**, 7285 (1998)].
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14. Membranes were prepared for binding assays as follows. Brain regions were dissected and immediately frozen in liquid nitrogen and were stored at −80°C for less than 1 week before use. Samples were placed on ice and homogenized by polytron in membrane preparation buffer [50 mM tris (pH 7.4), 1 mM EDTA, 3 mM $MgCl_2$], and crude membranes were prepared by centrifugation at 20,000*g* for 15 min at 4°C. Membranes were resuspended in either 50 mM tris-HCl (pH 7.4) for radioligand binding assays or in assay buffer [50 mM tris-HCl (pH 7.4), 100 mM NaCl, 3 mM $MgCl_2$, and 0.2 mM EDTA] containing 10 μM guanosine diphosphate for [$^{35}$S]GTP-$\gamma$-S binding assays. For both binding assays, reactions were terminated by rapid filtration over GF/B filters (Brandel Inc., Gaithersburg, MD) using a Brandel cell harvester. Filters were washed three times with ice-cold 10 mM tris-HCl (pH 7.4) and then counted in a liquid scintillation counter.
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25. We thank B. H. Koller for homologous recombinant mouse embryonic stem cells and chimeric mice, M. R. Capecchi for plasmid pIC19R/MCI-TK, and R. Hen for plasmid pD383. R.R.G. is a visiting scientist from the Institute of Pharmacology, Russian Academy of Medical Sciences, Moscow. Supported in part by NIH grants NS 19576 (M.G.C.) and HL16037, and by a cardiovascular unrestricted grant from Bristol-Myers Squibb (R.J.L.). R.J.L. and M.G.C. are Investigators of the Howard Hughes Medical Institute.

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# Induction of Nitric Oxide–Dependent Apoptosis in Motor Neurons by Zinc-Deficient Superoxide Dismutase

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**Mutations in copper, zinc superoxide dismutase (SOD) have been implicated in the selective death of motor neurons in 2 percent of amyotrophic lateral sclerosis (ALS) patients. The loss of zinc from either wild-type or ALS-mutant SODs was sufficient to induce apoptosis in cultured motor neurons. Toxicity required that copper be bound to SOD and depended on endogenous production of nitric oxide. When replete with zinc, neither ALS-mutant nor wild-type copper, zinc SODs were toxic, and both protected motor neurons from trophic factor withdrawal. Thus, zinc-deficient SOD may participate in both sporadic and familial ALS by an oxidative mechanism involving nitric oxide.**

Only 2% of ALS patients carry 1 of 60 different dominant mutations in Cu,Zn SOD, raising questions about how SOD might be involved in the majority of sporadic ALS patients expressing only wild-type (WT) SOD (*1*). Transgenic mouse experiments establish that ALS mutations increase a toxic gain-of-function of the protein that kills motor neurons (*2*). SOD is the major defense against superoxide ($O_2^{\cdot-}$), but WT SOD does not protect against the toxicity of ALS-SOD in mice or humans (*1*, *3*). Curiously, ALS-SODs can scavenge superoxide as efficiently as WT SOD when they contain their full complement of Cu and Zn (*4*). However, the mutations destabilize the SOD protein, which indirectly decreases Zn affinity by 5- to 50-fold compared with WT SOD (*5*, *6*). Mutations such as A4V (substitution of alanine at position 4 by valine), which cause rapid disease progression, yield SODs with the weakest affinity for Zn. Because Zn deficiency diminishes superoxide scavenging and increases tyrosine nitration by SOD (*6*), we examined how the altered redox properties of Zn-deficient SOD could be toxic to cultured motor neurons.

Loss of Zn visibly changed SOD from a "green" to a "blue" protein by altering the coordination of Cu through a shared histidine ligand (Fig. 1A). Reduction of the Cu to $Cu^+$ makes both forms of SOD colorless. The altered Cu coordination made Zn-deficient SOD a more efficient oxidant, able to oxidize ascorbate 3000-fold faster than Cu,Zn SOD, irrespective of whether the protein was WT or carried an ALS mutation (Fig. 1B). Other cellular reductants such as urate, glutathione, and cysteine could also reduce Zn-deficient SOD, but more slowly than ascorbate. Because such reduced SOD is slowly reoxidized by oxygen (*7*), Zn-deficient SOD can generate superoxide at the expense of cellular antioxidants. Cells still contain high concentrations of Cu,Zn SOD (*8*), which will quickly recapture superoxide leaking from reduced Zn-deficient SOD. However, nitric oxide (NO) can effectively compete with SOD for superoxide to produce the strong oxidant peroxynitrite ($ONOO^-$) (*9*). Peroxynitrite formation was assayed with the indicator dichlorodihydrofluorescein (DCDHF), which is oxidized by peroxynitrite but not by NO, superoxide, or hydrogen peroxide ($H_2O_2$) (*10*). Ascorbate plus Zn-deficient SOD oxidized DCDHF only in the presence of NO and under aerobic conditions (Fig. 1C). Adding a 1.5-fold excess of Zn to Zn-deficient SOD prevented DCDHF oxidation, showing that the Zn-deficient SOD can rapidly bind Zn to behave like Cu,Zn SOD in vitro. Oxidation of DCDHF did not involve $H_2O_2$ because catalase had no effect and addition of 20 μM $H_2O_2$ to Zn-deficient SOD did not oxidize DCDHF.

Importantly, WT Cu,Zn SOD did not pre-

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vent DCDHF oxidation when added together with Zn-deficient SOD (Fig. 1C). Because NO reacts at diffusion-controlled rates with other radicals, it may combine with superoxide produced in or near the active site to form peroxynitrite during the reoxidation of Zn-deficient SOD by oxygen.

$$SOD\text{-}Cu^{+} + O_2 \rightarrow SOD\text{-}Cu^{2+}\cdots OO^{\cdot -} + {}^{\cdot}NO \rightarrow$$

$$SOD\text{-}Cu^{2+}\cdots OONO^{-} \leftrightarrow SOD\text{-}Cu^{2+} + ONOO^{-}$$

A similar peroxynitrite intermediate has recently been observed during the rapid reaction of NO with oxygen bound to ferrous iron in *oxy*-myoglobin (*11*). Large excesses of Cu,Zn SOD would not affect peroxynitrite formation by this mechanism because superoxide may not be released as a free intermediate. These results offer one explanation for how sixfold overexpression of WT SOD fails to slow disease progression in ALS-SOD transgenic mice (*3*).

To determine whether the aberrant redox activity of Zn-deficient SOD could contribute to the toxic gain-of-function of SOD, we used liposomes to deliver Cu,Zn and Zn-deficient SOD to cultured motor neurons (Fig. 2). When replete with both metals, neither WT nor ALS-mutant Cu,Zn SODs were toxic and both substantially protected motor neurons deprived of trophic support from apoptosis (Fig. 2, A and B). When WT or four different ALS-SODs were made Zn-deficient, all initiated apoptosis in motor neurons cultured with brain-derived neurotrophic factor (BDNF) (Fig. 2C). Zn-deficient SODs did not increase apoptosis of motor neurons cultured without BDNF (Fig. 2D). Codelivery of WT Cu,Zn SOD with Zn-deficient SOD also did not protect cultured motor neurons.

Wild-type, Zn-deficient SOD was toxic to motor neurons despite its ability to rapidly rebind Zn in vitro (Fig. 1C), suggesting that incorporation of Zn into SOD can be limiting in motor neurons. In our culture conditions, the media contained about 1 μM Zn, which is higher than the extracellular Zn concentrations normally found in brain (*12*). Higher concentrations of Zn are toxic to neurons and may limit attempts to treat ALS with Zn supplementation (*12*).

Liposomal delivery of SOD apoprotein depleted of Cu and Zn did not affect motor neuron survival (Fig. 3A). Neither free Cu nor albumin-bound Cu delivered in liposomes was toxic to motor neurons (Fig. 3A). Two specific $Cu^{+}$ chelators also protected motor neurons from Zn-deficient SOD (Fig. 3B), which was consistent with their ability to remove Cu from reduced Zn-deficient SOD in vitro, but not from Cu,Zn SOD (*13*). Thus, Cu associated with Zn-deficient SOD was necessary to induce apoptosis in motor neurons, and toxicity was not due to the SOD protein itself.

Endogenous NO produced by motor neurons contributed to Zn-deficient, SOD-induced apoptosis. Nitric oxide synthase (NOS) inhibitors protected against Zn-deficient, SOD-induced death, whereas maintaining physiological

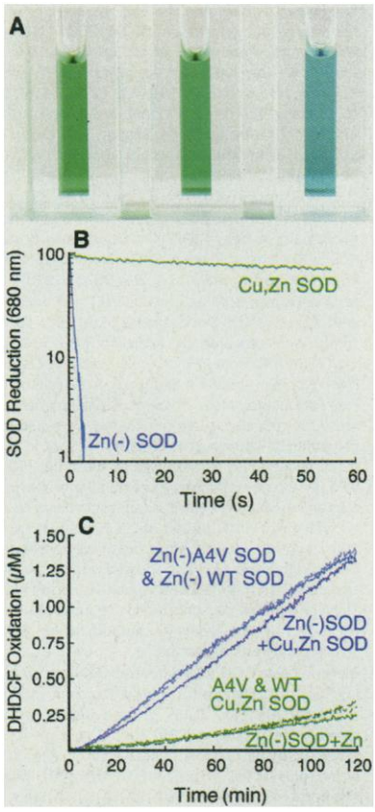


**Fig. 1.** (**A**) Wild-type Cu,Zn SOD and ALS mutant A4V Cu,Zn SOD (50 mg/ml) is green, whereas Zn-deficient A4V SOD is blue. Zn-deficient WT SOD also has the same blue spectrum. (**B**) Reduction of Zn-deficient A4V SOD (blue line) and Cu,Zn A4V SOD (green line) was monitored at 680 nm upon mixing with 1.5 mM ascorbate in a stopped flow spectrometer. Rates of reduction for Zn-deficient WT and A4V SODs were 900 and 1200 $M^{-1}\,s^{-1}$, respectively. The equivalent Cu,Zn SODs were reduced at rates of 0.28 and 0.43 $M^{-1}\,s^{-1}$. (**C**) Oxidation of DCDHF by Zn-deficient SOD plus NO. Each reaction contained 10 μM SOD, 5 μM ascorbate, 100 μM DETA-NONOate, and 100 μM DCDHF. DETA-NONOate generated 0.25 μM steady-state NO under these conditions. Rates were corrected for background oxidation of DCDHF by DETA-NONOate. Addition of Zn (40 μM) reduced the oxidation of DCDHF to that of WT SOD. Addition of 10 μM WT Cu,Zn SOD did not appreciably slow DCDHF oxidation by 10 μM WT, Zn-deficient SOD.

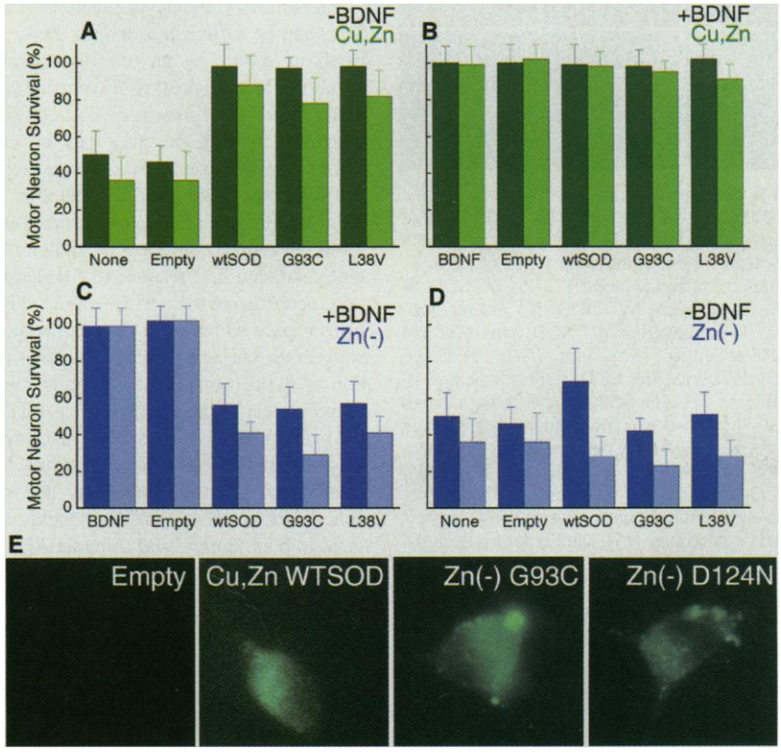


**Fig. 2.** Effects of Zn-deficient (blue) and Cu,Zn (green) WT and ALS-mutant SODs on motor neuron survival after 1 (dark shade) and 4 days (light shade). (**A** and **D**) Comparison of motor neuron survival after BDNF withdrawal, but after treatment, respectively, with Cu,Zn SOD or Zn-deficient SOD liposomes (*18*). (**B** and **C**) Comparison of motor neuron survival after culture with BDNF and treatment with either Cu,Zn SOD or Zn-deficient SOD liposomes. Values are the mean ± SD of at least two independent experiments performed in duplicate. Results from two ALS-SODs G93C and L38V are shown. Equivalent results were obtained with the Zn-deficient SOD mutants A4V, I111T, and D124N. All of the metal-replete Cu,Zn SODs had 100 ± 5% Cu and Zn, whereas Zn-deficient WT SOD had 102 ± 10% Cu and 9 ± 7% Zn, G93C had 85 ± 12% Cu and 17 ± 10% Zn, and L38V had 91 ± 10% Cu and 10 ± 10% Zn. (**E**) Intracellular incorporation of biotin-labeled SODs delivered with liposomes (*19*).

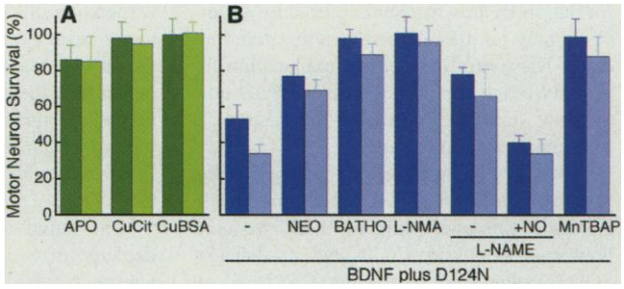


**Fig. 3.** (**A**) Motor neuron survival after treatment with either apoSOD (Cu <0.1%, Zn <0.1%), 50 μM Cu citrate, or Cu complexed with BSA entrapped in liposomes. (**B**) Treatment with Zn-deficient D124N SOD (*20*) (104% Cu and 6% Zn) induced motor neuron death. Cell death was prevented by the cuprous chelators bathocuprione (50 μM) and neocuprione (5 μM). Cell death was also prevented by 100 μM L-*N*-methylarginine (L-N MA) or 100 μM L-nitroarginine methyl ester (L-NAME). Addition of 20 μM DETA-NONOate reversed the protection by NOS inhibition. MnTBAP (100 μM) prevented cell death by Zn-deficient SOD. Values are the mean ± SD from three independent experiments performed in duplicate.

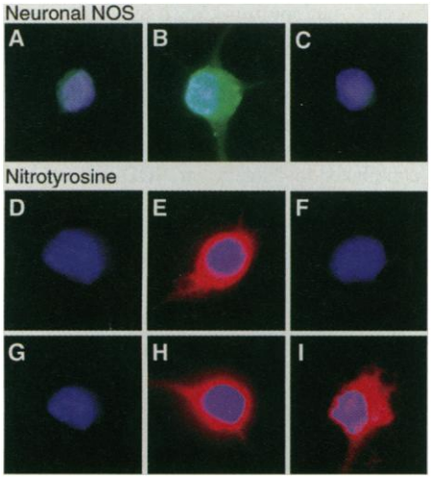


**Fig. 4.** Induction of neuronal NOS (**A** to **C**) and accumulation of nitrotyrosine (**D** to **I**) in motor neurons cultured with BDNF after treatment with liposomes containing different types of SOD. Liposomes contained (A and D) Cu,Zn WT SOD, (B and E) Zn-deficient WT SOD, (C) empty liposomes, (F) Zn-deficient WT SOD (plus 100 μM L-NAME added to the medium), (G and H) Cu,Zn and Zn-deficient L38V SOD, respectively, and (I) Zn-deficient D124N SOD. Nuclei were stained with DAPI (4′,6′-diamidino-2-phenylindole) in all sections (blue), whereas neuronal NOS and nitrotyrosine were stained with fluorescein- (green) and Cy3- (red) labeled secondary antibodies, respectively. Immunofluorescence staining was performed as described (*15*). Dilutions of polyclonal antibodies were 1:400 for neuronal NOS and 1:500 for nitrotyrosine (*21*). Images were captured electronically under identical conditions.

concentrations of NO (<100 nM) with an extracellular NO donor overcame this protection (Fig. 3B). Exogenous NO did not alter survival of motor neurons treated with Cu,Zn SOD liposomes. The superoxide and peroxynitrite scavenger, MnTBAP [manganese(III) 5,10,15,20-tetrakis) (4-benzoic acid)posphycin] (*14*) also prevented apoptosis induced by Zn-deficient SOD. Trophic factor–deprived motor neurons induce neuronal NOS expression and increase accumulation of nitrotyrosine, a footprint left by peroxynitrite (*15*). Motor neurons treated with Zn-deficient SOD in liposomes induced neuronal NOS and accumulated nitrotyrosine (Fig. 4). Both MnTBAP and NOS inhibition prevented the accumulation of nitrotyrosine as previously observed in motor neurons deprived of trophic factors (*15*). These results support a role for peroxynitrite formation in the induction of motor neuron apoptosis by Zn-deficient SOD. Nitrotyrosine has also been found in ALS-SOD transgenic mice as well as in sporadic and familial ALS patients (*16*).

In summary, failure to incorporate Zn into SOD can be sufficient to induce motor neuron death in culture by an oxidative mechanism involving NO. Whereas SOD mutants can generally fulfill the protective functions of SOD when replete with Zn, they may increase the risk of ALS because of their weaker affinity for Zn. Wild-type SOD could become toxic in sporadic ALS if either Zn uptake is disrupted or if a Zn chelator accumulates in motor neurons. One candidate Zn chelator is the structural protein, neurofilament L, which effectively competes with SOD for Zn in vitro and accumulates early in degenerating motor neurons (*6*). Deletion of the neurofilament L gene in mice profoundly delays paralysis in ALS-SOD mice (*17*). Our results suggest that inactivation of Zn-deficient SOD by Cu chelators may be therapeutic and offer the possibility that specific antioxidants such as MnTBAP could be beneficial in both familial and sporadic ALS. Common antioxidants such as ascorbate may not be effective because they can promote oxidant generation by Zn-deficient SOD.

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18. SOD were expressed as described (*6*) and biotinylated with EZ-Link NHS-Biotin (Pierce, Rockford, IL). Liposomes were prepared by extrusion with SOD at 20 mg/0.1 ml [M. Hope, M. Bally, G. Webb, P. Cullis, *Biochim. Biophys. Acta* **812**, 55 (1985); A. G. Estévez *et al.*, *Free Radical Biol. Med.*, in press]. Rat spinal motor neurons (200 cells per square centimeter) were cultured on polyornithine-laminin–coated 35-mm dishes in Neurobasal medium (*15*) and exposed to liposomes (50 nmol lipid per square centimeter). The number of motor neurons with neurites longer than four soma diameter treated with BDNF (100 pg/ml) at 24 hours was taken as 100% survival. Motor neuron death was prevented by caspase inhibitors (*15*) and showed nuclear condensation plus DNA fragmentation when visualized with the TUNEL (terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling) method, which together strongly suggests apoptosis.
19. Biotin-labeled SOD was detected 15 hours after adding liposomes. The cultures were fixed with 4% paraformaldehyde plus 0.1% glutaraldehyde in phosphate-buffered saline (PBS) for 20 min on ice. The slides were successively incubated with PBS, 50 mM lysine plus 0.1% Triton X-100 (pH 7.4), blocking solution [2% bovine serum albumin (BSA) plus 0.1% Triton X-100 in PBS], and fluorescein isothiocyanate–streptavidin (Life Technologies) at a 1:200 dilution in blocking solution. Images were captured with an Olympix cooled digital camera coupled to a IX-70 inverted Olympus microscope and processed identically with the Espirit software.
20. D124N is known to diminish Zn affinity [L. Banci *et al.*, *Eur. J. Biochem.* **196**, 123 (1991)], whereas D124Q occurs in ALS patients [C. E. Shaw *et al.*, *Ann. Neurol.* **43**, 390 (1998)]. Expression of D124N gave higher yields than ALS mutants, so it was used for many experiments before testing ALS-mutant SODs.
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[2] **Motor Neuron Degeneration in Mice That Express a Human Cu,Zn Superoxide Dismutase Mutation**

Mark E. Gurney; Haifeng Pu; Arlene Y. Chiu; Mauro C. Dal Canto; Cynthia Y. Polchow; Denise D. Alexander; Jan Caliendo; Afif Hentati; Young W. Kwon; Han-Xiang Deng; Wenje Chen; Ping Zhai; Robert L. Sufit; Teepu Siddique

*Science*, New Series, Vol. 264, No. 5166. (Jun. 17, 1994), pp. 1772-1775.

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[3] **Aggregation and Motor Neuron Toxicity of an ALS-Linked SOD1 Mutant Independent from Wild-Type SOD1**

Lucie I. Bruijn; Megan K. Houseweart; Shinsuke Kato; Karen L. Anderson; Scott D. Anderson; Eisaku Ohama; Andrew G. Reaume; Rick W. Scott; Don W. Cleveland

*Science*, New Series, Vol. 281, No. 5384. (Sep. 18, 1998), pp. 1851-1854.

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[5] **Mutations in Copper-Zinc Superoxide Dismutase that Cause Amyotrophic Lateral Sclerosis Alter the Zinc Binding Site and the Redox Behavior of the Protein**

Thomas J. Lyons; Hongbin Liu; Joy J. Goto; Aram Nersissian; James A. Roe; Janet A. Graden; Carla Cafe; Lisa M. Ellerby; Dale E. Bredesen; Edith Butler Gralla; Joan Selverstone Valentine

*Proceedings of the National Academy of Sciences of the United States of America*, Vol. 93, No. 22. (Oct. 29, 1996), pp. 12240-12244.

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[8] **Superoxide Dismutase is an Abundant Component in Cell Bodies, Dendrites, and Axons of Motor Neurons and in a Subset of Other Neurons**

Carlos A. Pardo; Zuoshang Xu; David R. Borchelt; Donald L. Price; Sangram S. Sisodia; Don W. Cleveland

*Proceedings of the National Academy of Sciences of the United States of America*, Vol. 92, No. 4. (Feb. 14, 1995), pp. 954-958.

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[8] **Undetectable Intracellular Free Copper: The Requirement of a Copper Chaperone for Superoxide Dismutase**

T. D. Rae; P. J. Schmidt; R. A. Pufahl; V. C. Culotta; T. V. O'Halloran

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[12] **Rapid Induction of Alzheimer Ab Amyloid Formation by Zinc**

Ashley I. Bush; Warren H. Pettingell; Gerd Multhaup; Marc d. Paradis; Jean-Paul Vonsattel; James F. Gusella; Konrad Beyreuther; Colin L. Masters; Rudolph E. Tanzi

*Science*, New Series, Vol. 265, No. 5177. (Sep. 2, 1994), pp. 1464-1467.

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[16] **Elevated Free Nitrotyrosine Levels, but not Protein-Bound Nitrotyrosine or Hydroxyl Radicals, throughout Amyotrophic Lateral Sclerosis (ALS)-Like Disease Implicate Tyrosine Nitration as an Aberrant in vivo Property of One Familial ALS-Linked Superoxide Dismutase 1 Mutant**

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[17] **Absence of Neurofilaments Reduces the Selective Vulnerability of Motor Neurons and Slows Disease Caused by a Familial Amyotrophic Lateral Sclerosis-Linked Superoxide Dismutase 1 Mutant**

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[17] **Protective Effect of Neurofilament Heavy Gene Overexpression in Motor Neuron Disease Induced by Mutant Superoxide Dismutase**

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