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Zinc Selectively Blocks the Action of *N*-Methyl-D-Aspartate on Cortical Neurons

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Large amounts of zinc are present in synaptic vesicles of mammalian central excitatory boutons and may be released during synaptic activity, but the functional significance of the metal for excitatory neurotransmission is currently unknown. Zinc (10 to 1000 micromolar) was found to have little intrinsic membrane effect on cortical neurons, but invariably produced a zinc concentration–dependent, rapid-onset, reversible, and selective attenuation of the membrane responses to *N*-methyl-D-aspartate, homocysteate, or quinolinate. In contrast, zinc generally potentiated the membrane responses to quisqualate or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate and often did not affect the response to kainate. Zinc also attenuated *N*-methyl-D-aspartate receptor–mediated neurotoxicity but not quisqualate or kainate neurotoxicity. The ability of zinc to specifically modulate postsynaptic neuronal responses to excitatory amino acid transmitters, reducing *N*-methyl-D-aspartate receptor–mediated excitation while often increasing quisqualate receptor–mediated excitation, is proposed to underlie its normal function at central excitatory synapses and furthermore could be relevant to neuronal cell loss in certain disease states.

A GROWING BODY OF EVIDENCE SUGgests that the transition metal zinc, a nutritive requirement with a widespread essential role in both plant and animal metabolism (*1*), may serve a specific signaling function in mammalian central excitatory neurotransmission. Chelatable Zn is present in high concentrations throughout mammalian brain, particularly in neocortical gray matter, pineal, and hippocampus (*2*). Ultrastructural studies have suggested that forebrain Zn is localized to synaptic vesicles in excitatory boutons (*3*). In the hippocampus, Zn is specifically localized in the terminals of the excitatory mossy fiber projection (*4*). Endogenous Zn is spontaneously released into the synaptic cleft (*5*), and a calcium-dependent increase in rate of Zn release can be evoked by electrical stimulation (*6*) or exposure to large amounts of potassium (*7*).

The hypothesis that synaptically released Zn participates in central excitatory neurotransmission is supported by several electrophysiological studies linking alterations in Zn availability to changes in neuronal circuit behavior. Zn application increases the firing of some cortical neurons (*8*) and prolongs the excitatory synaptic potential in olfactory cortical neurons (*9*). In addition, chelation of Zn with diethyldithiocarbamate (*10*) or dietary Zn depletion (*11*) has been reported to produce alterations in mossy fiber transmission.

However, evidence for an appropriate direct membrane action of Zn specific to excitatory synapses has been sparse. Previously described effects of Zn on neuronal membranes, including attenuation of voltage-dependent sodium (*12*) or calcium (*13*) conductances, and potentiation of postsynaptic γ-aminobutyric acid responses (*9*), represent possible avenues of Zn action but lack specificity to excitatory synapses. Most central excitatory synapses, including in particular the hippocampal mossy fiber connections, are likely mediated by the action of glutamate, or related compounds, on perhaps three subclasses of receptors, each defined by a specific agonist: *N*-methyl-D-aspartate (NMDA), quisqualate (Quis), and kainate (Kain) (*14*). We investigated the effect of Zn on the responses to each of these agonists in a cell culture system.

Dissociated mouse cortical cell cultures were prepared and maintained generally as previously described (*15, 16*). The only source of Zn in the culture medium was the serum (Hyclone, defined grade), resulting in an estimated Zn concentration of less than 2 μ*M* in the maintenance medium. Cultures between 15 and 24 days in vitro were mounted on the heated (35°C) stage of an inverted phase-contrast microscope and perfused continuously with a defined, Zn-free recording medium (*p*H 7.3) containing the following (in millimoles per liter): Na, 130; K, 5.4; Ca, 4; Cl, 143; glucose, 15; and Hepes, 10 (salts were from Baker, analyzed reagent grade). Magnesium was omitted from the medium (except as noted below) to facilitate study of responses mediated by the NMDA receptor (*17*); tetrodotoxin (1 μ*M*) was added routinely to reduce interference from spontaneous synaptic activity. Intracellular recordings were made from directly visualized neurons with the use of 4*M* potassium acetate–filled microelectrodes (40 to 100 megohms); current was passed through the recording electrode by means of a standard bridge circuit. Only neurons with stable resting potentials greater than −50 mV and an input time constant permitting unequivocal balancing of the bridge circuit were included in the study.

Pressure ejection (*18*) of 25 to 100 μ*M* NMDA, 100 to 200 μ*M* Kain, or 10 to 50 μ*M* Quis onto cortical neurons reliably produced depolarizing responses associated with increases in membrane conductance (Fig. 1A). Consistent with other data (*19*), this NMDA response could be selectively blocked by 100 to 1000 μ*M* 2-amino-5-phosphonovalerate (*16, 20*). Delivery of 100 μ*M* to 1 m*M* Zn alone (as $ZnCl_2$) generally produced no change in membrane potential or conductance (Fig. 1A), although occasionally (four times in 21 trials in one series), a small 2- to 5-mV transient hyperpolarization was noted (Fig. 3D).

Pressure ejection of 500 μ*M* to 1 m*M* Zn for several seconds always reversibly attenuated (16 of 16 cells) and sometimes completely blocked (8 of 16 cells) the membrane depolarization and conductance increase produced by subsequent application of NMDA (Fig. 1A), resulting in a mean reduction of 85% ± 4% (SEM) in the baseline NMDA depolarization amplitude (Fig. 1B). This attenuation was highly selective for NMDA responses. The same concentrations of Zn reversibly potentiated the response to Quis in most cells tested (17 of 21) (Fig. 1A) while having no effect in a few (4 of 21) cells tested; the mean increase in Quis depolarization amplitude was 26% ± 4% (Fig. 1B). Some potentiation in

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the Quis response was also produced in five of five cells by 100 μ*M* Zn; no potentiation was seen with pressure ejection of recording medium alone. Zn (500 μ*M* to 1 m*M*) had a variable effect on Kain responses, often producing no change (6 of 12 cells) (Fig. 1A) but occasionally increasing (2 of 12) or decreasing (4 of 12) those responses; virtually no change in the mean kainate depolarization was seen (Fig. 1B).

The dose-response relation for Zn depression of NMDA responses was determined by comparing NMDA depolarization amplitudes before (control) and immediately after application of varying concentrations of Zn on a given cortical neuron. A small attenuation of NMDA responses was observed with 10 μ*M* Zn, and a near total abolition of NMDA responses occurred with 1 m*M* Zn; the half-maximal inhibitory concentration (IC_{50}) for this action was 100 μ*M* (Fig. 2). A Hill plot of the dose-response data revealed a slope of 1.2 (inset to Fig. 2).

In three to five other cells each, 1 m*M* Zn also reversibly attenuated the membrane responses produced by the endogenous NMDA agonists, 3 m*M* quinolinate (Quin) (*21*) or 100 μ*M* L-homocysteate (HCA) (*22*), while potentiating the response to 100 μ*M* α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), a putative quisqualate agonist (*23*) (Fig. 3A).

The ability of Zn to attenuate NMDA receptor–mediated neuroexcitation was reminiscent of the recently described action of several other divalent cations, including physiological concentrations of Mg, to produce a voltage-dependent block of NMDA-activated channels (*17*). In fact, 500 to 1000 μ*M* Mg (as $MgCl_2$) could grossly mimic the ability of Zn to attenuate the subsequent response to NMDA (Fig. 3B); a 45% ± 12% (SEM) reduction in NMDA responses was seen in a series of four cells. However, Mg did not mimic the ability of Zn to potentiate Quis responses (Fig. 3B) (15 cells). Furthermore, a more detailed comparison of Mg and Zn blockade of NMDA responses at different membrane potentials revealed a difference in the voltage dependence of the two blockades. In the absence of both Mg and Zn, NMDA depolarization amplitudes increased steadily with membrane hyperpolarization, likely reflecting the increased driving force for inward cation movement at progressive negative potentials (Fig. 3C, left). When 1 m*M* Mg was coejected with NMDA, the response amplitudes increased with membrane hyperpolarization only above approximately −50 mV; below that potential, response amplitudes paradoxically decreased with further membrane hyperpolarization (*17*) (Fig. 3C, middle). However, when 1 m*M* Zn was coejected with NMDA, this paradoxical decrement was not seen, although responses often tended to plateau above approximately −50 to −60 mV (Fig. 3C, right).

It was experimentally convenient to obtain NMDA responses in a Mg-free recording solution, but some NMDA responses could be obtained in the presence of 1 m*M* Mg (added to the bathing solution and all pressure ejection pipettes). The presence of this physiological concentration of Mg did not abolish the above described effects of Zn on excitatory amino acid responses. Zn at 500 μ*M* still reduced the amplitude of depolarizing responses to 200 to 500 μ*M* NMDA in seven of seven neurons tested (mean decrease 70% ± 3%) and increased the amplitude of Quis depolarizations in seven of eleven neurons (mean increase 14% ± 5%) (Fig. 3D). Zn at 100 μ*M* had minimal effect on Quis responses but still reduced NMDA responses in five of seven neurons (mean decrease 31% ± 10%).

NMDA, Quis, and Kain are all neurotoxic to central neurons and likely manifest their neurotoxic properties through activation of their respective receptor subtypes. Thus, we next attempted to determine whether Zn exerted the same selectivity in altering agonist-induced toxicity as in altering neuroexcitation.

Cultures were exposed for 5 minutes to 500 μ*M* NMDA, 500 μ*M* Quis, or 5 m*M* Kain in a tris-buffered physiological salt solution (containing 0.8 m*M* Mg) as previously described (*15*), with or without the addition of 500 μ*M* $ZnCl_2$. This exposure to Zn was found in previous experiments to be below the threshold needed to produce neurotoxic injury by itself (*24*) (Fig. 4). Exposure to NMDA was followed within minutes by neuronal swelling easily detectable under phase-contrast microscopy and, after 16 to 24 hours, by widespread neuronal disintegration (Fig. 4). Despite its potential intrinsic neurotoxicity, addition of 500 μ*M* Zn to the exposure solution blocked both the acute neuronal swelling

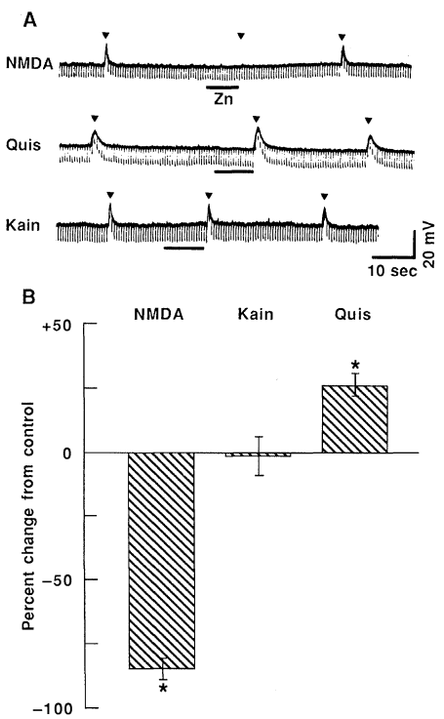

Fig. 1. Selectivity of Zn on responses at the three putative excitatory amino acid receptor subtypes. (**A**) Representative intracellular recordings from three cortical neurons show agonist responses (arrowheads) elicited by pressure ejection before, immediately after, and approximately 30 seconds after pressure ejection of Zn (horizontal bar). Downward deflections at 1-second intervals were produced by injection of constant hyperpolarizing current pulses through the recording electrode; the size of the deflections reflects (inversely) cell membrane conductance. Recording medium was used as the carrier in all pressure ejection pipettes. The response to 100 μ*M* NMDA [resting potential (RP), −52 mV] was reversibly abolished by 500 μ*M* Zn, whereas the response to 20 μ*M* Quis (RP, −62 mV) was reversibly augmented by 500 μ*M* Zn, and the response to 100 μM Kain (RP, −60 mV) was unchanged by 1 m*M* Zn. (**B**) Percentage change (mean ± SEM) in the baseline response to each agonist immediately after pressure ejection of 500 μ*M* to 1 m*M* Zn. The experimental paradigm was the same as in (A) above. The number of cells tested was NMDA, 16; Kain, 12; and Quis, 21. The asterisk indicates a significant difference ($P < 0.01$) compared with control (Wilcoxon signed rank test, two-tailed).

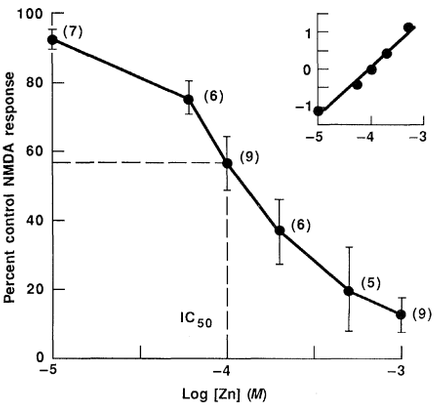

Fig. 2. Log concentration-response relation for blockade of NMDA depolarization by Zn. For each cell tested, the depolarization amplitude produced by 100 μ*M* NMDA immediately after Zn ejection was expressed as a percentage of the control NMDA depolarization in the same cell; the plot shows the mean percentage ± SEM (number of cells in parentheses) at each Zn concentration. Assuming that 1 m*M* Zn produced the maximum attenuation of NMDA depolarization (to 13% of control), the IC_{50} of Zn is 100 μ*M*. Inset: Hill plot of the same data, in which abscissa is log[Zn] and the ordinate is $\log[E/E_{max} - E]$, where E is the mean percentage reduction in the control NMDA response and E_{max} is the reduction produced by 1 m*M* Zn. The line, fitted by linear regression, has a slope of 1.2.

and the late cell death produced by NMDA (Fig. 4). Zn had no protective effect on the neurotoxicity produced by exposure to 500 μM Quis or 5 mM Kain (Fig. 4). In another experiment, 500 μM Zn also blocked the neurotoxicity produced by exposure to 100 μM HCA for 5 minutes: lactate dehydrogenase (LDH) release (*25, 26*) was 125 ± 6 unit/ml (SEM, $n = 5$; mean background of 35 unit/ml subtracted) in cultures exposed to HCA alone, but only 23 ± 10 unit/ml ($n = 5$) in cultures exposed to HCA in the presence of Zn (different at $P < 0.01$; two-tailed t test).

This report describes the novel finding that Zn produces a rapid, reversible, and selective blockade of the membrane depolarization and conductance increase produced by NMDA on cultured cortical neurons. Since Zn also blocks neuronal excitation mediated by the endogenous NMDA agonists HCA and Quin, but not by the non-NMDA agonists Quis, AMPA, or Kain, this blockade likely reflects a postsynaptic action of Zn at the NMDA receptor or channel. The observed Hill slope of 1.2 further suggests that such a postsynaptic action does not require cooperativity between multiple Zn ions.

Zn could act at the NMDA receptor to reduce its affinity for agonist, or the efficiency of coupling to its channel—whether that channel is a distinct molecular entity or a functional substate of a single glutamate channel complex (*27*). Alternatively, Zn could act directly at the channel, perhaps obstructing it in a fashion similar to the action of Mg, but permeating sufficiently at hyperpolarized membrane potentials (with subsequent relief of block) to account for the observed absence of a negative NMDA slope conductance (*28*). Additional study will be required to distinguish among these possibilities.

Pressure ejection of only 100 μM Zn substantially reduced a subsequent NMDA response, whether or not Mg was also present; the effective Zn concentration produced by this pulse was likely less than 100 μM, since some dilution by the subsequent agonist pulse must occur. Such concentrations of Zn are probably attainable in vivo with synaptic release of endogenous stores. Assaf and Chung (*7*) calculated that the Zn released upon excitation of hippocampal slices by 24 mM potassium could produce a uniform extracellular Zn concentration of 300 μM; localization of released Zn to the synaptic zone could result in peak concentrations well above that amount.

We propose that the ability of Zn to specifically modulate the postsynaptic response to excitatory amino acid transmitters underlies its normal function at central excitatory synapses. Corelease of endogenous Zn with glutamate would be expected to reduce the resultant proportion of NMDA channels relative to Quis or Kain channels activated by that broad-spectrum agonist. Since NMDA channels have special properties—including high Ca permeability (*29*), activation by membrane depolarization (due to relief of Mg block; see above), and triggering of Ca spikes (*30*)—this reduction could serve to dynamically alter the nature of excitatory neurotransmission. A buildup of extracellular Zn with repetitive excitatory synaptic activity, for example, might down-regulate NMDA channel activation, countering the Mg-induced tendency of NMDA channels to amplify depolarizing change,

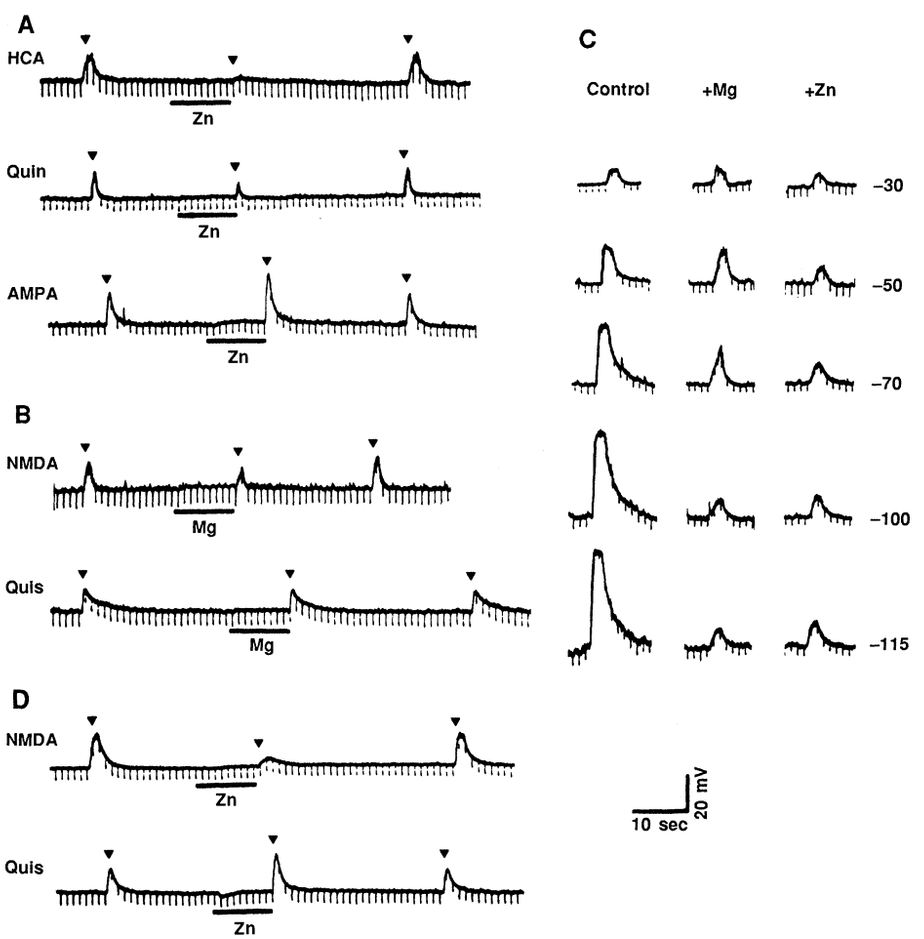

Fig. 3. Comparison of Zn and Mg on excitatory amino acid responses. (**A**) Intracellular recordings show the effect of 1 mM Zn (horizontal bars) on responses to 100 μM HCA (RP, −63 mV), 3 mM Quin (RP, −66 mV), and 100 μM AMPA (RP, −59 mV) (arrowheads). High Quin concentrations were required to produce reproducible responses (*16*). (**B**) Effect of 1 mM Mg (horizontal bars) on responses to 25 μM NMDA (RP, −70 mV) and 20 μM Quis (RP, −70 mV). Mg produced reversible attenuation of the NMDA depolarization but did not affect the Quis depolarization. (**C**) Voltage dependence of Zn and Mg attenuation of NMDA responses compared on a single neuron. The bathing solution contained neither Zn nor Mg. Responses to pressure ejection of 50 μM NMDA alone (no Mg or Zn) (Control), 100 μM NMDA + 1 mM Mg but no Zn (+Mg), and 100 μM NMDA + 1 mM Zn but no Mg (+Zn) were elicited at the indicated membrane potentials. The membrane was polarized by passage of steady current through the recording electrode, while bridge balance in relation to a superimposed current pulse was continuously monitored. To improve "space clamp," the intracellular electrodes were filled with 2M cesium acetate instead of potassium acetate, and NMDA was ejected near the cell body (and thus electrically close to the intracellular electrode). Each puffer pipette was tested in turn, with the two others removed away to minimize the possibility of any cross-contamination (for example, through introduction of Mg during the Zn trial). The responses shown for NMDA alone (Control) were obtained last, confirming adequate membrane voltage control even during the latter part of the recording. Control experiments demonstrated that 1 mM Zn alone had no intrinsic effect on membrane potential or conductance over the range −36 mV to −115 mV (except for occasional small hyperpolarizations possibly related to pressure ejection). (**D**) Effect of 500 μM Zn (horizontal bars) on responses to 400 μM NMDA (RP, −63 mV) and 20 μM Quis (RP, −65 mV) in the presence of 1 mM Mg in the recording medium (and in all pressure ejection pipettes). A larger NMDA concentration was chosen to overcome the partial blockade of NMDA responses produced by Mg.

and could thus provide an important negative-feedback brake on synaptic excitation. Reduction of Ca entry through NMDA channels could account for the recent observation that Zn reduces paired-pulse potentiation in rat hippocampal mossy fibers (*31*). A possible role for Zn in regulating lasting synaptic changes such as long-term potentiation, a phenomenon putatively linked to learning, which may be mediated by NMDA receptors (*32*), is an intriguing subject for future investigation.

Furthermore, endogenous Zn might serve a protective function, preventing normal levels of excitatory synaptic activity from becoming neurotoxic. A defect leading to reduced Zn release at excitatory synapses could lead to gradual NMDA receptor–mediated neuronal death—for example, producing the special pattern of neuronal loss seen in Huntington's disease (*16, 33*). Maneuvers specifically directed toward enhancing synaptic Zn release might provide a new therapeutic approach to reducing NMDA receptor-mediated brain injury (*34*) or seizures (*35*).

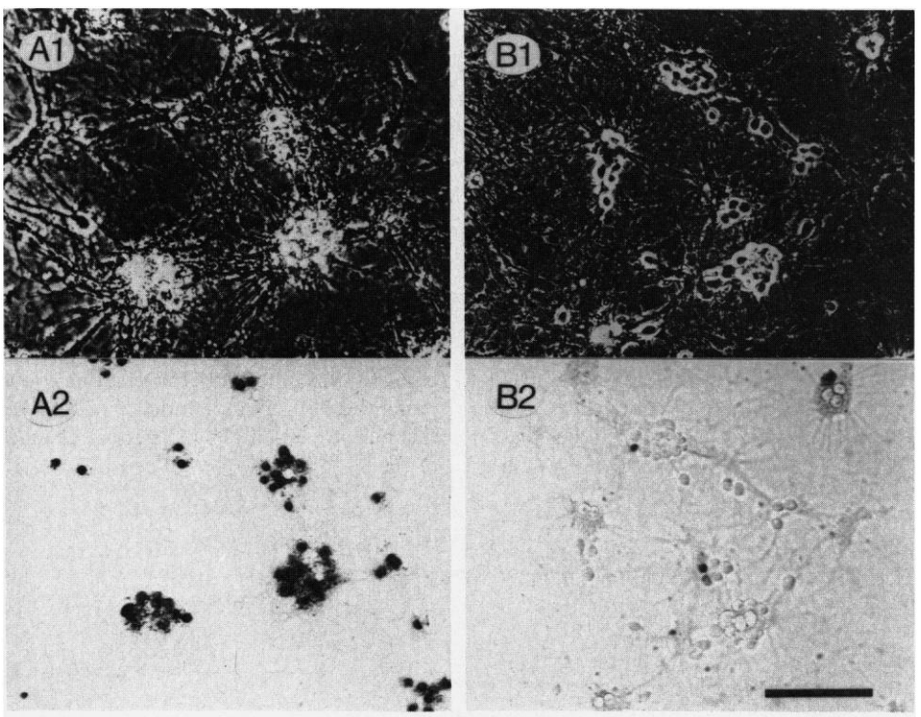

Fig. 4. Blockade of NMDA neurotoxicity by Zn. (**A1** and **B1**) Phase-contrast and (after a 5-minute incubation in 0.4% trypan blue dye) (**A2** and **B2**) bright-field micrographs of representative fields in matched sister cultures, taken 24 hours after exposure to 500 μ*M* NMDA for 5 minutes, either alone (A1 and A2) or in the presence of 500 μ*M* Zn (B1 and B2). Scale bar, 100 μm. (**C**) Neuronal injury or loss was accompanied by the efflux of LDH activity to the bathing medium, an easily measured index (*25*) that correlates quantitatively with the extent of excitatory amino acid–induced neuronal cell damage (*26*). Bars depict mean + SEM ($n = 5$ or 6) LDH concentration (in conventional units per milliliter) in the medium 20 hours after a 5-minute exposure to the indicated agonist, either without (open bars) or with (shaded bars) 500 μ*M* Zn; control bars represent the background LDH efflux found in wash controls (no agonist). Cultures exposed to 500 μ*M* NMDA showed a large LDH efflux, reflecting extensive neuronal damage, which was decreased to near background level by the addition of Zn. In contrast, the neurotoxicity induced by 500 μ*M* Quis or 5 m*M* Kain was not decreased by Zn. A high concentration of Kain was necessary to produce detectable neuronal damage, although the extent of damage with Kain was still considerably less than with the lower concentrations of NMDA or Quis used. A 5-minute exposure to 500 μ*M* Zn alone in the control condition (shaded bar) was not neurotoxic. The asterisk indicates a significant effect of Zn ($P < 0.01$) compared with the same neurotoxin in the absence of Zn (two-tailed *t* test with Bonferroni correction for multiple comparisons).

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A Chicken Transferrin Gene in Transgenic Mice Escapes X-Chromosome Inactivation

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Mammalian X-chromosome inactivation involves a coordinate shutting down of physically linked genes. Several proposed models require the presence of specific sequences near genes to permit the spread of inactivation into these regions. If such models are correct, one might predict that heterologous genes transferred onto the X chromosome might lack the appropriate signal sequences and therefore escape inactivation. To determine whether a foreign gene inserted into the X chromosome is subject to inactivation, transgenic mice harboring 11 copies of the complete, 17-kilobase chicken transferrin gene on the X chromosome were used. Male mice hemizygous for this insert were bred with females bearing Searle's translocation, an X-chromosome rearrangement that is always active in heterozygous females (the unrearranged X chromosome is inactive). Female offspring bearing the Searle's translocation and the chicken transferrin gene had the same amount of chicken transferrin messenger RNA in liver as did transgenic male mice or transgenic female mice lacking the Searle's chromosome. This result shows that the inserted gene is not subject to X-chromosome inactivation and suggests that the inactivation process cannot spread over 187 kilobases of DNA in the absence of specific signal sequences required for inactivation.

X-CHROMOSOME INACTIVATION IN mammalian females represents a unique kind of gene regulation in two respects. (i) The active and inactive states of each gene are maintained in the same nucleus, in contrast to the typical tissue-specific genes in which both copies of a gene are either active or inactive. (ii) The phenomenon involves not a battery of dispersed genes but most of the genes of a chromosome. X-chromosome inactivation (XCI) probably is initiated at a single site on the chromosome (*1*). The inactive state then spreads along the chromosome, encompassing all but the limited X-Y pairing and recombination region. In X-autosome translocations, the inactive state may spread into the autosome, may fail to do so, or may "skip" proximal autosomal regions while spreading into more distal ones (*2*).

A key question in our understanding of the XCI process is whether specific regulatory DNA sequences are required to render certain chromosomal regions susceptible to XCI and other regions immune. We report here a study of XCI in transgenic mice having the complete chicken transferrin gene, including 2.2 kb of 5′ and 3.7 kb of 3′ flanking sequence, on the X chromosome. McKnight *et al.* described the preparation of these mice and showed that 11 copies of the chicken transferrin gene were located on the X chromosome and were preferentially expressed in liver (*3*). We used complementary DNA (cDNA) solution hybridization techniques (Table 1) to determine the relative expression of the chicken transferrin gene in hemizygous males and in heterozygous females. If the transferrin insert were subject to XCI, the livers of heterozygous females should be a mixture of cells having the transferrin gene on the active X and those having the gene on the inactive X, whereas all liver cells of a male would express the inserted gene. Thus heterozygous females should produce a lower overall quantity of message than the males. Although our data showed no significant difference in transferrin gene expression between males and females (Table 1), variation in our assay system did not permit us to exclude the hypothesis of a twofold difference in expression between the available males and females.

To definitively show whether the chicken transferrin gene on the mouse X chromosome was subject to XCI, we took advantage of the Searle's translocation (*4*), a reciprocal exchange between the X chromosome and chromosome 16. When heterozygous in females, both segments of the rearranged X in Searle's translocation are active, whereas the normal chromosome is nonrandomly inactivated in all cells (*5*). We could therefore produce female mice heterozygous for Searle's translocation with the transferrin gene on the normal X chromosome and expect that the chromosome bearing the chicken transferrin gene would be inactive.

We mated males hemizygous for the chicken transferrin gene to females lacking the transferrin insert but heterozygous for the Searle's translocation with the unrearranged X chromosome marked with the *Tabby* (*Ta*) gene. All female offspring should have the chicken transferrin gene on an otherwise normal X chromosome, and this was confirmed by hybridization of the transferrin gene probe to dot-blotted DNA samples prepared from tail (*6*). Females inheriting the normal X chromosome instead of the Searle's rearrangement were identified by the *Ta* heterozygous phenotype, whereas females containing the Searle's translocation would appear phenotypically normal (*7*). The phenotypically normal female mice were killed, and livers were assayed for chicken transferrin gene expression. All three Searle's females tested showed levels of transferrin gene expression that were not significantly different from levels observed in transgenic males, demonstrating that the chicken transferrin gene insert was not inactivated when on the inactive X chromosome (Table 1). We cannot rule out the possibility that the level of expression of the transferrin gene insert on the inactive X is somewhat lower than that on the active X, or that some of the 11 copies of the gene are active while others are not. However, it is clear that complete inactivation does not occur.

In this report, we have demonstrated that 11 copies of the complete chicken transferrin gene, introduced onto the X chromosome of transgenic mice, escaped complete XCI. A gene inserted into the X chromosome might escape XCI because (i) it is located in the limited X-Y pairing and recombination region of the X chromosome, which normally escapes XCI, (ii) the insert-

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