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Metalloneurochemistry and the Pierian Spring: ‘Shallow Draughts Intoxicate the Brain’

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Abstract

Metal ions perform critical and diverse functions in nervous system physiology and pathology. The field of metalloneurochemistry aims to understand the mechanistic bases for these varied roles at the molecular level. Here, we review several areas of research that illustrate progress toward achieving this ambitious goal and identify key challenges for the future. We examine the use of lithium as a mood stabilizer, the roles of mobile zinc and copper in the synapse, the interplay of nitric oxide and metals in retrograde signaling, and the regulation of iron homeostasis in the brain. These topics were chosen to demonstrate not only the breadth of the field, but also to highlight opportunities for discovery by studying such complex systems in greater detail. We are beginning to uncover the principles by which receptors and transmitters utilize metal ions to modulate neurotransmission. These advances have revealed exciting new insights into the intricate mechanisms that give rise to learning, memory, and sensory perception, while opening many new avenues for further exploration.

Keywords

bioinorganic chemistry; neurochemistry; metalloproteins; signal transduction

1. Introduction

Alexander Pope memorably professed in *An Essay on Criticism*, “A little learning is a dangerous thing; Drink deep, or taste not the Pierian Spring: There shallow draughts intoxicate the Brain, And drinking largely sobers us again.”^[1] His admonition to avoid superficiality in scholarly pursuits is particularly relevant to studies of living things, and especially the human brain, an organ of unfathomable complexity.

Metalloneurochemistry is the field concerned with delineating the physiological and pathological functions of metal ions in the nervous system. Metal ions seem to be involved at some level in most brain functions. As in the rest of the body, they confer structural stability and catalytic activity to proteins required for metabolism and other fundamental life

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Dedication – to Harry on the occasion of your 80th birthday. Thanks for many decades of good humor, sage leadership, and outstanding science.

processes. But in the nervous system, metal ions also play important but often poorly understood roles in synaptic transmission, signal transduction, cognition, learning, memory formation, and sensory perception. Dysregulation of metal ion homeostasis has profound consequences for brain health, and is almost certainly a contributing factor to neurological diseases such as Alzheimer's, traumatic brain injury, ischemic stroke, epilepsy, and attention deficit hyperactivity disorder.^[2] Metal ions and their complexes can also be of therapeutic value for treating certain mental disorders, especially bipolar disorders.

Although the term metalloneurochemistry was formally introduced only recently, work in this area has a rich history.^[3] Neurotoxic effects of lead, for instance, have been known since antiquity. The Greek poet Nicander (c. 2nd century BCE) wrote of the "hateful brew" of white lead, which sometimes induces hallucinations, fatigue, and paralysis.^[4] Over time, neurophysiological properties of other trace elements, including mercury, iodine, and cobalt were identified,^[5] although it was not until the 20th century that even a cursory understanding of how these metals affect biochemical pathways began to emerge.^[6]

We now know that lead is a potent neurotoxin because it interferes with *N*-methyl-D-aspartate (NMDA) receptor activity by altering its localization in the synapse and changing the expression patterns of its subunits.^[7] The NMDA receptor is one of several calcium channels that mediate a majority of excitatory neurotransmission events in the brain.^[8] Calcium is the most studied metal in neuroscience, and many intricacies of how it functions in various signaling pathways have been determined.^[9] Despite these successes and great advances in molecular biology, including the advent of omics biology, the introduction of sophisticated gene editing technologies such as CRISPR/Cas, and recent improvements in single neuron imaging, unraveling the molecular mechanisms by which metals act in the central nervous system remains a formidable challenge.

As we enter what Rafael Yuste and George Church have termed "the new century of the brain," the century in which they call upon neuroscientists to determine the precise mechanisms and neural signaling patterns that give rise to thought and control behavior, metalloneurochemists are poised to make substantial contributions.^[10] Here, we present five vignettes of research that illustrate the scope of metalloneurochemistry. These topics were chosen to display the diversity of the field and give the reader a sense of the many wonderful opportunities for future exploration.

2. What is the Role of Zinc in the Synapse?

Although most zinc ions in the body are tightly bound to proteins, pools of loosely bound, "mobile," or "chelatable" zinc accumulate in the brain, mammary gland, pancreas, and prostate.^[11] In the brain, vesicular stores of mobile zinc are concentrated in a subset of glutamatergic neurons in regions that are strongly engaged in sensory perception and memory formation, including the amygdala, hippocampus, neocortex, and pyriform cortex.^[12] During some forms of excitatory synaptic transmission, Zn^{2+} is co-released with glutamate into the synaptic cleft, where it can inhibit or potentiate a variety of ligand-gated ion channels.^[12] In this manner, Zn^{2+} not only participates in neural signaling, but also modulates long-term potentiation (LTP), the process through which synaptic strength

increases in response to a stimulus.^[13] New analytical techniques have greatly improved our ability to study such processes and a full picture of zinc biochemistry in the synapse is now beginning to emerge.

Among these tools is a family of highly selective fluorescent probes that can be used to detect mobile zinc in live cells and tissue. These sensors are prepared from small molecule or protein scaffolds and feature diverse excitation and emission profiles, a wide range of zinc binding affinities, and unique subcellular accumulation properties.^[14] Typically, these constructs fluoresce exclusively in the presence of Zn^{2+} , facilitating spatiotemporal imaging of mobile zinc. Quantitative measurements of zinc concentrations can be made with available intra- and extracellular ratiometric sensors. In addition to these chemical tools, a valuable resource for studying synaptic zinc is the ZnT3 knockout mouse.^[15] This protein mediates transport of zinc into synaptic vesicles, and ZnT3 knockout mice lack vesicular zinc.^[16] Although the mice suffer from some physiological impairment, including diminished learning and memory in older animals, they are valuable as negative controls to ensure that synaptic effects attributed to mobile zinc arise exclusively from vesicular sources.^[17]

One of the greatest obstacles to studying synaptic zinc has been the lack of a reliable extracellular chelator to intercept it rapidly on the timescale of excitatory postsynaptic current (EPSC) generation and selectively over other potential metal ions.^[18] The introduction of ZX1, a membrane-impermeable zinc chelator with high selectivity for Zn^{2+} over other biologically relevant metals, has substantially addressed this problem.^[13] The 1 nM dissociation constant ($K_{\text{d-Zn}}$) for the Zn^{2+} complex of ZX1 is similar to that of the widely-used chelating agent CaEDTA ($K_{\text{d-Zn}} = 2$ nM) and much lower than that of tricine ($K_{\text{d-Zn}} = 2.8$ μM), a buffer commonly used to chelate the ion.^[19] Moreover, competition experiments employing fluorescent sensors revealed that ZX1 binds Zn^{2+} rapidly, with a second order rate constant (k_2) of $4 \times 10^5 \text{ M}^{-1}\cdot\text{s}^{-1}$, which is nearly 200 times faster than either of the other agents.^[19] The combination of these factors makes ZX1 a superior candidate for studying synaptic zinc compared to CaEDTA or tricine. For example, whereas 100 μM ZX1 can effectively compete with the high affinity zinc binding site of the NMDA receptor GluN2A subunit, tricine cannot—even at concentrations as high as 10 mM.^[19]

The NMDA receptor (Figure 1) is an obligate heterotetramer, typically comprising two GluN1 and two GluN2 subunits, that forms a calcium channel after activation by L-glutamate and either glycine or D-serine, with release of a bound Mg^{2+} ion.^[8] The GluN1 subunit has eight isoforms that result from different splicing of a single gene. A GluN2 subunit can be any one of four proteins, designated GluN2A through GluN2D, that are each encoded by separate genes.^[20] Of the four GluN2 variants, GluN2A and GluN2B bind zinc with low nanomolar and micromolar affinities, respectively.^[21] Zinc binding stabilizes the closed conformation of the entire receptor, preventing opening of the calcium channel. In this way, zinc can regulate NMDA receptor activity and modulate excitatory neurochemical signaling in a concentration-dependent manner.

The concentration of zinc in a synapse after an excitatory event is notoriously difficult to determine. Estimates of zinc concentrations in the synaptic cleft immediately following

exocytosis span several orders of magnitude, from 10 nM to >100 μ M, and depend on the number of zinc vesicles that fuse to the presynaptic outer membrane.^[12, 22] This so-called 'phasic' zinc can then inhibit NMDA receptors if the local concentration is near or exceeds the zinc binding affinity of the particular receptor subunit assembly.^[23] Depending on the zinc concentration in the resting synapse, it is possible that 'tonic' zinc can inhibit NMDA receptors, particularly those containing the high zinc affinity GluN2A subunit.^[23] Other models have been advanced, including one that predicts the existence of a veneer of zinc that might accumulate on pre- and post-synaptic membrane proteins.^[18, 24]

Experiments using ZX1 to study the role of synaptic zinc in the hippocampus revealed that zinc released in response to electrophysiological stimulation inhibits postsynaptic NMDA receptors.^[13] Furthermore, in hippocampal mossy fiber-CA3 (MF-CA3) synapses, there is evidence to suggest that vesicular zinc not only inhibits postsynaptic LTP, but also promotes presynaptic LTP.^[13] Subsequent work with NR2A H128S knock-in mice, which express a mutated version of the GluN2A subunit lacking the high-affinity zinc-binding site,^[25] also demonstrated that endogenous zinc reduces NMDA receptor mediated LTP at the MF-CA3 synapse.^[22]

Recent studies employing ZX1, ZnT3 knockout mice, and ratiometric sensors have shown that tonic zinc and phasic zinc can also inhibit extrasynaptic NMDA receptors through physiologically relevant mechanisms in the dorsal cochlear nucleus (DCN), a region of the brain that is strongly engaged in auditory processing.^[19] Spillover of glutamate from the synapse activates extrasynaptic NMDA receptors in response to short trains of synaptic stimuli.^[19] The phasic release of zinc that accompanies glutamate during such trains of presynaptic stimulation attenuates extrasynaptic NMDA receptor activity.^[19] Higher rates of presynaptic stimulation induce release of glutamate that activates more extrasynaptic NMDA receptors, which do not appear to be inhibited by phasic zinc.^[19] These receptors do, however, seem to be modulated by tonic zinc, which is in low nanomolar levels in the extrasynaptic space.^[19] Unraveling the functional consequences of these regulatory processes for neural signaling could profoundly alter our understanding of neuroprotective mechanisms and is under active investigation.

These same general techniques have also been used to demonstrate that synaptically released vesicular zinc inhibits the AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor.^[26] Like the NMDA receptor, the AMPA receptor is an ionotropic glutamate receptor that forms a transmembrane ion channel in the presence of L-glutamate.^[27] Although reports in the literature draw conflicting conclusions as to whether or not physiological concentrations of zinc can modulate AMPA receptor activity, many of these discrepancies are attributable to the use of non-ideal zinc chelators.^[26] For example, application of 10 mM tricine to parallel fiber synapses in the DCN had no effect on AMPA receptor-mediated EPSCs, whereas 100 μ M ZX1 potentiated the currents in wildtype but not ZnT3 knockout mice.^[26] Similar results were observed in the hippocampus, supporting the conclusion that endogenous zinc is a general modulator of synaptic transmission in glutamatergic synapses.^[26] Experiments in mice reveal that sustained exposure to loud sounds can reduce presynaptic vesicular zinc levels in the DCN and implicate more complex mechanisms for controlling AMPA receptor synaptic plasticity.^[26]

There are many other types of ion channels and receptors that are commonly found in the central nervous system, and zinc affects several of them, including metabotropic glutamate receptors, receptors for acetylcholine, adenosine, serotonin, dopamine, catecholamine, and melanocortin, and channels for Na^+ , K^+ , Ca^{2+} , and Cl^- .^[28] The effects of zinc on these proteins can be complicated. For example, at concentrations between 10 nM and 1 μM , zinc potentiates glycine receptors through an allosteric mechanism, but the effects become inhibitory at higher concentrations.^[29] Physiologically, it appears that tonic zinc enhances glycinergic neurotransmission in the DCN and prevents spontaneous firing of fusiform cells, perhaps to attenuate pathological effects of spontaneous activity.^[29]

As new studies emerge that reveal the breadth of zinc function, it is clear how much more there is to learn. One key question involves the degree of generalizability of results obtained in one region of the brain to others, even for similar processes. As described above, loud sounds can alter presynaptic zinc levels in the DCN, and similar observations of experience-dependent changes in zinc levels have been made in other parts of the brain engaged in sensory perception, such as the somatosensory cortex.^[30] Do these results point to a general mechanism for controlling sensory inputs? Synaptic zinc is released in regions of the olfactory bulb in tissue slices stimulated with electrophysiological pulses.^[31] Does vesicular zinc modulate postsynaptic receptors in the olfactory bulb during olfaction the same way it does in the DCN during audition? In addition to acting on receptors directly, synaptic zinc can also alter endocannabinoid synthesis and thus modulate neurotransmission through a completely separate pathway.^[32] What other mechanisms might be implicated? As we formulate hypotheses to address these fundamental questions, it will become necessary to confront the challenge of leveraging the knowledge to develop treatments for neurological disorders.

3. How Does Lithium Stabilize Moods?

Lithium is among the most effective long-term treatments for bipolar disorder and mania.^[33] Communities with elevated lithium concentrations in the public water supply tend to have lower suicide rates, and advocates have called for addition of lithium to municipal water supplies for mental health prophylaxis.^[34] Despite its well-documented success as a mood stabilizer and long history of clinical use, the biochemical mechanisms through which lithium acts remain poorly understood. Here we discuss some of the most compelling hypotheses that have been advanced to account for its activity.

The early history of lithium use in the 19th century has been compared to that of the person who ate the first oyster.^[35] Nobody would have expected lithium salts to have psychopharmacological effects, but physicians still investigated lithium compounds during the 19th century: by 1859, Li_2CO_3 was a recommended, though dubious, treatment for gout; by the early 1870s, LiBr was used as a hypnotic and anticonvulsant; and by the 1890s, Li_2CO_3 was used in Denmark as a prophylactic and antidote for depression.^[35] Although lithium therapy continued sporadically in the early 20th century—particularly in the south of France—the introduction of lithium to clinical psychiatry is generally credited to John Cade, who, in 1949, reported the case histories of ten manic patients whom he had successfully treated with lithium citrate or carbonate salts.^[35–36]

Initial theories accounting for the mechanism of action of lithium have largely been discredited. For example, LiBr was thought to be more effective than other bromide salts, not because of any specific activity of the lithium cation, but rather because of the small atomic mass of lithium: gram for gram LiBr has a higher bromide percentage than any other bromide salt.^[36a] Cade realized that lithium was the active agent and speculated that manic attacks arose from lithium ion deficiencies in the body; nevertheless, there is no evidence to support such a hypothesis.^[36a]

Bipolar disorder was once thought to be the consequence of changes in membrane permeability or ionic effects on neurotransmission.^[37] Therefore, investigators researched the effects of lithium on small molecule neurotransmitters, particularly catecholamine and acetylcholine.^[38] Even though lithium can affect the behavior and distribution of a variety of neurotransmitters by altering not only the pathways through which they are synthesized and released, but also processes controlling their uptake, theories involving these mechanisms have largely been disregarded.^[38] Similarly, a once dominant hypothesis concerning the effects of lithium on sodium-potassium pumps has also been supplanted by evidence that aberrations in signaling pathways can dramatically affect synaptic plasticity and glutamatergic neurotransmission, and they might be the root cause of bipolar disorder.^[37] Lithium appears to enhance glutamate signaling—possibly that mediated by AMPA receptors—in the CA1 region of the hippocampus, which could affect overall neuronal excitability, efficiency, and plasticity.^[37]

With respect to intracellular signaling cascades, lithium can perturb several pathways related to inositol, glycogen synthase kinase-3 (GSK-3), and protein kinase C (PKC), among others.^[39] Lithium modulates the activity of inositol-1-monophosphatase (IMPase) by noncompetitively inhibiting the binding of a Mg^{2+} ion to the enzyme and uncompetitively inhibiting substrate binding.^[40] IMPase dephosphorylates myo-inositol-1-phosphate to give myo-inositol, a substrate for the production of phosphatidylinositol.^[41] A series of enzymes, including phospholipase C, convert phosphatidylinositol to inositol-1,4,5-trisphosphate (IP_3), which is a key regulator of intracellular Ca^{2+} stores.^[41] Furthermore, some of the phosphorylated derivatives of phosphatidylinositol and other molecules associated with this pathway, such as 1,2-diacylglycerol, participate in other signal transduction pathways that could affect neural function.^[39, 41] Together, these effects have led to the development of the inositol-depletion hypothesis, which is derived from the observation that lithium inhibition of IMPase will cause a reduction in the pool of free inositol available for cell signaling.^[41] Although this hypothesis has several attractive features, particularly given striking results in numerous *in vitro* experiments, there is no data yet to suggest that this model is clinically relevant.^[41]

The protein GSK-3 is a serine/threonine kinase that phosphorylates at least 50 different proteins involved in cell signaling, homeostasis, and several diseases, including Alzheimer's, schizophrenia, and Huntington's.^[39] Lithium inhibits Mg^{2+} binding to the GSK-3 β 1 splice variant through a competitive mechanism and inhibits substrate binding through a noncompetitive mechanism.^[39] Because GSK-3 β can phosphorylate microtubule-associated proteins (MAPs) that are important for maintaining cytoskeletal structures, including tau and MAP-1B, lithium therapy might induce changes in neuronal cytoskeletal architecture.^[38]

Unfortunately, no compelling clinical evidence exists for the involvement of GSK-3 in bipolar disorder.^[41]

Recent genetic work has revealed that lithium response in patients is correlated with the brain-derived neurotrophic factor (BDNF) system.^[42] BDNF plays critical roles in regulating plasticity and LTP in some hippocampal synapses and may, with other proteins, contribute to bipolar mood disorders.^[37] Genome-wide studies are currently being conducted by the Consortium on Lithium Genetics and may reveal more proteins that are associated with lithium efficacy.^[42] In all likelihood, there are many factors that contribute to the mood stabilizing effects of lithium, and it is even possible that different mechanisms will be important in different areas of the brain or even in different synapses.

For all that we have learned about lithium biochemistry, we have not yet been able to find a clinically relevant model. Collectively, these lessons are instructive even if frustrating. For example, it is clear that even with detailed knowledge of how lithium interacts with a given neuronal protein at a molecular level, there is no guarantee that it will behave the same way in vivo. Biochemical pathways do not exist in isolation, and inhibition of a key enzyme in one cascade does not prevent a cell from compensating through an otherwise orthogonal mechanism. The case of lithium illustrates many problems confronting metalloneurochemists and demonstrates the need for developing better ways to study metals in live animals.

4. Are Changes in Brain Iron Homeostasis a Cause or Effect of Neurodegeneration?

It has only been a few decades since iron was recognized as an important player in neurochemistry. Dysregulation of iron metabolism is now believed to be a central event in the development of neurodegenerative disorders.^[43] Iron accumulation in specific brain areas is linked to both genetic and non-genetic diseases.^[44] Despite significant advances in imaging and quantitation techniques, our understanding of the mechanisms of iron regulation in the brain remains limited. There is disagreement about whether dysregulation of iron homeostasis is a cause or an effect of neurodegeneration. We discuss here key aspects of brain iron metabolism and disease.

Iron is the most abundant transition metal in humans. Even though the total amount of iron in the body ranges from 3.5 to 5 g,^[45] only a trace amount is present in the brain, with the highest concentrations in the substantia nigra pars compacta (SNpc).^[44b] Iron is necessary in the central nervous system (CNS) for mitochondrial respiration, myelin production, and the synthesis of dopaminergic neurotransmitters.^[43, 46] Because the brain capillary endothelial cells (BCECs) forming the blood-brain barrier prevent direct iron uptake from blood, iron crosses the blood-brain barrier in a multistep process. Circulating transferrin (Tf) sequesters Fe³⁺ in the plasma at concentrations of ~40 μ M.^[46] The first stage of iron uptake in the brain involves binding of Tf to transferrin receptor 1 (TfR1) expressed at the luminal side of BCECs, followed by receptor-mediated endocytosis.^[46–47] The subsequent steps of iron release at the abluminal side of BCECs remain a subject of debate. Two theories have emerged, differing in the involvement of divalent metal transporter 1 (DMT1). In the DMT1-

independent model, Tf-bound Fe^{3+} is transported into endosomes across BCECs and directly released into the brain interstitial fluid (ISF).^[47] In the DMT1-dependent model, Tf-bound Fe^{3+} is reduced to Fe^{2+} , released from endosomes in the cytosol of BCECs via DMT1, and finally exported into the ISF through ferroportin (Fpn), the only known iron exporter.^[43, 46] Once in the ISF, iron may bind to circulating Tf produced by the choroid plexus, or it may be coordinated by ascorbate, citrate, and ATP secreted by astrocytes, the latter forming the pool of nontransferrin-bound iron (NTBI). The mechanisms of iron transport into astrocytes and microglia from the ISF have not yet been elucidated, whereas oligodendrocytes import iron from extracellular ferritin (Ft).^[43] Given that neurons express TfR1, DMT1, and Fpn, they most likely acquire the bulk of their iron from circulating Tf in a manner similar to that of the BCEC DMT1-dependent pathway (Figure 2).^[43, 46]

The low concentration of Tf in ISF ($<0.5 \mu\text{M}$) is consistent with the theory that NTBI ($\sim 1 \mu\text{M}$) is also a source of iron uptake for neurons,^[46] probably via DMT1 and other divalent metal transporters. It remains unclear, however, how Fe^{3+} is dissociated from citrate/ascorbate/ATP and reduced to Fe^{2+} at the outer membrane surface prior to uptake by DMT1.^[46] Irrespective of the uptake mechanism, iron is imported in the Fe^{2+} form, accumulating into the cytosolic labile iron pool (LIP, Figure 2). Much lower amounts of cytosolic Fe^{2+} are oxidized to Fe^{3+} and preferentially stored by Ft in neurons compared to other cell types, suggesting that most intracellular iron is taken up for rapid utilization.^[46] As in the case of BCECs, neuronal iron export is mediated by Fpn, which is assisted by membrane-anchored ceruloplasmin, a copper-based enzyme facilitating the rapid oxidation of Fe^{2+} to Fe^{3+} and subsequent binding by circulating Tf.^[43, 44b, 46] Fpn is further stabilized by the amyloid precursor protein (APP), transported to the membrane by the microtubule-associated protein tau (Figure 2).^[43]

Regulation of the LIP occurs under the action of two iron response proteins (IRP1,2), of which IRP2 is preferentially expressed in the CNS.^[43] IRPs are critical for maintaining iron homeostasis while neurons are exposed to the elevated iron levels that accompany normal aging. Excessive Fe^{2+} accumulation in the LIP exacerbates its ability to produce reactive oxygen species (ROS) through Fenton chemistry, ultimately leading to cell death.^[43, 44b, 46] Independent of its role in triggering ROS production, cytosolic iron promotes a specific cell death pathway termed ferroptosis, the cellular mechanisms of which are still under investigation.^[48] In healthy neurons, IRPs respond to increases in the LIP by (i) upregulation of Ft and APP and (ii) downregulation of TfR1 and DMT1, promoting iron storage/export and reducing iron uptake, respectively (Figure 2). The requirement of both IRPs for normal physiological function was demonstrated in mice by the embryonic lethality caused by deletion of both proteins.^[43] Targeted deletion of the gene encoding IRP2 resulted in excessive iron accumulation and neurodegeneration.^[44a] Absence of IRP2 may therefore be considered a primary cause of neural impairment. Disruptions in the IRP regulatory machinery were also identified in the two most prevalent age-related neurodegenerative disorders, Alzheimer's (AD) and Parkinson's (PD) diseases, as explained below.

In AD, tau hyperphosphorylation and subsequent aggregation reduces the pool of soluble tau, disrupts APP transport to the membrane, prevents APP association with Fpn, and ultimately reduces iron efflux from neurons.^[43] This sequence was confirmed by recent

studies showing Tf desaturation^[49] and downregulation of ceruloplasmin and APP in the plasma of AD patients,^[50] observations consistent with impaired iron export. Thus, iron accumulation appears to be an effect of AD progression. Another pathological feature of AD is the accumulation of aggregated amyloid beta peptide (A β), formed by amyloidogenic cleavage of APP.^[2d] A critical link between iron and AD pathology was established by the discovery that translation of APP is directly regulated by intracellular iron through an iron-responsive element in the 5'-untranslated region of its mRNA, which specifically binds to IRPs.^[51] Consequently, elevated iron levels upregulate translation of APP and increase the amount of protein that can undergo cleavage to A β . Moreover, iron promotes aggregation of both A β and hyperphosphorylated tau. The oxidative damage of A β in AD is actually attributed to its affinity toward iron, the metal-free peptide exhibiting significantly lower toxicity.^[52]

PD is pathologically characterized by the loss of dopaminergic neurons in the SNpc and the aggregation of α -synuclein. Recent advances in the development of imaging and quantitation methods that can detect iron in specific brain regions have confirmed iron accumulation in neurons and glia of the SNpc of PD patients.^[43] Iron increase alone, however, cannot explain the observed cell death in this particular region. Laser ablation-inductively coupled plasma-mass spectrometry and immunohistochemical studies attributed the pathological vulnerability of the SNpc in PD to microscopic overlap of iron with dopamine.^[53] Sustained IRP1 activity, upregulation of DMT1, and reduced ferroxidase activity of ceruloplasmin are all associated with PD and account for the observed increase in the LIP.^[43] Furthermore, a recent study connected the decrease in expression of APP in substantia nigra dopaminergic neurons with nitric oxide-induced modifications of IRP2, indicating a possible role for nitrosative stress as an upstream event in PD pathology.^[54]

Irrespective of whether it is a pathological cause or effect, accumulation of iron in neurons is a consistent observation across the entire spectrum of neurodegenerative disorders. Therefore, it offers a unique opportunity for treatment through iron chelation therapy. An ideal iron chelator for clinical application should (i) form a nontoxic complex with iron, (ii) be sufficiently liposoluble to permeate the blood-brain barrier and inner mitochondrial membrane, (iii) be neutral and of low (<500 Da) molecular weight, and (iv) selectively bind Fe³⁺ over Fe²⁺ in order not to disrupt the activity of other metalloproteins containing divalent metals such as zinc and copper.^[44b] Significant therapeutic progress was initially achieved for treatment of AD with the use of desferrioxamine and clioquinol, but each of the two chelators has drawbacks in poor blood-brain barrier permeability and neurotoxicity, respectively.^[43, 44b] A recently developed chelator with improved blood-brain barrier penetration is deferiprone, which is currently in clinical trials and has the potential to become the first disease-modifying treatment for PD.^[43] Future directions aimed at improving the clinical efficacy of iron chelation focus on the use of prochelators, natural products, nanoparticle-based delivery systems across the blood-brain barrier, and gene therapy.^[44b, 46]

Changes in iron levels outside the brain have little or no effect on brain iron homeostasis.^[43] Therefore, iron-related neurodegeneration is most likely triggered by genetic or non-genetic factors within the CNS. An important consideration when studying the neuropathology of

iron is that an increase in total brain iron alone does not necessarily induce oxidative stress, but it is rather a local alteration in iron homeostasis that is responsible for the development of disease. Iron accumulation in some brain regions may effect iron depletion in others, further impairing the neuronal function. Moreover, the mechanisms of iron uptake, storage, and utilization are specific to each cell type in the brain.^[44b] Evidence accumulated through decades of research leaves no doubt that dysregulation of iron metabolism occurs in most neurodegenerative disorders, genetic and non-genetic alike. The gaps in our understanding of the molecular mechanisms of iron trafficking and regulation in the brain hamper our ability to devise treatment strategies for these diseases. Future research on iron neurochemistry should first aim to elucidate the biological pathways of iron transport across BCECs, uptake and regulation by astrocytes and glia cells, and neuronal import from the NTBI pool. Once these mechanisms are understood, the efforts should focus on identifying the causes of aberrant iron metabolism specific to each disorder. Iron chelation therapy has thus far ameliorated the symptoms of AD, PD, and a few other diseases, but it is unlikely that it will succeed alone in curing them. Elucidation of the molecular mechanisms of iron metabolism in the brain and the pathological causes of its alteration will inform the development of targeted and more efficacious therapeutic approaches. Until then, the debate over whether brain iron accumulation is the cause or consequence of neurodegeneration continues.

5. How Does a Redox Active Metal Control Neurochemical signaling?

Copper is a redox active metal that can cycle between Cu^+ and Cu^{2+} oxidation states in biological systems.^[55] Like zinc, copper serves as a catalytic cofactor in a number of proteins but can also accumulate into pools of loosely bound ions that can participate in cell signaling pathways.^[55] Similar to iron, dysregulation of copper homeostasis has been implicated in a variety of neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and Niemann-Pick type C disease.^[56] Excess copper accumulation in the brain and other organs can lead to Wilson's disease.^[56] Copper deficiencies are a hallmark of Menkes disease, which results from mutations in ATP7A, a copper-binding ATPase that transports copper into the Golgi apparatus from the cytoplasm and also participates in synaptogenesis.^[56] Less attention has been given to the role of copper in neurotransmission. Copper can be released into the synaptic cleft and interact with a variety of synaptic proteins to modulate excitatory and inhibitory neurotransmission, as well as alter neuroproteostasis.^[57]

Whole-cell patch clamp electrophysiological recordings of cultured hippocampal neurons in the presence and absence of CuCl_2 indicate that Cu^{2+} is a noncompetitive antagonist of the NMDA receptor with an IC_{50} value of $0.27 \mu\text{M}$.^[58] Experimental data suggest that Cu^{2+} is able to inhibit the receptor in the absence of agonist in a voltage-independent manner, which would rule out the possibility of a mechanism in which Cu^{2+} mimics Ca^{2+} and permeates the channel.^[58] Instead, Cu^{2+} probably binds to the extracellular portion of the receptor.^[58] Intriguingly, in contrast to Zn^{2+} , the extent of Cu^{2+} -mediated NMDA receptor inhibition depends on the agonist concentration.^[58] Similar work in cultured rat cortical neurons revealed that Cu^{2+} can also reversibly inhibit AMPA/kainate receptors with an IC_{50} value of $\sim 4 \mu\text{M}$.^[59] Acid-sensing ion channels (ASICs) in cultured rat hypothalamic neurons can also

be inhibited by Cu^{2+} .^[60] ASICs are proton-gated sodium channels that contribute to a variety of processes throughout the nervous system, including mechanosensation, pain perception, learning, and memory.^[60] It appears that Cu^{2+} modulates these receptors through a noncompetitive, reversible mechanism with an IC_{50} value of $\sim 47 \mu\text{M}$.^[60]

Experiments with olfactory neurons from Chilean toads demonstrated that Cu^{2+} modulates neuronal excitability in a biphasic fashion.^[61] Application of Cu^{2+} in concentrations between 1 and 100 nM resulted in a concentration-dependent increase in the rate of neuronal firing, but addition of higher concentrations of Cu^{2+} between 1 and 5 μM led to a decrease in firing rate.^[61] These results, which mirror others observed with much higher concentrations of Zn^{2+} , have been attributed to differential occupancy of high and low affinity binding sites within sodium channels.^[61] Ultimately, the physiological relevance of these observations depends on the presence of sufficient inhibitory concentrations of synaptic Cu^{2+} , estimates of which span several orders of magnitude.^[59]

The invention of small molecule fluorescent probes for studying labile Cu^+ pools has provided new opportunities to study copper biochemistry.^[55] These probes can selectively detect Cu^+ in biological samples ranging from algae to cultured cells to mice.^[55] In particular, the probe CopperFluor-3 has been used to great advantage to identify pools of endogenous Cu^+ that modulate spontaneous activity in retinal and hippocampal neural circuit development.^[62] These findings raise questions about the biologically active redox states of metal ion signaling agents. Because reactive oxygen species and changes in intracellular reduction potentials can alter metal oxidation states, it is essential that future studies aim to understand how these factors modulate neurotransmission.

6. How Do Metals Affect Neurotransmission and Retrograde Signaling by Nitric Oxide?

Besides their intrinsic functions, metals in the brain mediate the activity and signal transduction pathways of a variety of chemical messengers. We discuss here a case that exemplifies the complex interplay between metals and other signaling agents, namely, the biosynthesis and neuronal activity of nitric oxide.

Nitric oxide (NO) is an inorganic diatomic radical displaying an amazingly rich chemistry and biology.^[63] Identified three decades ago as the endothelium-derived relaxing factor,^[64] this small gaseous signaling molecule has since been the subject of fascinating and intense research. The physiological functions of NO are associated with a wide range of processes in the nervous, vascular, and immune systems.^[63, 65] In the brain, NO is implicated, *inter alia*, in neuroprotection, hearing, olfaction, motor function, learning, and memory formation.^[66] The synthesis of NO is tightly regulated by metal ions, and its utilization largely relies on metalloproteins. The detailed mechanisms of these transformations, however, are only beginning to be understood.

Nitric oxide synthases (NOSs) are complex enzymes that produce nitric oxide in vivo by the five-electron oxidation of L-arginine to citrulline and NO.^[67] Among the three isoforms in humans, the constitutive neuronal NOS (nNOS) is the most abundant in the CNS.^[66c]

Metals are required at each step of NO production and signal transduction. First, the reductase domain of nNOS is regulated by complexation of Ca^{2+} to a calmodulin (CaM) cofactor.^[65a, 66c, 67a] Phosphorylation at various sites alters the sensitivity of nNOS toward Ca^{2+} /CaM binding in opposite ways, e.g., decreasing it by phosphorylating serine 847^[68] or increasing it by modifying serine 1412.^[69] Second, the active site of the oxygenase domain comprises a cysteine (Cys)-ligated iron(II) porphyrin, similar to that encountered in cytochrome P450.^[65a, 67a] Third, the enzyme is active only in the homodimer form, assembled with the aid of a zinc-bridged tetrathiolate unit in which two Cys residues from each monomer coordinate a central Zn^{2+} ion.^[65a, 67b] *S*-Nitrosation of these key Cys residues under aerobic conditions, either by an exogenous NO donor or excess endogenous NO, results in Zn^{2+} dissociation and loss of enzymatic activity.^[65a, 70] Therefore, *S*-nitrosation of the $\text{Zn}(\text{Cys})_4$ unit may act as a self-regulatory mechanism of nNOS, preventing excessive NO synthesis and associated nitrosative stress. Additional regulation is provided by the membrane association of nNOS with the NMDA receptor subunit GluN2B, which ensures proximity of the enzyme to the site of Ca^{2+} entry in the cell.^[66c, 67b] Fourth, the only known biological receptor for NO is the Fe(II) heme site of soluble guanylyl cyclase (sGC), which binds NO with remarkable affinity ($K_d \sim 20 \text{ pM}$)^[71] and selectivity ($>10,000$ -fold) over dioxygen.^[66c] sGC is responsible for conversion of GTP to the second messenger cyclic GMP (cGMP), which can exert direct actions on cyclic nucleotide-gated ion channels and phosphodiesterase enzymes, or activate protein kinase G (PKG).^[66c] Finally, other important biological metal targets of NO have been identified, such as the heme a_3 group of mitochondrial cytochrome *c* oxidase, the iron-sulfur clusters of metalloproteins, and the zinc centers of metallothionein.^[63, 65a, 72] Even though the foregoing NO-induced protein modifications were characterized *in vitro*, the mechanisms and physiological relevance of these processes *in vivo* are far from being understood,^[63] owing at least in part to the difficulty in tracking and quantitating biological NO.

A particular aspect of neurotransmission by nitric oxide that has attracted considerable interest relates to the potential function of NO as a retrograde synaptic messenger. Activity-dependent regulation of synapses by retrograde signaling involves “on demand” neurotransmitter release from a postsynaptic neuron and subsequent activation of presynaptic targets.^[73] NO satisfies several of the criteria established for a retrograde messenger.^[73–74] The proposed retrograde signaling pathways of NO, summarized in Figure 3, encompass and recapitulate all of the previously mentioned interactions with metal ions.

The putative signaling cascade is initiated when presynaptically released glutamate opens NMDA receptors on the postsynaptic terminal, allowing Ca^{2+} influx and binding to CaM, which in turn activates nNOS to produce NO from L-arginine. Gaseous NO freely diffuses out of the postsynaptic neuron and travels across the synapse into the presynaptic terminal, where it either binds to the metal centers of sGC and/or other metalloenzymes, or *S*-nitrosates cysteine residues of proteins. NO-bound sGC promotes the conversion of GTP to cGMP and subsequent activation of PKG (Figure 3). The combined actions of NO, cGMP, and PKG eventually inhibit voltage-gated $\text{K}^+/\text{Ca}^{2+}$ channels and stimulate presynaptic neurotransmitter release at either excitatory (glutamatergic) or inhibitory (GABAergic) terminals.^[66c, 73] The molecular mechanisms of transduction in the presynaptic component of NO retrograde signaling are far from being elucidated, however. The difficulties in

proving NO to be a retrograde messenger primarily relate to practical limitations, as discussed below.

As an integral feature of its retrograde signaling function, NO is associated with the induction of long-term potentiation (LTP) or depression (LTD) in various regions of the brain.^[74–75] Most of the evidence supporting the role of NO in neurotransmission has been accumulated in vitro, however, and consists of electrophysiology data. Experiments are typically performed by external application of extreme stimuli such as high concentrations of NO donors, potassium ions, or nNOS inhibitors, creating a physiologically irrelevant environment.^[73] Differences in cellular strains and experimental protocols hamper reproducibility and independent validation. Furthermore, the many biological sites of NO action make it unfeasible to manipulate individual presynaptic targets to alter neurotransmitter release, a required criterion for establishing a retrograde signaling pathway.^[73] Lastly, controversy still remains over the possible production of NO in presynaptic neurons.^[76] Taken together, these factors have produced inconclusive and contradictory results spanning decades of research, as illustrated by studies of NO-induced hippocampal LTP.^[73] Initial reports^[74–75] strongly suggested that inhibition of nNOS and application of extracellular NO scavengers disrupts induction of LTP in the CA1 region of the hippocampus, but recent results^[75e] contradict this 25-year old hypothesis. Given these controversies, we suggest that future research in this area should focus on answering two important questions: (i) what, if any, is the physiological function of retrograde signaling by NO? and (ii) what is the relative contribution of the retrograde component of NO neurotransmission to synaptic plasticity? Clearly new strategies and tactics are required to address these questions.

Progress in validating the biological routes by which NO relays its neural signals has traditionally been hindered by the lack of suitable instrumentation and detection methods in live cells. For decades, NO was detected extracellularly, indirectly, and often inaccurately.^[77] Studies of NO signaling pathways are still burdened by the “ignorance of exactly where the NO comes from and where it acts.”^[66c] In order to unequivocally establish the roles of NO in retrograde signaling and synaptic LTP/LTD, the following guidelines^[73] are advised: (1) perform studies under more physiologically relevant conditions; (2) minimize reliance on extreme stimuli; (3) establish sufficiency of NO in modulating synaptic transmission; (4) quantitate intrasynaptic NO release; (5) improve spatiotemporal resolution of NO detection. Meeting these challenges, especially the last two, will be greatly facilitated by the design and implementation of improved fluorescent probes for direct NO sensing. Fluorescence imaging is the only non-intrusive technique with the potential to achieve the required selectivity, sensitivity, subcellular localization, analyte quantitation, and dynamic monitoring of NO signaling events at neuronal synapses. Even though immense progress was already achieved with the discovery of CuFL1^[78] and Cu₂FL2E,^[79] the first fluorescent sensors that detect endogenous NO directly with nM sensitivity, an unmet need remains in the field for probes that emit in the near-infrared and are ratiometric, organelle-targeted, and reversible. Finally, the recent emergence of nitroxyl (HNO) as a signaling agent endowed with unique physiological actions in the CNS^[80] opens additional avenues of research in neurochemistry. Because the chemistry and biological activity of HNO are distinct from those of NO,^[81] a requirement has risen for new

generations of reporters able to discriminate the two with spatiotemporal resolution in biological milieu. The recent development of HNO-selective amperometric,^[82] reaction-^[83] and metal-based^[84] fluorescent sensors should be valuable in untangling the signaling pathways of NO and HNO, two closely related messengers in the brain.

7. Outlook

Metal ions play fundamental roles in the central nervous system and we are only beginning to understand how they contribute to health and disease. The vignettes presented above offer only a glimpse of a broad and exciting field, for which we suggest some general topics for those interested in pursuing metalloneurochemistry. First, there is a need for better analytical tools to study metal ions and other neurotransmitters in complex, living organisms with high spatial and temporal resolution. Methods to accurately determine concentration and speciation in live tissue are critical if we are to fully understand these systems. Second, metal ions participate in a tightly regulated network. Changes in the concentration or oxidation state of one metal can cause unexpected—and often unpredictable—changes in the activity of other metals. Part of this network is depicted in Figure 4, which shows the interdependence of several important metals in mammals.^[85] As research continues, a fuller picture of how these ions interact with each other will continue to materialize. Because none of these pathways exist in isolation, it is incumbent upon researchers to avoid tunnel vision and relate their work to this larger framework.

Third, and perhaps most important, the immense complexity of the brain requires that all results obtained from simple model studies be considered in the context of a dynamic living system. It is the obligation of all metalloneurochemists to exercise caution when extrapolating data collected under non-physiological conditions to draw conclusions about in vivo behavior. For these reasons, we should avoid shallow draughts and instead drink deeply from the Pierian spring.

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Biographies

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Andrei Loas obtained a B.Eng. degree in Chemical Engineering from the Polytechnic University of Bucharest, Romania (2007). He further pursued a Ph.D. in Materials Science and Engineering at New Jersey Institute of Technology (2012), working under the guidance of Dr. Sergiu M. Gorun on hydrogen-free metal-organic oxidation catalysts. His current research as a Postdoctoral Associate in the laboratory of Dr. Stephen J. Lippard at MIT is focused on developing fluorescent probes to investigate the biological functions of nitric oxide and nitroxyl.

Stephen J. Lippard is the Arthur Amos Noyes Professor of Chemistry at the Massachusetts Institute of Technology. His research interests span the fields of inorganic chemistry, biochemistry, and neuroscience. He earned a bachelor's degree at Haverford College and received his Ph.D. from MIT in 1965. After spending a year as a postdoctoral fellow at MIT, he then joined the faculty at Columbia University, where he was promoted to full professor in 1972. In 1983, he returned to MIT as professor of chemistry, where he served as head of the chemistry department from 1995 to 2005.

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Figure 1. Structure of the NMDA receptor. A surface representation of the heterotetrameric NMDA receptor is shown in gray depicting the amino terminal, ligand binding, and transmembrane domains. The carboxy terminal domain has been omitted. Representative GluN1 and GluN2B subunits are shaded pink and blue, respectively. Glycine (green spheres) and glutamate (orange spheres) binding sites are also shown. Figure modified from PDB 4PE5 and rendered in PyMOL (Schrödinger, Portland, OR). Adapted from reference 8.

Figure 2.

General mechanisms of neuronal iron homeostasis. Abbreviations: Tf = transferrin, TfR1 = transferrin receptor 1, DMT1 = divalent metal transporter 1, LIP = labile iron pool, Ft = ferritin, Fpn = ferroportin, APP = amyloid precursor protein, CP = ceruloplasmin, IRP1,2 = iron response proteins. Adapted from references 43, 44, and 46.

Figure 3.

Proposed retrograde signaling pathways of NO. Abbreviations: NMDAR = NMDA receptor, nNOS = neuronal nitric oxide synthase, CaM = calmodulin, VGCC = voltage-gated calcium channel, VGKC = voltage-gated potassium channel, sGC = soluble guanylyl cyclase, cGMP = cyclic GMP, PKG = protein kinase G. Adapted from references 72 and 73.

Figure 4.

A partial network of metal ion interdependence. Arrows indicate a relationship whereby changes in concentration of the starting element alter the activity or distribution of the linked element, or in which an increase in concentration of the linked element abrogates the activity of the starting element. Adapted from reference 84.