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Copper Homeostasis in the CNS

A Novel Link Between the NMDA Receptor and Copper Homeostasis in the Hippocampus

Michelle L. Schlief and Jonathan D. Gitlin*

Edward Mallickrodt Department of Pediatrics,
Washington University School of Medicine, St. Louis, MO

Abstract

Copper is an essential nutrient that plays a fundamental role in the biochemistry of the central nervous system, as evidenced by patients with Menkes disease, a fatal neurodegenerative disorder of childhood resulting from the loss-of-function of a copper-transporting P-type adenosine triphosphatase (ATPase). Despite clinical and experimental data indicating a role for copper in brain function, the mechanisms and timing of the critical events affected by copper remain poorly understood. A novel role for the Menkes ATPase has been identified in the availability of an *N*-methyl-D-aspartate (NMDA) receptor-dependent, releasable pool of copper in hippocampal neurons, suggesting a unique mechanism linking copper homeostasis and neuronal activation within the central nervous system. This article explores the evidence that copper acts as a modulator of neuronal transmission, and that the release of endogenous copper from neurons may regulate NMDA receptor activity. The relationship between impaired copper homeostasis and neuropathophysiology suggests that impairment of copper efflux could alter neuronal function and thus contribute to rapid neuronal degeneration.

Index Entries: Copper; NMDA receptor; CNS; hippocampus; P-type ATPase; Menkes disease

Copper Homeostasis

Copper, an essential transition metal, plays a critical role in diverse metabolic pathways in all aerobic organisms *(1)*. Recent data permits a comprehensive outline of mammalian cellular copper metabolism (Fig. 1). Copper enters the plasma membrane by the copper transporter ctr1, a high-affinity multimeric protein that transports copper in a metal-specific and saturable fashion *(2)*. Genetic ablation of ctr1 in mice results in profound growth and developmental defects and mid-gestation death *in*

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*Author to whom all correspondence and reprint requests should be addressed. E-mail: gitlin@kids.wustl.edu

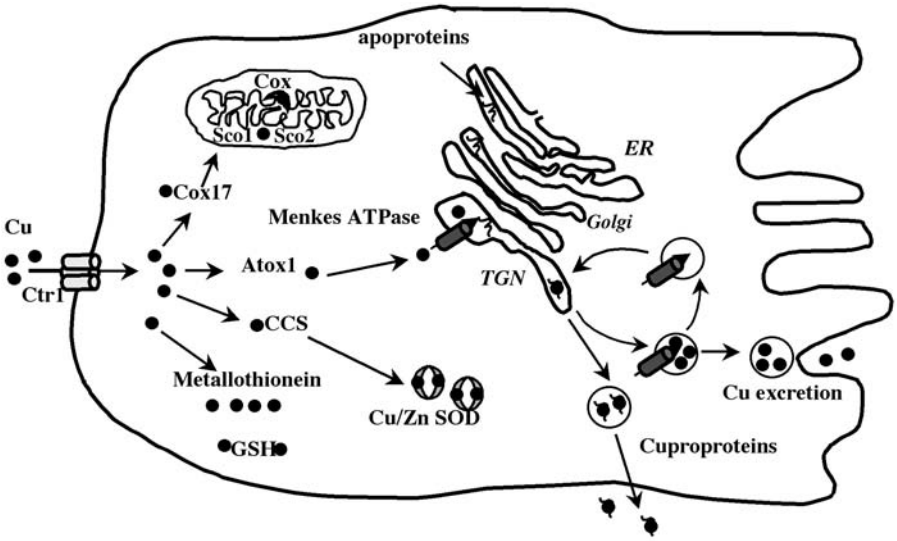

Fig. 1. Model of intracellular copper homeostasis in a polarized epithelial cell, illustrating the specific proteins known to be involved and the pathways utilized. The localization of the adenosine triphosphatase in the trans-Golgi network and the movement of these transporters from this compartment underlies the rapid post-translational mechanism of copper homeostasis.

utero, indicating a critical role for copper homeostasis in embryonic development *(3,4)*. Once in the cytoplasm, unique cellular mechanisms have evolved that permit the intracellular trafficking and compartmentalization of copper, ensuring an adequate tissue supply while avoiding cellular toxicity *(5)*. Under normal physiological conditions, the amount of free or available copper within the cell is extraordinarily restricted *(6)*.

The trafficking of copper to specific enzymes within the cell is mediated by copper chaperone proteins. These metallochaperones function to provide copper directly to specific cellular pathways, while protecting it from intracellular scavenging *(7)*. Chaperones have been demonstrated to deliver copper to their respective targets through direct protein–protein interactions, inserting copper into the active site of the cuproenzyme or transport protein *(8)*. The chaperone atox1 targets copper to both the Menkes and Wilson copper-transporting P-type adenosine triphosphatase (ATPases), which in turn deliver copper to the secretory pathway, whereas cox17 transports copper to the mitochondria for incorporation into cytochrome c oxidase *(10)*; and CCS shuttles copper to Cu/Zn superoxide dismutase. Interestingly, several disease causing mutations impair the interaction between atox1 and the copper-transporting ATPases. Studies have revealed a direct role for atox1 in trafficking intracellular copper to the secretory pathway of mammalian cells, and demonstrates the critical role of this cuprochaperone in perinatal copper homeostasis *(11)*.

Copper enters the secretory pathway through the Menkes and Wilson copper-transporting P-type ATPases. These transporters contain eight transmembrane domains, six copper-binding MXCXXC motifs in the amino-terminus that accept copper and assist in delivering it to the channel *(12)*, a transmembrane CPC motif involved in copper transport across the membrane, and a canonical ATP-binding domain. Copper-transporting ATPase orthologs have been found in plants, bacteria, and yeast *(13–15)*. Molecular characterization of cellular copper metabolic pathways has revealed these ATPases are the major determinant of cellular

copper homeostasis. The copper-transporting ATPases function to both deliver copper to secreted cuproenzymes and participate in copper homeostasis via relocalization in response to increases in intracellular copper. Copper-dependent trafficking of these ATPases provides a sensitive posttranslational mechanism for maintaining intracellular copper homeostasis and avoiding toxicity *(16).* Recent studies have determined that the response of Menkes and Wilson ATPases to increases in intracellular copper provides a critical component of copper homeostasis within epithelial and hepatic cells.

Copper canonically serves as a cofactor for catalytic electron transfer by a small but diverse set of cuproenzymes required for processes critical to neuronal function including: cytochrome *c* oxidase, involved in mitochondrial respiration; superoxide dismutase, involved in antioxidant defense; dopamine β-hydroxylase, involved in catecholamine production; and peptidylglycine α-amidating monooxygenase, involved in neuropeptide; and hormone processing *(17).* The ability of copper to cycle between the cuprous (I) and cupric (II) state gives rise to this utility. Interestingly, a noncanonical role for copper has recently been discovered. The plant molybdenum cofactor biosynthesis enzyme CNX1 (Cnx1), which is critical for the formation of the molybdenum cofactor (Moco), binds copper and delivers it to the Moco precursor as a way of protecting reactive sulphur atoms before molybdenum coordination *(18).* Copper complexed to the reactive sulphur groups protects them and provides a mechanism to assist in the insertion of molybdenum into the cofactor as the metals are exchanged.

Because of the redox reactive nature of the copper ion and the potential for oxidative damage within the cell, copper homeostasis is tightly regulated. When regulatory mechanisms fail, for instance during chronic copper overload, a dangerous state may develop. Labile ions become available to participate in a Fenton reaction, which may result in oxidative damage to proteins and lipids, and enhancement of the formation of toxic-free radicals *(19).* In conditions of copper excess, it has also been found that copper-bound metallothionein can accumulate in aggregates within the cell, which may be toxic to the local environment *(20).* As redox reactive copper may be a source of reactive oxygen species implicated in the pathogenesis of several neurodegenerative diseases *(21),* understanding normal brain copper homeostasis may be critical to understanding these disorders.

Copper in the Central Nervous System

Central nervous system (CNS) development and fitness is particularly dependent on copper metabolism and homeostasis *(22).* Genetic disruption of copper homeostasis can lead to neurodegeneration within the cerebral cortex, thalamus, cerebellum, and hippocampus, in addition to marked Purkinje cell abnormalities, increased perinatal mortality, severe growth retardation, and congenital malformations including microphthalmia *(11,23,24).*

In the adult human nervous system, acquired copper deficiency owing to excessive zinc consumption, gastrectomy, or idiopathic malabsorption is associated with sensory ataxia owing to ascending sensory tract dysfunction and neuropathy of the dorsal column, with resultant lower limb spasticity and abnormal sensations in the extremities. Although resolution of the copper deficiency prevents further deterioration, residual neurological deficits generally persist *(25).* Moreover, a fatal condition arises in children with a genetic lesion that results in failure to transport copper across the placenta, the gastrointestinal tract and the blood–brain barrier as a result of a defect in the gene encoding the Menkes copper-transporting P-type ATPase. Patients with Menkes disease are characterized by a number of phenotypic conditions, including demyelination and neurodegeneration of the cerebral cortex, thalamus, cerebellum, and hippocampus. No life-saving treatment options are currently available and patients typically die within the first decade of life *(26, 27).*

Copper toxicity because of Wilson disease or idiopathic toxicosis can be likewise fatal if untreated, demonstrating the cellular susceptibility to copper overload, particularly in the brain. Although copper accumulation may lead to injury in many different tissues, resulting in diverse clinical manifestations, most individuals with Wilson disease will present with evidence of liver or CNS involvement. Signs and symptoms of Wilson disease are rarely observed before the age of 3 yr, presumably reflecting the considerable capacity of the liver to store excess copper. Neurological features may include Parkinsonian symptoms, consistent with the neuropathological findings of basal ganglia involvement. Psychiatric symptoms are also common and may include personality changes such as irritability and low threshold to anger, depression, cognitive deterioration, psychosis, and schizophrenia-like symptoms *(28)*.

Copper is initially absorbed from the diet into the blood stream within the small intestine. When the blood stream reaches the liver, copper is taken up by hepatocytes and excess copper is secreted into the bile. Copper from the blood stream is transported to all tissues, including the brain. Much of the copper in the CNS is tightly bound for catalytic use by cuproproteins, for instance, the K_d value of superoxide dismutase for copper is 6 fM *(6)*. Although free copper ions as such do not likely exist, a subset of intracellular copper is thought to be loosely bound to small molecules such as histidine and glutathione. This pool likely forms the basis of the chelatable copper found within the cell. This population of labile copper could potentially represent a pool available for release, and for assumption of a neuromodulatory role. However, it remains formally possible that copper tightly bound to a cuproenzyme could be released quickly after a change in conditions.

It has been reported that copper is released synaptically in some populations of brain neurons *(29)*. Reports of total copper levels in various brain regions have been generated utilizing atomic absorption spectroscopy, and have revealed that the average gray matter concentration is in the high micromolar range, at least an order of magnitude greater than the concentrations of several non-amino acid neurotransmitters and most neuropeptides *(30)*. Utilizing atomic absorption spectroscopy to examine release of copper from cortical synaptomes following brief depolarization, the concentration of copper in the synaptic cleft was calculated to reach local concentrations in the micromolar range *(31)* as compared with the low millimolar concentrations of glutamate found in the synaptic cleft during excitatory synaptic transmission *(32)*. However, this technique does not distinguish between the various pools of copper that may exist in cells, making it difficult to calculate the proportion of copper tightly bound by cuproproteins vs that which may be part of a labile pool. Some information about the localization of chelatable copper in the CNS has been generated using Timm's modified sulphide silver staining, a histochemical approach that detects chelatable Cu^{2+}, Zn^{2+}, and Fe^{2+}; subsequent treatment with trichloracetic acid is reported to selectively reveal copper, and is also referred to as the Danscher modification. Using this method, labile copper pools were detected in the rat CNS within the soma of cortical pyramidal and cerebeller granular neurons, and in neuropil within the cerebral cortex, hippocampus, red nucleus, cerebellum, and spinal cord *(33)*. Additionally, copper has been detected on synaptic terminal membranes of locus ceruleus afferents *(34)*.

Effects of Copper on Neuronal Excitability

Exogenous copper application affects the excitability of some populations of neurons. The question is still open, however, as to whether synaptically released endogenous copper plays a significant role in normal synaptic physiology. Exogenous applications of micromolar concentrations of copper produce an antagonistic effect on both *N*-methyl-D-aspartate (NMDA)- and γ-aminobutyric acid-mediated currents in isolated olfactory bulb mitral/tufted cells and

interneurons. Copper also blocks glycine-mediated currents under nondesensitizing conditions, although it has no effect on the desensitized component of these currents. These effects are not voltage-dependent *(35)*. Studies in cultured mouse and rat hippocampal and cortical neurons have similarly found that copper acts as a noncompetitive antagonist at NMDA receptors, suggesting that copper interacts preferentially with agonist-bound receptors *(36,37)*. As stimulation of the NMDA receptor controls permeability to Ca^{2+}, modulation of receptor activity by copper could modulate activation of calcium-dependent cascades that contribute to important synaptic modifications *(38–40)*. Indeed, studies examining the effect of copper on synaptic plasticity in the CA1 region of rat hippocampus have demonstrated that long-term potentiation is abrogated by low micromolar copper concentrations *(41,42)*.

Copper has also been shown to reduce the rate of repetitive firing of action potentials through action on transient and delayed rectifier-type potassium channels, and to inhibit voltage-gated calcium channels, thereby exerting an inhibitory effect on neurotransmitter release *(43)*. Additionally, micromolar copper has been found to activate the two-pore-domain, baseline K^+ channel TREK-1 *(44)*. TREK-1 is expressed in hippocampal excitatory neurons and localizes both to synaptic and nonsynaptic sites. TREK-1 channel activity, which is also elicited by hypoosmolarity, acidic pH, and by a number of general anesthetics, results in a reduction in neuronal excitability *(45)*. Taken together, these data raise the possibility that during times of low copper status, neurotransmission at some synapses may not function properly.

Intriguingly, we have recently shown that synaptic NMDA receptor activation results in the rapid and reversible trafficking of Menkes ATPase both to the somato-dendritic and axonal compartments of cultured hippocampal neurons, independent of the intracellular copper concentration (Fig. 2). Chelation of intracellular Ca^{2+} was also found to abrogate this trafficking signal, whereas blocking voltage-gated calcium channels and voltage-gated sodium channels had no effect. Thus, Ca^{2+} entry through the NMDA receptor activates a calcium-dependent biochemical cascade leading to Menkes ATPase trafficking, independent of action potential-mediated activity, and demonstrating a previously unrecognized link between copper and calcium homeostasis in mammalian cells *(46)*. To clearly determine that the glutamate receptor activity-dependent trafficking of Menkes ATPase is independent from copper-dependent trafficking, all of these studies were performed in the presence of the copper-specific chelator bathocuproine sulfonate *(47)*.

As trafficking of the catalytically active Menkes ATPase is a dominant force maintaining intracellular copper homeostasis in non-neuronal cell types, the effect of NMDA receptor stimulation on copper homeostasis was examined in hippocampal neurons, utilizing metabolic labeling with radioactive ^{64}Cu to follow the copper pool. These studies revealed that trafficking of Menkes ATPase following NMDA receptor activation is associated with rapid release of copper from hippocampal neurons (Fig. 3). This process was also independent of action potential-mediated activity, as antagonism of voltage-gated sodium channels did not inhibit copper release following stimulation. The removal of protein from the efflux solution by acetone or trichloroacetic acid precipitation did not alter the amount of detectable ^{64}Cu in the media following glutamate-glycine treatment, indicating that the released copper was most likely either free in solution or loosely bound to small molecules, and not incorporated into specific cuproproteins. Although the absolute amount of copper released in our experiments can not be determined using ^{64}Cu, previous analysis of extracellular copper concentrations following brief depolarization of cortical synaptosomes demonstrates the physiological significance of these findings *(31,47)*.

Menkes ATPase is directly required for this copper efflux as similar studies in hippocampal neurons derived from mice lacking a functional Menkes ATPase demonstrated no copper release. Interestingly, copper release occurs more

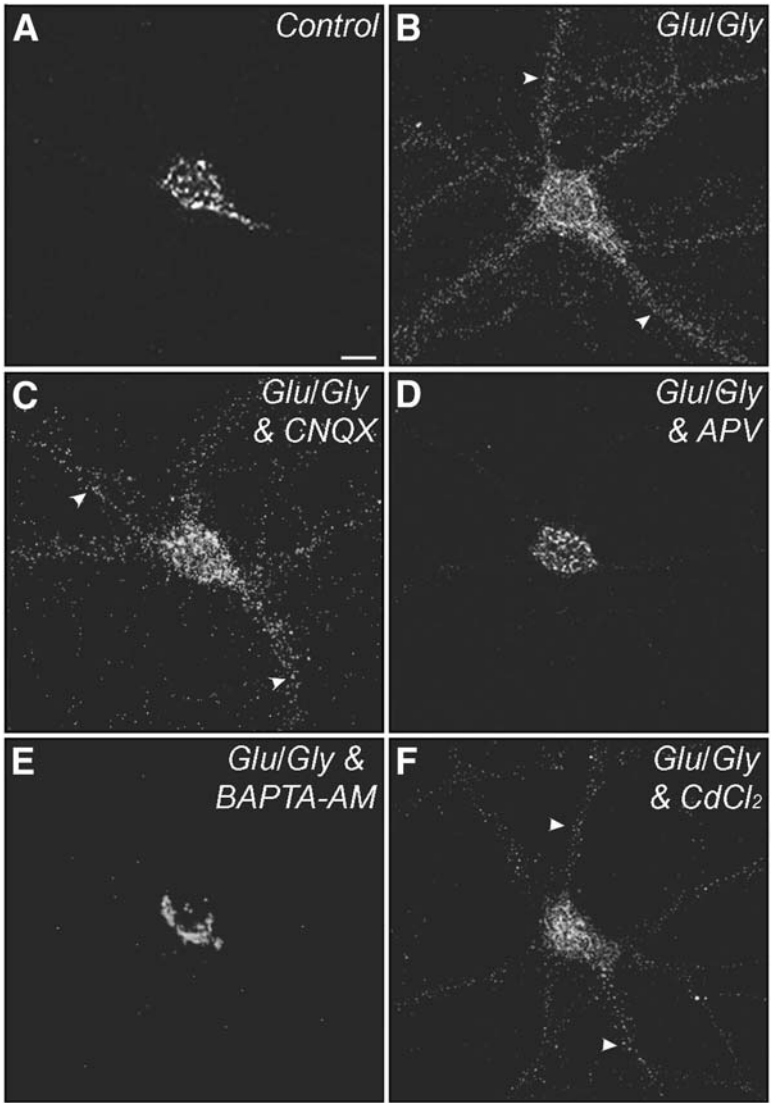

Fig. 2. *N*-methyl-D-aspartate receptor-dependent trafficking of Menkes adenosine triphosphatase (ATPase). Neurons were treated for 24 h with 200 μ*M* of the copper chelator bathocuproine sulfonate and then exposed to extracellular solution with 2 m*M* Mg^{2+} **(A)**, 50 μ*M* glutamate and 5 μ*M* glycine in Mg^{2+} free extracellular solution **(B)**, or pretreated with the indicated drugs and then exposed to 50 μ*M* glutamate, 5 μ*M* glycine and the indicated drugs in Mg^{2+} free extracellular solution **(C–F)**. Neurons were treated with the AMPA receptor antagonist CNQX **(C)**, the NMDA receptor antagonist APV **(D)**, the cell permeable Ca^{2+} chelator BAPTA-AM **(E)**, or the voltage-gated calcium channel antagonist $CdCl_2$ **(F)**. Cells were fixed and immunolabeled for Menkes for analysis by confocal microscopy. Arrowheads (B, C, F) indicate Menkes ATPase outside late Golgi and in processes (Scale bar: 10 μm). (Reproduced with permission from ref. *47*.)

rapidly than Menkes ATPase trafficking, suggesting the existence of a readily releasable copper pool accumulated by previous Menkes ATPase trafficking events. This establishes a novel role for Menkes ATPase in the availability of an NMDA receptor-dependent, releasable pool of copper in hippocampal neurons and demonstrates a unique mechanism linking cop-

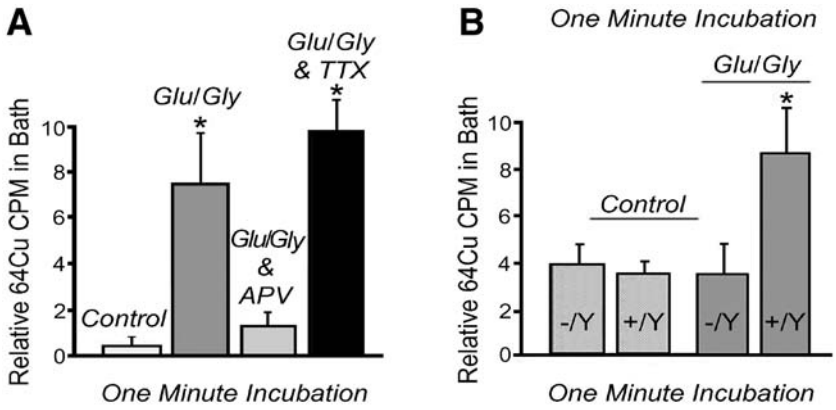

Fig. 3. Copper efflux from hippocampal neurons. **(A)** Neurons were incubated with 300 μCi of ^{64}Cu for 6 h, washed, and exposed to extracellular solution with 2 m*M* Mg^{2+} (control), 50 μ*M* glutamate and 5 μ*M* glycine or glutamate-glycine in combination with the *N*-methyl-D-aspartate receptor antagonist APV (Glu/Gly + APV). The bath solution was analyzed for ^{64}Cu for the first minute of efflux, and the relative counts per minute (cpm) were calculated. **(B)** Mouse hippocampal neurons were isolated and cultured at P0 from male offspring of *Mo^{br}* heterozygous female, wild-type male matings. Individual neuronal cultures were prepared from each pup, followed by metabolic labeling with ^{64}Cu, treatment with ECS with 2 m*M* Mg^{2+} (control) or 50 μ*M* glutamate and 5 μ*M* glycine. Measurement of ^{64}Cu for the first minute of efflux was taken, and relative cpm was calculated. (Modified with permission from ref. *47*.)

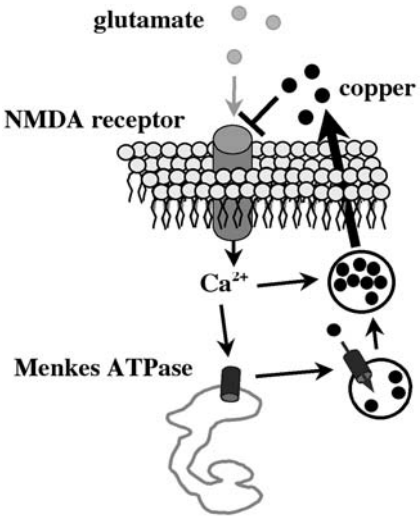

Fig. 4. Model of *N*-methyl-D-aspartate (NMDA) receptor-mediated copper homeostasis in hippocampal neurons. Ca^{2+} entry through the NMDA receptor results in the trafficking of Menkes adenosine triphosphatase (ATPase), which accumulates copper into a membrane bound compartment, forming and replenishing a novel pool of potentially releasable copper. NMDA receptor activation also results in the release of copper from this readily releasable copper pool. Copper released from the neuron may act to functionally block NMDA receptors and thus limit Ca^{2+} entry into the cell, limiting the potential for excitotoxic damage. Experiments indicate that the population of NMDA receptors responsible for trafficking of Menkes ATPase and copper release are neuronal in origin and synaptically localized *(47)*.

per homeostasis and neuronal activation within the CNS *(47)*.

We hypothesize that Ca^{2+} entry through the NMDA receptor results in the trafficking of Menkes ATPase, which accumulates copper into a membrane bound compartment, forming and replenishing a novel pool of potentially releasable copper (Fig. 4). NMDA receptor activation also results in the release of copper from this readily releasable copper pool. Copper released from the neuron could act endogenously to functionally block NMDA receptors and thus limit Ca^{2+} entry into the cell, in a manner akin to exogenously applied copper *(35,36,43,47)*. The mechanism of this copper block is currently unknown. However,

S-nitrosylation of the NMDA receptor, in which a molecule of nitrogen oxide reacts with Cys399 on the extracellular face of the NMDA receptor to functionally downregulate receptor function, requires the catalytic removal of an electron from the nitrogen monoxide species *(48).* Copper is a potent electron acceptor, and has been shown to promote nitrosylative events in vitro, and in other systems in vivo *(49,50),* placing synaptically released copper in the unique position to potentially catalyze NMDA receptor *S*-nitrosylation. The loss of secretable copper in Menkes disease may thus result in the decreased ability of the neuron to modulate NMDA receptor activation, and may thus lead to an increase in Ca^{2+} entry. Future studies will address the sensitivity of hippocampal neurons derived from wild-type or Mo^{br} Menkes mutant littermate male mice to excitotoxic insult. An increased sensitivity to excitotoxicity could explain the seizures and neurodegenerative damage in Menkes patients. This mechanism is a novel model of pathogenesis in Menkes disease, and potentially reveals new targets for the treatment of patients.

Summary

The increasing recognition of the disregulation of metal homeostasis in human CNS disease has highlighted gaps in our current understanding of the normal biology in this area. Currently, the role of copper in CNS disease is poorly understood. It is suggested that further elucidation of the mechanisms of copper homeostasis within the CNS may be critical to understanding the role of copper in fundamental neurobiological processes, as well as the pathophysiology of neurodegenerative disease.

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