er development and longer mean adult life-span than the wild type. It appears therefore that mutations in the Clk genes have similar effects on development and on life-span as has lowered temperature.

Two groups of strains have a very distinct relation between adult life-span and the length of development: one group includes *gro-1 clk-2* and *clk-3*; *gro-1* double mutants and the second includes all strains containing either *daf-2* or *age-1*. At any given temperature, *gro-1 clk-2* and *clk-3*; *gro-1* double mutants have a much longer development and a shorter adult life-span than the wild type (Fig. 3). In contrast, all strains containing either *daf-2* or *age-1* have a much longer mean adult life-span, relative to development, than the wild type (Fig. 3). The *daf-2 clk-1* and *age-1 fer-15*; *gro-1* strains, which are part of the second group, display the effects of both of the life-extending mutations they contain. These strains show the typical lengthening of adult life-span due to *daf-2* or *age-1*, and in addition, *clk-1* or *gro-1* lengthen their development and increase their life-spans even further.

We propose that the Clk genes are involved in the general control of timing in *C. elegans*, perhaps constituting parts of a general physiological clock. Because, in Clk mutants, life appears to proceed at a slower pace, our results are consistent with the "rate of living" theory of aging (*1*, *16*). Conceivably, the slower rate of living of Clk mutants could be accompanied by a lowered rate of metabolism. However, the possibly slower metabolic rates of Clk mutants are probably not due to a defect in a key metabolic enzyme, but rather due to an altered control of metabolism. All Clk mutations are fully maternally rescued for almost all timing defects (*5*, *6*). This means that homozygous mutant progeny of a heterozygous mother have a nearly wild-type phenotype. It is unlikely that a gene coding for a key metabolic enzyme, required in even moderate amounts in every cell throughout life, could display this form of inheritance. This result, along with certain other aspects of the *clk-1* phenotype (*5*), suggests that the Clk genes are probably regulatory genes that may, among other things, control metabolic rate. Although how the slower rate of living seen in Clk mutants leads to a longer life-span is unknown, a reduced metabolic rate could lead to a slower production of reactive by-products of metabolism and, thus, to more gradual aging.

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18. We thank W. Lai for her expert help and T. Barnes and J. Ewbank for helpful discussion and for critical reading of the manuscript. Some nematode strains used in this work were provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR). This work was supported by a Medical Research Council of Canada grant to S.H. and by fellowships to B.L. from the J. W. McConnell Foundation and from the Fonds pour la Formation de Chercheuss et l'Aide à la Recherche (FCAR), Québec.

The Role of Zinc in Selective Neuronal Death After Transient Global Cerebral Ischemia

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Zinc is present in presynaptic nerve terminals throughout the mammalian central nervous system and likely serves as an endogenous signaling substance. However, excessive exposure to extracellular zinc can damage central neurons. After transient forebrain ischemia in rats, chelatable zinc accumulated specifically in degenerating neurons in the hippocampal hilus and CA1, as well as in the cerebral cortex, thalamus, striatum, and amygdala. This accumulation preceded neurodegeneration, which could be prevented by the intraventricular injection of a zinc chelating agent. The toxic influx of zinc may be a key mechanism underlying selective neuronal death after transient global ischemic insults.

Chelatable zinc (Zn^{2+}) is present in presynaptic vesicles of central excitatory neurons (*1*) and is released with synaptic activity or membrane depolarization (*2*). Al-

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though the precise role of synaptically released Zn^{2+} is not known, Zn^{2+} blocks currents mediated by N-methyl-D-aspartate (NMDA) (*3*, *4*) and by γ-aminobutyric acid (GABA) (*3*) as well as voltage-gated calcium channels (*5*). In addition, exposure to excessive extracellular Zn^{2+} is neurotoxic to cortical neurons; this toxicity may be mediated in part by Zn^{2+} influx through glutamate receptor- or voltage-gated Ca^{2+} channels (*6*). Presynaptic Zn^{2+} translocates into selectively vulnerable hippocampal hi-

lar neurons degenerating after transient forebrain ischemia (7) and into limbic or cortical neurons degenerating after brain seizures induced by kainate (8) or perforant path stimulation (9); hence, Zn^{2+} may play a role in the pathogenesis of neuronal degeneration in these conditions. However, whether such Zn^{2+} translocation is a cause or a marker of these neuronal deaths has not been established. We set out to answer three questions: Does Zn^{2+} influx into selectively vulnerable hippocampal neurons precede the neurodegeneration induced by transient ischemic insults? Does chelation

of extracellular Zn^{2+}, sufficient to prevent Zn^{2+} influx, reduce this neuronal death? Finally, can Zn^{2+} influx be implicated in the selective vulnerability of other brain neurons?

Staining of Long-Evans rat brains with the Zn^{2+}-specific fluorescent dye N-(6-methoxy-8-quinolyl)-$para$-toluenesulfonamide (TSQ) revealed dense fluorescence in the mossy fiber axon terminals of dentate granule neurons in the hilus and CA3 regions of the hippocampus, as well as in the stratum radiatum and stratum oriens of CA1 (10) (Fig. 1A). Neuronal or glial cell bodies

did not exhibit Zn^{2+} fluorescence. Twenty-four hours after a 10-min period of forebrain ischemia (11), Zn^{2+} fluorescence in presynaptic fibers was reduced, and new fluorescence appeared in some hilar neuronal cell bodies (7) (Fig. 1B). Subsequent acid fuchsin staining (12) on the same section revealed an essentially one-to-one correlation between neuronal degeneration and this apparent translocation of Zn^{2+} fluorescence (7) (Fig. 1C). Seventy-two hours after ischemia, Zn^{2+} fluorescence in presynaptic fibers had returned to normal, but selectively degenerating CA1 pyramidal neurons also displayed
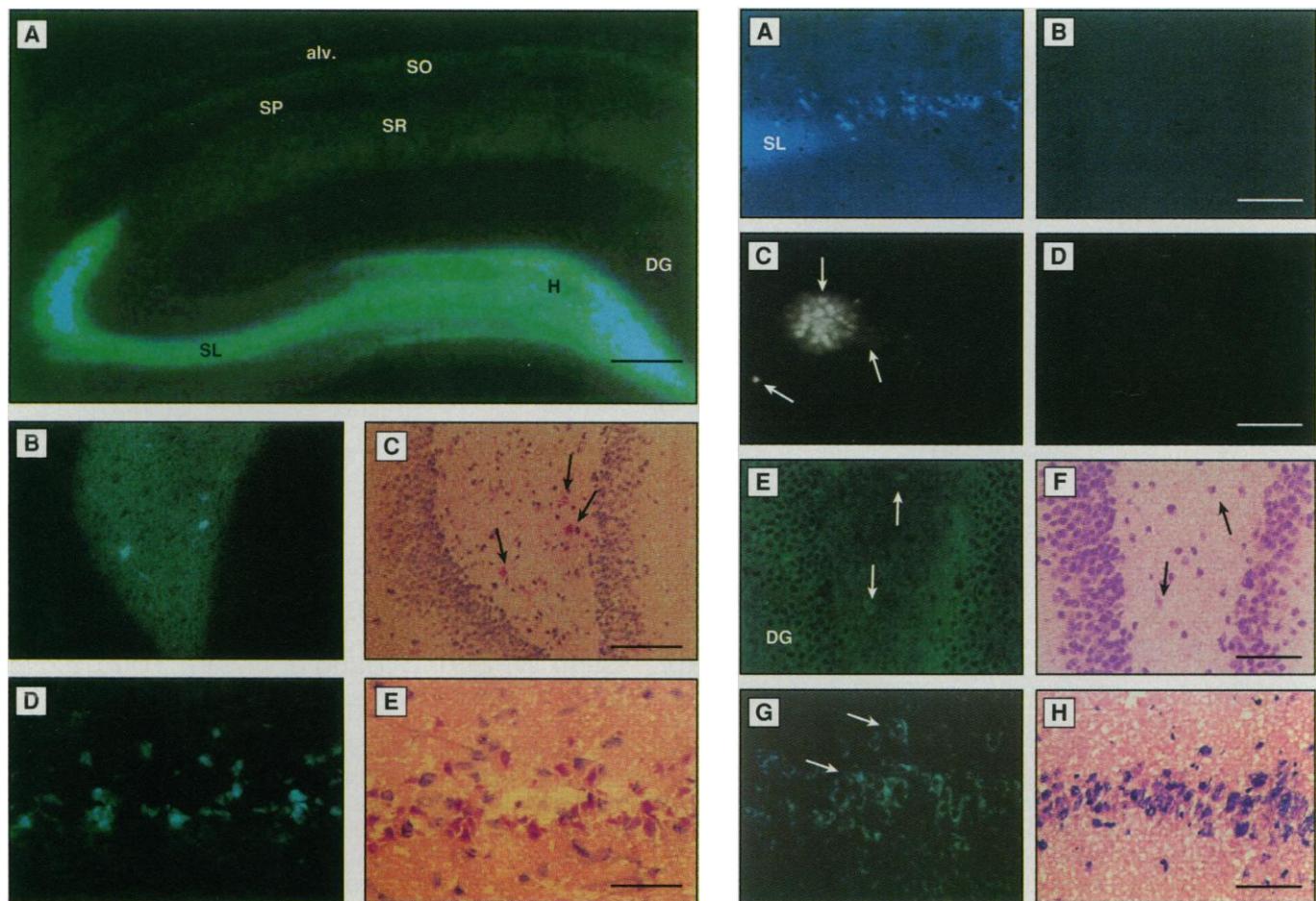
Fig. 1 (left). TSQ staining and neuronal degeneration in the hippocampus. (**A**) Fluorescent photomicrograph of normal rat hippocampus after staining with TSQ. showing dense fluorescence in the hilus (H) and the stratum lucidum (SL) of CA3. In addition, TSQ staining is seen in the stratum radiatum (SR) and the stratum oriens (SO) of CA1 (DG, dentate gyrus). No Zn^{2+} fluorescence is seen in the stratum pyramidale (SP) or alveus (alv.) of CA1 (dark bands). (**B**) Twenty-four hours after a 10-min period of forebrain ischemia, TSQ fluorescence is reduced in presynaptic terminals and is newly apparent in the cell bodies of some hilar neurons. (**C**) The same hippocampal section as in (B), with subsequent acid fuchsin staining. All the TSQ-fluorescent neurons in (C) exhibited ischemic acidophilic changes (pink cytoplasm, arrows). (**D**) Seventy-two hours after a 10-min period of ischemia, dense TSQ staining appeared in degenerating CA1 pyramidal neurons. (**E**) The same section as in (D), with acid fuchsin staining. All the CA1 neurons with Zn^{2+} fluorescence showed acidophilic changes. Scale bars, 800 μm (A); 200 μm (B and C); 100 μm (D and E). **Fig. 2 (right).** (**A** to **D**) Specificity of TSQ as a Zn^{2+} indicator, as shown by fluorescent photomicrographs. TSQ staining of adjacent hippocampal sections of a rat, 72 hours after a 10-min period of ischemia, without (A) or with

(B) pretreatment with dithizone (11), shows that dithizone treatment removed TSQ fluorescence from CA1 neuronal cell bodies as well as from the stratum lucidum (SL) of CA3. TSQ staining of cultured cortical neurons, degenerating after 24 hours of exposure to a combination of 1 μM AMPA and 20 μM Zn^{2+} (C) or 15 μM NMDA (D), shows the specificity of the appearance of TSQ fluorescence in Zn^{2+}-induced neuronal death. TSQ fluorescence was prominent in most cultured cortical neurons in (C) (arrows) but not in (D). (**E** to **H**) Zn^{2+} translocation precedes degenerative changes in the hippocampus. TSQ staining of the hippocampal hilus, 30 min after a 10-min period of ischemia (E), shows faint Zn^{2+} fluorescence in the cytosol of certain hilar neurons (arrows). In (F), the section shown in (E) was subsequently stained with acid fuchsin; the neurons with Zn^{2+} fluorescence did not yet exhibit acidophilic degeneration (arrows). TSQ staining of hippocampal CA1, 24 hours after a 10-min period of ischemia (G), shows Zn^{2+} fluorescence mostly in the cytoplasm and possibly some nuclei (arrows). In (H), another section from the same rat as in (G) was stained with acid fuchsin, which revealed that neurons did not yet exhibit acidophilic changes. Scale bars, 200 μm (A, B, E, and F); 100 μm (C, D, G, and H).

Zn^{2+} fluorescence (Fig. 1, D and E).

Exposure to dithizone, a specific cell-permeable Zn^{2+} chelator (1, 13), abolished TSQ fluorescence in the degenerating neurons (Fig. 2, A and B), which confirmed that this fluorescence reflected an increase in intracellular Zn^{2+}. TSQ fluorescence was prominent in cultured cortical neurons (14) degenerating after exposure to Zn^{2+} plus α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) (Fig. 2C) (6), but not after exposure to NMDA (Fig. 2D), ionomycin, Fe^{3+}, or staurosporine (15);
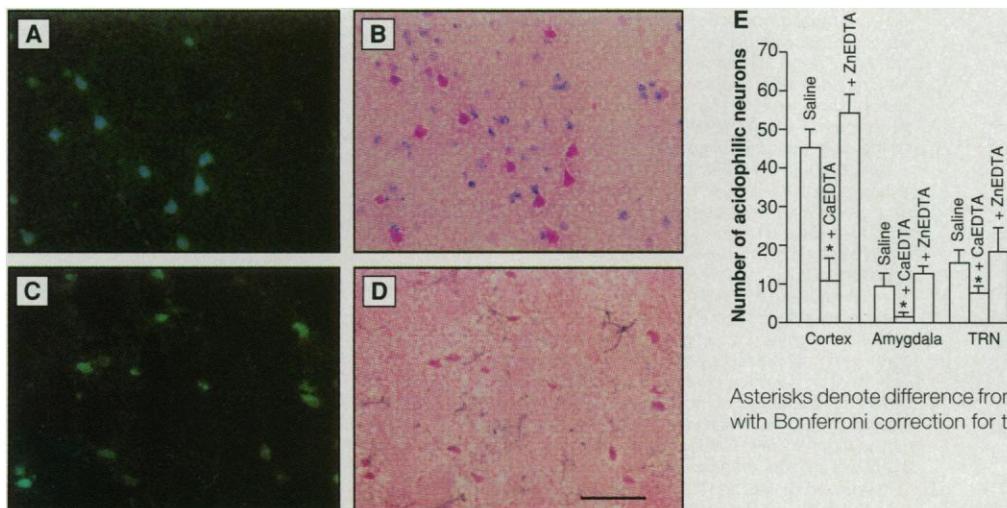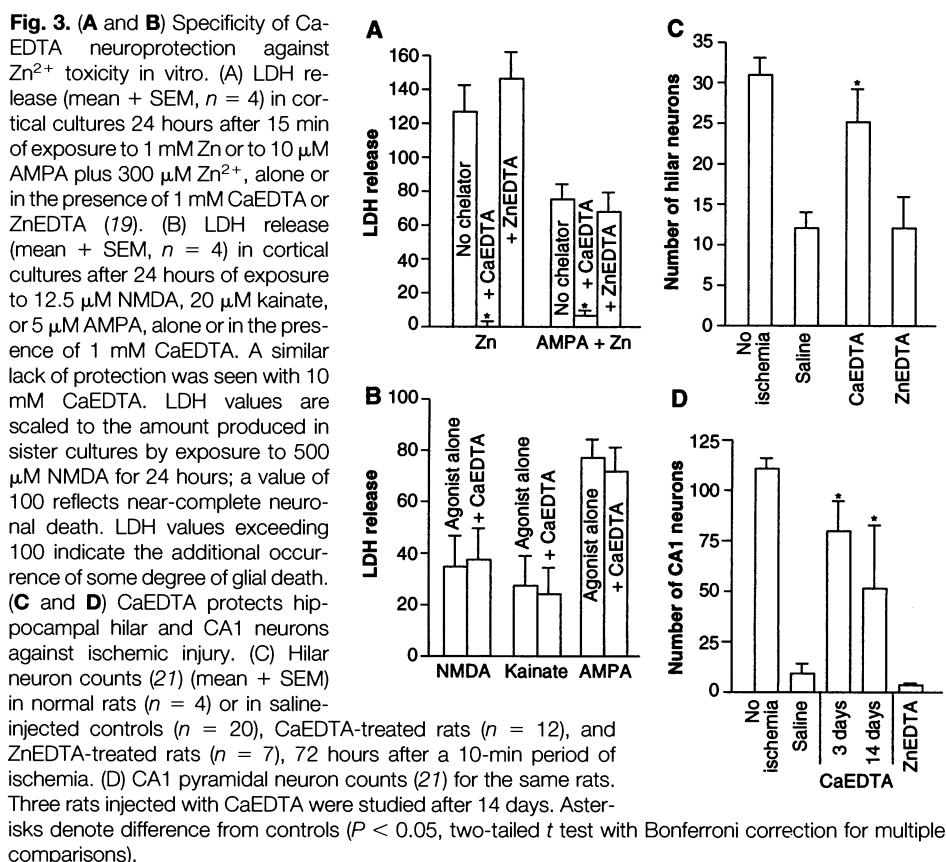
these findings suggest that Zn^{2+} accumulation was not a nonspecific accompaniment of cell death (for example, as a result of protein-bound Zn^{2+} released into the cytosol). Kainate-induced neuronal death in the cerebellum, where synaptic Zn^{2+} is not present, is not accompanied by the appearance of TSQ fluorescence (8).

To see whether Zn^{2+} influx preceded ischemic neurodegeneration, we studied brains 30 min after a 10-min period of forebrain ischemia. TSQ fluorescence was decreased in the neuropil and was already

apparent in certain hilar neuronal cell bodies (Fig. 2E). Acid fuchsin staining revealed that these fluorescent neurons did not yet exhibit degenerative changes (Fig. 2F). Similarly, Zn^{2+} fluorescence was already apparent in some CA1 pyramidal neuronal cell bodies 24 hours after ischemia (Fig. 2G), a time when these neurons remained morphologically and functionally intact (Fig. 2H) (16).

To block the influx of extracellular Zn^{2+} into postsynaptic neurons, we used the cell membrane–impermeant chelator disodium EDTA saturated with equimolar Ca^{2+} (CaEDTA). EDTA has a higher affinity for Ca^{2+} than for Mg^{2+}, but it has a much higher affinity for Zn^{2+} than for either Ca^{2+} or Mg^{2+} (17). Thus, CaEDTA binds extracellular Zn^{2+} without reducing extracellular Ca^{2+} (18) or meaningfully reducing extracellular Mg^{2+}. Addition of 1 mM CaEDTA to the bathing medium blocked the death of cultured cortical neurons induced by 15 min of exposure to either 1 mM Zn^{2+} or the synergistic combination of 300 μM Zn^{2+} and 5 μM AMPA (Fig. 3A) (19). In contrast, the non-Zn^{2+} chelator ZnEDTA (1 mM) showed no protective effect. CaEDTA did not reduce the Ca^{2+}-overload excitotoxicity induced by 24 hours of exposure in vitro to NMDA, kainate, or AMPA (Fig. 3B), nor did it reduce hippocampal neuronal death induced by NMDA injection in vivo (20). Although CaEDTA binds Fe^{2+} or Cu^{2+}, it does not reduce the neurotoxicity of these metals (20).

To examine whether Zn^{2+} influx was required for ischemic selective hippocampal neuronal death, we injected CaEDTA into the lateral ventricles 30 min before transient forebrain ischemia (11). Injection of CaEDTA, but not of ZnEDTA or saline, markedly reduced both Zn^{2+} influx into hilar or CA1 pyramidal neurons and the degeneration of these neurons after 72

Fig. 3. (A and **B)** Specificity of Ca-EDTA neuroprotection against Zn^{2+} toxicity in vitro. (A) LDH release (mean + SEM, $n = 4$) in cortical cultures 24 hours after 15 min of exposure to 1 mM Zn or to 10 μM AMPA plus 300 μM Zn^{2+}, alone or in the presence of 1 mM CaEDTA or ZnEDTA (19). (B) LDH release (mean + SEM, $n = 4$) in cortical cultures after 24 hours of exposure to 12.5 μM NMDA, 20 μM kainate, or 5 μM AMPA, alone or in the presence of 1 mM CaEDTA. A similar lack of protection was seen with 10 mM CaEDTA. LDH values are scaled to the amount produced in sister cultures by exposure to 500 μM NMDA for 24 hours; a value of 100 reflects near-complete neuronal death. LDH values exceeding 100 indicate the additional occurrence of some degree of glial death. **(C** and **D)** CaEDTA protects hippocampal hilar and CA1 neurons against ischemic injury. (C) Hilar neuron counts (21) (mean + SEM) in normal rats ($n = 4$) or in saline-injected controls ($n = 20$), CaEDTA-treated rats ($n = 12$), and ZnEDTA-treated rats ($n = 7$), 72 hours after a 10-min period of ischemia. (D) CA1 pyramidal neuron counts (21) for the same rats. Three rats injected with CaEDTA were studied after 14 days. Asterisks denote difference from controls ($P < 0.05$, two-tailed t test with Bonferroni correction for multiple comparisons).

Fig. 4. Other brain areas. **(A)** TSQ staining of parietal cortex (layer V), 72 hours after a 10-min period of ischemia in saline-injected control rats. **(B)** The same section as in (A) after acid fuchsin staining. **(C)** Thalamic reticular nucleus with TSQ staining, 3 hours after a 10-min period of ischemia. **(D)** The same section as in (C) after acid fuchsin staining. **(E)** Numbers of neurons that stained with acid fuchsin (mean + SEM, $n = 7$ to 20) in a 500 μm by 500 μm area of perirhinal cortex, amygdala, and thalamic reticular nucleus (TRN), 72 hours after a 10-min period of ischemia, in rats injected with saline (control), CaEDTA, or ZnEDTA. Asterisks denote difference from respective controls ($P < 0.05$, two-tailed t test with Bonferroni correction for two comparisons). Scale bar, 100 μm.

hours (Fig. 3, C and D) (*21*).

Neurons in certain brain areas outside the hippocampus—the thalamic reticular nucleus, amygdala, striatum, and neocortical layers II, III, and V—also selectively degenerate after transient global ischemia (*11, 16, 22*). Seventy-two hours after ischemia, degenerating neurons in the neocortex, thalamic reticular nucleus, amygdala, and striatum all exhibited TSQ fluorescence (Fig. 4, A to D) (*23*). Intraventricular injection of CaEDTA reduced neuronal degeneration in all regions (Fig. 4E).

Our observations suggest that the classical phenomenon of selective neuronal death in several brain regions after transient brain ischemia may be mediated substantially by the toxic transsynaptic movement of Zn^{2+} from presynaptic terminals into postsynaptic neurons (*6–8*). Causality is supported by three arguments. First, intracellular Zn^{2+} accumulation within degenerating neurons was specific to neuronal death induced by Zn^{2+} influx. A one-to-one correlation between Zn^{2+} accumulation and ischemic neuronal degeneration was observed in vivo, whereas neuronal deaths induced in vitro by excitotoxicity, a Ca^{2+} ionophore, Fe^{3+}, or even staurosporine (which induces apoptosis) (*14*) were not associated with intracellular Zn^{2+} accumulation. Second, intracellular Zn^{2+} accumulation preceded neuronal degeneration. Third and most important, Zn^{2+} chelation reduced ischemic neuronal degeneration.

CaEDTA is not specific for Zn^{2+} and can also chelate endogenous copper and iron (*18*). However, cytoprotective chelation of these other endogenous metals can be excluded. ZnEDTA, which chelates Cu^{2+}, did not reduce ischemic neuronal death, and EDTA chelation did not block Cu^{2+} neurotoxicity (*20*). Similarly, EDTA chelation of iron does not limit its toxic participation in radical generation through the Fenton reaction (*24*), and Fe^{3+}-induced neuronal death was not attenuated by CaEDTA (*20*).

The idea that Zn^{2+} toxicity contributes to ischemic selective neuronal death does not necessarily conflict with other studies that implicate excitotoxicity in this death (*25*). Both processes might be required to induce lethal injury. It is even possible that these processes are intertwined; the neuroprotective effect of glutamate antagonists in transient global ischemia may be attributable in part to reduced Zn^{2+} influx through NMDA receptor–gated or voltage-gated Ca^{2+} channels (*6*). Further study will be required to delineate the mechanisms underlying Zn^{2+}-induced neuronal death. Zn^{2+} reacts with the thiol and imidazole moieties of many proteins and could produce widespread alterations in cell biology, including disruption of tubulin assembly or

overactivation of Ca^{2+}-activated enzymes such as protein kinase C (*26*). In any case, countermeasures specifically aimed at reducing toxic Zn^{2+} influx might constitute a useful strategy for protecting brain neurons against global ischemic insults.

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20. CaEDTA (1 mM) did not reduce the death of cultured cortical neurons induced by 48 hours of exposure to 200 μM Cu^{2+} or 100 μM Fe^{3+}. NMDA (150 nmol) was injected into the hippocampus alone or with 100 mM CaEDTA in 0.5 μl of phosphate-buffered saline. CaEDTA did not reduce this NMDA-induced neuronal death in vivo.
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23. In all brain areas examined, there was a one-to-one correlation between Zn^{2+} fluorescence and selective neuronal degeneration. Examination of thalamic reticular nucleus at early time points revealed that neuronal TSQ fluorescence was already present 30 min after ischemia, before the onset of neurodegenerative changes.
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