Divalent metal transporter 1 (DMT1) regulation by Ndfip1 prevents metal toxicity in human neurons

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The regulation of metal ion transport within neurons is critical for normal brain function. Of particular importance is the regulation of redox metals such as iron (Fe), where excess levels can contribute to oxidative stress and protein aggregation, leading to neuronal death. The divalent metal transporter 1 (DMT1) plays a central role in the regulation of Fe as well as other metals; hence, failure of DMT1 regulation is linked to human brain pathology. However, it remains unclear how DMT1 is regulated in the brain. Here, we show that DMT1 is regulated by Ndfip1 (Nedd4 family-interacting protein 1), an adaptor protein that recruits E3 ligases to ubiquitinate target proteins. Using human neurons we show that Ndfip1 is upregulated and binds to DMT1 in response to Fe and cobalt (Co) exposure. This interaction results in the ubiquitination and degradation of DMT1, resulting in reduced metal entry. Induction of Ndfip1 expression protects neurons from metal toxicity, and removal of Ndfip1 by shRNAi results in hypersensitivity to metals. We identify Nedd4–2 as an E3 ligase recruited by Ndfip1 for the ubiquitination of DMT1 within human neurons. Comparison of brains from Ndfip1−/− with Ndfip1+/+ mice exposed to Fe reveals that Ndfip1−/− brains accumulate Fe within neurons. Together, this evidence suggests a critical role for Ndfip1 in regulating metal transport in human neurons.

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The brain is a specialized organ that requires metals ions for a number of important cellular processes. As such, the brain contains a relatively high concentration of a number of metals such as Fe, Zn, and Cu (in the order of 0.1–0.5 mM) (1). Importantly, the concentrations of these metals are potentially toxic under stress conditions without the requirement of exogenous uptake through ingestion. It is therefore crucial that the brain has highly efficient homeostatic mechanisms in place to prevent aberrant metal toxicity. Over the last decade, growing evidence suggests that the misregulation of metals within the brain is involved in the neuro-pathology of a number of disorders, such as Parkinson’s and Alzheimer’s diseases (2). Metal ions are suggested to have two distinct roles in the pathophysiology of brain disorders. Firstly, redox active metals such as Cu, Fe, Mg, and Co can result in metal-catalyzed protein oxidation that leads to protein damage and denaturation (3). Secondly, metal-protein associations can result in protein aggregation and the formation of insoluble protein bodies (4). In addition to the role metals can play in disease states, it has also now become clear that at times of stress, such as in trauma or stroke, neurons become vulnerable to uncontrolled entry of excess metals (3). It is therefore critical that the brain is able to mount cellular defense mechanisms against sudden surges of metal toxicity.

Normal metal uptake occurs via transferrin-bound Fe that is incorporated into cells by an endocytotic process initiated by transferrin receptor 1 (TfR1) (5). Non-protein-bound metals can also enter cells through a promiscuous metal transporter, divalent metal transporter 1 (DMT1), previously known as DCT1, NRAMP2, or Slc11a2 (6, 7), that is known to actively transport Fe, Zn, Mn, Co, Cd, Cu, Ni, and Pb, via a proton-coupled mechanism (8). DMT1 is predicted to have 12 transmembrane segments and is expressed in neurons, allowing for the incorporation of metals from the extracellular environment and/or the recycling endosomes. A spontaneous mutation at position G158SR in DMT1 results in deficits in Fe adsorption in both the mk mouse and the Belgrade rat, giving rise to microcytic, hypochromic anemia (9, 10). These mutant mice also suffer from neurodegeneration in a Parkinsonian model, pointing to an important role for DMT1 in the regulation of metals in the brain (11). Transcriptional control of DMT1 expression through Fe response elements has been described previously (12); however, little is known about the post-translational control mechanisms regulating DMT1 protein levels within the brain. Lessons from yeast studies have demonstrated that the DMT1 homolog, known as Smf1, is degraded by ubiquitination after binding to Bsd2 through an E3 ligase mechanism (13, 14).

In a previous study, we demonstrated that Nedd4 family-interacting protein 1 (Ndfip1) is upregulated in cortical neurons following traumatic brain injury, and this over-expression is protective against neuronal apoptosis in vitro (15). The precise mechanisms of neuroprotection by Ndfip1 remains to be elucidated but given the role of its yeast homologue Bsd2 in metal homeostasis, it is of interest to discover if similar mechanisms apply in neuroprotection, particularly in humans. To test this hypothesis, we used a culture model involving human primary cortical neurons, together with a human neuronal cell line. We show that metal ions upregulate Ndfip1 in human neurons, and induction of Ndfip1 expression is protective against Co or Fe toxicity. Conversely, knock down of the Ndfip1 mRNA results in increased sensitivity of cells to Co and prevents them from downregulating DMT1. We demonstrate that Ndfip1-binding to DMT1 is instrumental for ubiquitination and downregulation of DMT1 and identify Nedd4–2 as a ubiquitin ligase for polyubiquitination of DMT1 under metal-induced stress. Lastly, we show that Ndfip1−/− mice have an increased accumulation of Fe within the brain when exposed to acute metal toxicity.

Results

Ndfip1 in Human Neurons Is Upregulated in Response to Co and Fe Exposure. Neuronal exposure to metal ions induces a number of biochemical responses that can result in cellular toxicity. To test the function of Ndfip1 under such conditions, CoCl2 was administered to the human neuroblastoma cell line SH-SY5Y. This cell line has...
a basal level of Ndfip1 expression (Fig. 1A, control lane). Addition of CoCl₂ in increasing molar concentrations caused an upregulation of Ndfip1 in a dose-response manner up to lethal concentrations (Fig. 1A). Similarly, cells exposed to 200 μM of FeCl₂ also showed an upregulation of Ndfip1 (Fig. 1A). Ndfip1 is also present at basal levels in cultured primary cortical neurons from 18-week-old human embryos (Fig. 1B, control lane). Exposure of these neurons to 200 μM CoCl₂ over 18 h showed upregulation of Ndfip1 by approximately two-fold compared to the basal level of the protein (Fig. 1B). We observed that the increased levels of Ndfip1 protein was a result of increased protein expression rather than a change in protein stability (Fig. S1). Immunocytochemistry using polyclonal antibodies showed upregulation of Ndfip1 in cultured human primary neurons following exposure to CoCl₂ for 24 h (Fig. 1C).

**Ndfip1 Promotes the Survival of Neurons from Metal Toxicity.** The results from the metal ion treatment suggested that upregulation of Ndfip1 in human neurons may provide a critical defense strategy against metal poisoning. To test this further, an inducible expression system for Ndfip1 was developed wherein the Ndfip1 gene was driven off the yeast Gal4/UAS transcription system (Fig. 2A). As previously described this system relies on the ability of Hsp90 to drive off the yeast Gal4/UAS transcription system (Fig. 2A) but upregulated (arrowheads) when exposed to 200 μM Co for 18 h. Representative blots from at least three independent experiments shown. (C) Immunocytochemistry with anti-Ndfip1 antibodies indicate basal levels of Ndfip1 expression (control) in cultured human primary cortical neurons (11 DIV) but upregulated (arrowheads) when exposed to 200 μM CoCl₂. (Scale bar, 10 μm.)

![Fig. 1.](image1.png)  
**Fig. 1.** Ndfip1 is upregulated in neurons in response to metal exposure. (A) SH-SY5Y cell lysates after exposure to increasing concentrations of Co for 18 h results in increased Ndfip1 expression as seen in western blots. A similar result was observed when cells were treated with Fe. (B) Treatment of human primary cortical neurons with Co also upregulates Ndfip1 when treated with 200 μM Co for 18 h. Representative blots from at least three independent experiments shown. (C) Immunocytochemistry with anti-Ndfip1 antibodies indicate basal levels of Ndfip1 expression (control) in cultured human primary cortical neurons (11 DIV) but upregulated (arrowheads) when exposed to 200 μM CoCl₂. (Scale bar, 10 μm.)

![Fig. 2.](image2.png)  
**Fig. 2.** Ndfip1 protects human neurons from metal toxicity. (A) Tamoxifen-inducible expression construct of Ndfip1-Flag was created and used in a stable line of SH-SY5Y cells (anti-Flag blot shown). (B) Flow cytometry profile of SH-SY5Y cells treated with Co for 18 h. Different distributions of alive (quadrant 1), apoptotic (quadrant 2), and apoptotic plus necrotic populations (quadrant 3) between control and treated cells are shown. Cells treated with Co are dying through an apoptotic pathway. (C) Flow cytometry analysis of SH-SY5Y cells induced to express exogenous Ndfip1 protein protects against Co toxicity, compared with uninduced controls. (D) Flow cytometry analysis indicates that human embryonic cortical neurons induced to express exogenous Ndfip1 are protected against Co toxicity, compared to uninduced controls. (E) SH-SY5Y cells stained for Fe uptake show that cells induced to express Ndfip1 prevented Fe uptake compared with uninduced controls. Two-way Anova tests (Bonferroni posttests) indicate significant protection by Ndfip1 at higher concentrations of Co in (C) and (D) (P < 0.05* and P < 0.001***, = SEM). Each flow cytometric analysis represents three independent experiments.

Our results indicate that Ndfip1 is protective against metal poisoning; however, it was unclear whether this was due to a reduction in metal accumulation or the ability of the cells to tolerate higher concentrations of metals. Using inducible Ndfip1 SH-SY5Y
cells, we stained for cellular Fe levels after treatment with FeCl₂. In the absence of Ndfip1 induction, Fe staining was visible at 150 μM with higher levels observed at 300 μM leading to cell death (Fig. 2E, top row). In cells induced to express Ndfip1, few cells contained any Fe staining at 150 μM concentration. Only at 300 μM was Fe staining observed at levels seen in uninduced cells at 150 μM (Fig. 2E, bottom row). This result suggests that Ndfip1 acts to limit the amount of metals that can enter the cell.

Removal of Ndfip1 Promotes Neuronal Death from Metal Toxicity. The above experiments with both primary cortical neurons and a neuronal cell line indicate that Ndfip1 is upregulated in response to metals, and is protective against metal-induced apoptosis. To investigate this further, a lentiviral shRNAi construct was used to transiently knock down Ndfip1 in SH-SY5Y cells (Fig. 3A). Control cells were infected with lentiviral particles containing a non-functioning shRNAi. Infected cells (and control infections) were treated with increasing concentrations of CoCl₂ (0–500 μM) and compared for cell death using flow cytometry and staining with annexin V and propidium iodide (Fig. 3B). The results reveal effects that are contrary to that of Ndfip1 induction described above (Fig. 2C), and show that cells containing Ndfip1 shRNAi are more susceptible to Co-induced apoptosis at low concentrations of CoCl₂ (up to 300 μM). Interestingly, no significant difference was observed between Ndfip1 shRNAi and control shRNAi cells at higher concentrations (400–500 μM) of CoCl₂ (Fig. 3B). Nonetheless, the knock-down data mirrors the Ndfip1-induction result, wherein at lower concentrations (0–300 μM) of CoCl₂, induction of Ndfip1 has no protective effect on SH-SY5Y cells (Fig. 2C). One possible explanation is that endogenous Ndfip1 levels are protective at lower concentrations of CoCl₂, therefore removal of Ndfip1 renders these cells susceptible to lower CoCl₂ concentrations. At higher concentrations of CoCl₂ (500 μM), the endogenous Ndfip1 appears to be as ineffective as the Ndfip1 shRNAi cells in protecting against cell death.

Ndfip1 Interacts with DMT1 and Facilitates Its Ubiquitination in Neurons. The data above demonstrate that Ndfip1 can protect neurons (from human fetal brain tissue and a neuronal cell line) from death arising from metal toxicity by limiting the amount of metal entry. A critical issue is to understand the possible mechanism behind this protective function. In yeast, Bsd2 (homolog of Ndfip1) binds and degrades the metal transporter Smf1 (homolog of DMT1) (13, 14). To explore this relationship in human neurons, immunohistochemistry and immunoprecipitation studies were carried out. Tissue sections of the human cortex show co-extensive staining of Ndfip1 and DMT1 (Fig. 4A), implying that these two proteins are compartmentalized in similar locations within the cell. To explore binding between these two proteins, human cortical neurons were used for immunoprecipitation with antibodies to Ndfip1 and DMT1, before and after treatment with CoCl₂. The results show that lysates immunoprecipitated with DMT1 antibodies also contain Ndfip1 (Fig. 4B). Conversely, immunoprecipitation with Ndfip1 antibodies brought down DMT1; however, this band is substantially reduced when the cells were pretreated with CoCl₂ (Fig. 4C), suggesting that DMT1 levels are reduced in the presence of Co. This data provides evidence that DMT1 is a binding partner for Ndfip1 in human neurons.

Since Ndfip1 is known to play a pivotal role in targeting proteins for E3 ligase ubiquitination (18), it is of interest to examine whether or not binding of Ndfip1 to DMT1 results in ubiquitination of DMT1. To examine this, SH-SY5Y cells were subjected to increasing concentrations (0–400 μM) of CoCl₂ and western blotting for DMT1. The results indicated that DMT1 was downregulated as CoCl₂ concentrations increased (Fig. 4A). If Ndfip1 is required for the degradation of DMT1, then cells deficient in Ndfip1 should not be able to downregulate DMT1 in the presence of Co. To test this, SH-SY5Y cells containing shRNAi directed toward Ndfip1 were exposed to CoCl₂ for 18 h before western blotting for DMT1. Cells with Ndfip1 shRNAi were unable to degrade DMT1 in 400 μM CoCl₂ whereas control shRNAi cells were able to efficiently degrade DMT1 (Fig. 4B). Conversely we postulated that upregulation of Ndfip1 would therefore result in the degradation of DMT1. Using the inducible Ndfip1 SH-SY5Y cell line, cells administered 4-hydroxy tamoxifen to induce Ndfip1 expression show a decrease in the levels of DMT1 present (Fig. 4C, lane 2) compared with the non-induced control. Given the function of Ndfip1 in ubiquitination, MG132 was used to determine whether the degradation of DMT1 was proteasome-dependent. With the proteasome blocked by MG132, DMT1 was not downregulated despite Ndfip1 induction (Fig. 4C, lane 4). This suggests Ndfip1-mediated ubiquitination and degradation of DMT1 is proteasome-dependent. To examine DMT1 ubiquitination by Ndfip1, we induced SH-SY5Y cells with 4-hydroxy tamoxifen for 16 h before the cells were harvested. Lysates were immunoprecipitated for DMT1 and blotted for polyubiquitination. Without induction of Ndfip1, immunoprecipitated DMT1 is associated with a basal level of polyubiquitination. This data provides evidence that DMT1 is a binding partner for Ndfip1 in human neurons.
ubiquitination (Fig. 5D, lane 1). Following induction of Ndfip1, the level of ubiquitinated DMT1 is increased (Fig. 5D, lane 2, cell lysate lanes indicate similar loading levels).

**Ndfip1 Protects Against Fe Toxicity and Its Effect Is Comparable to a Known Anti-Oxidant, Ebselen.** Thus far, we observed that Fe and Co both upregulate Ndfip1 (Fig. 1) and induced expression of Ndfip1 protects neurons from toxic levels of Co (Fig. 2). Given that DMT1 is known to transport a number of metal ions, we postulated that Ndfip1 should protect against other metals. Iron is of particular importance in metabolic processes of neurons and currently implicated in a number of brain pathologies. To test Ndfip1 protection against iron, SH-SY5Y cells were exposed to increasing concentrations of FeCl₂ with and without inducible expression of Ndfip1. Flow cytometry analysis of apoptotic cells revealed similar protective effects compared to Co, with two-fold protection against Fe-induced death at concentrations above 200 μM (Fig. 6A). It has been reported that the regulation of Fe transport by DMT1 can be controlled by the drug Ebselen (19) that is currently the subject of a phase III clinical trial in ischemic stroke (20). To compare the efficacy of Ndfip1 relative to Ebselen, the same FeCl₂ assay was conducted on SH-SY5Y cells exposed to Ebselen (Fig. 6B). The results showed that Ebselen was equally protective at low concentration of Fe, but at higher concentrations of Fe, Ebselen was less effective compared to Ndfip1 (Fig. 6A and B).

**The E3 Ligase Nedd4–2 Is Recruited by Ndfip1 To Ubiquitinate DMT1.** The experiments in the previous sections indicated that Ndfip1 binds to DMT1 under normal and stressed conditions; and that Ndfip1 is not only upregulated by metal ions, but in their presence, DMT1 levels are diminished, most likely by protein ubiquitination. It is therefore of interest to ascertain which E3 ligase mediates ubiquitination since Ndfip1 by itself is unable to catalyze ubiquitin tagging. DMT1 does not contain a PPxY motif that is classically required for binding to members of the Nedd4 family of E3 ligases (21). However, Ndfip1 is known to strongly bind to several Nedd4 family proteins (22). To address this, immunoprecipitation assays were conducted on human cortical neurons using a DMT1 antibody and western blots probed for the Nedd4 E3 ligases, Nedd4–2, and Itch. The experiments in the previous sections indicated that Ndfip1 binds to DMT1 under normal and stressed conditions; and that Ndfip1 is not only upregulated by metal ions, but in their presence, DMT1 levels are diminished, most likely by protein ubiquitination. It is therefore of interest to ascertain which E3 ligase mediates ubiquitination since Ndfip1 by itself is unable to catalyze ubiquitin tagging. DMT1 does not contain a PPxY motif that is classically required for binding to members of the Nedd4 family of E3 ligases (21). However, Ndfip1 is known to strongly bind to several Nedd4 family proteins (22). To address this, immunoprecipitation assays were conducted on human cortical neurons using a DMT1 antibody and western blots probed for the Nedd4 E3 ligases, Nedd4–2, and Itch. The results showed that Nedd4–2, but not Itch, was immunoprecipitated with DMT1 antibodies (Fig. 7A). Taken together these results suggest that Ndfip1, DMT1, and Nedd4–2 all form a complex that can result in the polyubiquitination of DMT1.

**Mouse Brains Show an Accumulation of Fe in Neurons.** If Ndfip1 can regulate DMT1 levels within neurons, then mice lacking Ndfip1 should accumulate excess metals due to misregulation of DMT1. Ndfip1<sup>−/−</sup> mice were generated by disruption of...
Brains sections from both Ndfip1−/− mice showed increased levels of Fe within the brain when compared with wild-type littermates. Ndfip1−/− mice show an increased level of DMT1 protein it is of interest to know whether other metal entry pathways were disrupted due to the misregulation of DMT1. The transferrin receptor is the major Fe entry pathway in most cells. Western blot analysis with TIR antibody showed that in Ndfip1−/− mice there is a decrease in the levels of TIR when compared with wild-type littermates (Fig. S2). Further studies are required to determine whether there is compensation of TIR levels due to the increased DMT1 levels.

Discussion

Transition metals are an essential requirement for brain function. However, the nervous system contains a number of metals at concentrations that if perturbed, acutely or chronically, can lead to cellular toxicity. The effect of excess metals within neurons is well-documented, with reports showing an increase in free radicals or the aggregation of proteins (3, 4). However, there is limited information on the nature of direct molecular and biochemical responses to excess metals in human neurons. The present study has shed light on this process by identifying Ndfip1 as a key protein in the cellular defense mechanism against metal toxicity.

Recently, DMT1 has been implicated in the neuropathology of excitotoxic cell death and also Parkinson’s disease (11, 25). During brain trauma or stroke, excitotoxic neuronal death occurs due to the uncontrolled release of glutamate or aspartate. Activation of glutamate-NMDA receptors has been found to stimulate nNOS which results in the activation of Dxtax1, this in turn can induce Fe uptake via interaction between PAP7 and DMT1 (25). Increased DMT1 activity leading to excessive Fe uptake was causally linked to cellular toxicity from reactive oxygen species. Excitotoxic neuronal death could be alleviated by using cell-permeable Fe chelators, pointing to a strong link between excess Fe and NMDA-induced neurotoxicity. In Parkinson’s patients, it was also found that dopaminergic neurons accumulate DMT1 (11). Additionally, mouse models of Parkinson’s disease demonstrate a causal link between Fe accumulation and neuronal death (11). Together, these disease models point to DMT1 misregulation as a focal mechanism for dysregulated metal uptake, leading to neuronal death.

The above suggests that post-translational control of DMT1 is a critical regulatory system in the prevention of metal accumulation and neuronal apoptosis. Our central hypothesis was framed around Ndfip1 for two reasons. First, previous work from this laboratory had identified Ndfip1 to be a critical protein for improving neuronal survival during stress in vitro and associated with neuronal survival in vivo following traumatic brain injury (15). Ndfip1 is endogenously present in cortical neurons at low levels, most likely residing in the Golgi complex. Upon activation by injury or stress, Ndfip1 is dramatically upregulated in neurons extending to the cytoplasm and neuronal processes. Importantly, upregulation of Ndfip1 is consistently and robustly associated with neuronal survival, and neurons undergoing apoptosis do not show Ndfip1 upregulation. Second, the yeast homolog of Ndfip1 has been previously shown to be critical for defending against toxicity by transition metals (13).

Given the evolutionary divergence between yeast and man, it is remarkable that Ndfip1 and its yeast homolog Bsd2 share similar mechanistic traits in protecting cells against metal poisoning. Importantly, both yeast and humans require an E3 ligase to catalyze degradation of the metal transporter by protein ubiquitination. In the case of humans we have found the E3 ligase is Nedd4–2, a mammalian relative of yeast Rsp5. As in yeast, the mammalian metal transporter DMT1 lacks a PPxY motif that is required for binding to Nedd4 ligases; this is circumvented by binding of DMT1 to Ndfip1 whose PPxY motifs are capable of binding to WW domains of Ndfip1 gene with a gene trap vector, causing truncation of the

The transferrin receptor is the major Fe entry pathway in most cells. Western blot analysis with TIR antibody showed that in Ndfip1−/− mice there is a decrease in the levels of TIR when compared with wild-type littermates (Fig. S2). Further studies are required to determine whether there is compensation of TIR levels due to the increased DMT1 levels.

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domains of Nedd4–2 (18). It appears that multiple members of the Nedd4 family are capable of binding to Ndfip1 for E3 ligase activity; in non-neuronal cells, Ndfip1 modulates Fe entry by interacting with the E3 ligase, WWP2, for DMT1 regulation (20). Hence, proteolysis against toxicity by metals through the ubiquitination of metal transporters is an ancient and highly conserved mechanism. Ubiquitination of proteins is a potent mechanism used for regulating protein stability, protein activity, and subcellular localization (27). The nervous system uses this versatile process in the development and migration of neurons, elaboration of synapses, and maintenance of neuronal circuits (28). Another aspect of the present study is the demonstration that protein ubiquitination by Ndfip1 via Nedd4–2 is instrumental for improving the survival of neurons under stress from metal toxicity, and as previously reported, for traumatic brain injury (15). In particular, the involvement of Nedd4 family of E3 ligases is significant, given that this family of genes was originally cloned in the developing nervous system (29), and its function in the nervous system has recently gained prominence. For example, in Drosophila, Nedd4 binding and ubiquitination of Commissureless has important effects in regulating axon guidance by the Commissureless target, Roundabout (30). In the rat dorsal ganglion, Nedd4–4 binding and ubiquitination of TrkA receptor selectively influences the survival of NGF—burden of BDNF-dependent neurons (31). The present study adds a further dimension to this important mechanism in the nervous system by emphasizing the role of Nedd4 adaptors such as Ndfip1. So while many proteins may not be recognized by Ndfip4 ligases due to the lack of the recognition motif PPXY, these proteins may bind to Ndfip1 and be avidly presented for ubiquitination by Nedd4 proteins. The list of known Ndfip1 targets remains small; apart from DMT1, Ndfip1 is linked to the ubiquitination of JunB (23). The use of adaptors such as Ndfip1 can endow greater versatility to the system; by adding a higher level of selectivity and access to targets that may otherwise be inaccessible to Nedd4. Since Ndfip1 can also be ubiquitinated by Nedd4 (22), the process may be rapidly turned off by degradation of Ndfip1.

In conclusion, we have shown that human neurons in culture are susceptible to metal toxicity in cellular assays involving increasing concentrations of environmental Fe and Co. This serves as an in vitro paradigm of brain injury paralleling stroke and brain trauma where transient levels of metals are known to flood the injury site. We have identified DMT1 as the key player in the entry of metal ions in the brain, and this process may be reversed by DMT1 degradation. Regulation of DMT1 levels is achieved by ubiquitination, via binding of DMT1 to the Nedd4–adaptor, Ndfip1. We demonstrate that increasing Ndfip1 in neurons provides effective defense against high concentrations of metal. In addition to these acute assays, we also show that Ndfip1 is critical for the longer-term regulation of metal in the brain. In mice lacking Ndfip1, Fe is abnormally concentrated in cortical neurons suggesting a housekeeping role for Ndfip1 in metal homeostasis.

Materials and Methods

Human Embryonic Cortical Neuron Cultures. Human cortices from 18-week-old embryos were obtained from NSW Fetal Tissue Consortium. Brain tissue was collected in HEPES buffered MEM and all procedures were conducted under sterile conditions. Tissue was dissociated and plated in neurobasal media supplemented with 1 × B27 (Invitrogen) and 0.5 mM L-glutamate. Neurons were seeded at 4 × 10⁴ cells per plate, and cultured with 5% CO₂ at 37 °C. Transfection of neurons was performed using Amaxa according to the manufacturers protocol. Inducible Ndfip1 plasmids (2 μg total) were added and cells electroporated using the mouse hippocampal neuron protocol.

Cell Culture and Lentiviral Infection. See SI Materials and Methods.

Metal Toxicity and Apoptosis Quantification by Flow Cytometry. See SI Materials and Methods.

Cobalt Uptake and Staining of SH-SY5Y Cells. See SI Materials and Methods.

Western Blots and Immunoprecipitation Assays. See SI Materials and Methods.

Immunohistochemistry. See SI Materials and Methods.

Histology and Brain Fe Measurements. See SI Materials and Methods.

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13. Keiser SM, et al. (2008) NDFIP1, an Nedd4–2-dependent E3 ligase, modulates Fe homeostasis in non-neuronal cells, Ndfip1 modulates Fe entry by interacting with the E3 ligase, WWP2, for DMT1 regulation (20). Hence, proteolysis against toxicity by metals through the ubiquitination of metal transporters is an ancient and highly conserved mechanism. Ubiquitination of proteins is a potent mechanism used for regulating protein stability, protein activity, and subcellular localization (27). The nervous system uses this versatile process in the development and migration of neurons, elaboration of synapses, and maintenance of neuronal circuits (28). Another aspect of the present study is the demonstration that protein ubiquitination by Ndfip1 via Nedd4–2 is instrumental for improving the survival of neurons under stress from metal toxicity, and as previously reported, for traumatic brain injury (15). In particular, the involvement of Nedd4 family of E3 ligases is significant, given that this family of genes was originally cloned in the developing nervous system (29), and its function in the nervous system has recently gained prominence. For example, in Drosophila, Nedd4 binding and ubiquitination of Commissureless has important effects in regulating axon guidance by the Commissureless target, Roundabout (30). In the rat dorsal ganglion, Nedd4–4 binding and ubiquitination of TrkA receptor selectively influences the survival of NGF—burden of BDNF-dependent neurons (31). The present study adds a further dimension to this important mechanism in the nervous system by emphasizing the role of Nedd4 adaptors such as Ndfip1. So while many proteins may not be recognized by Ndfip4 ligases due to the lack of the recognition motif PPXY, these proteins may bind to Ndfip1 and be avidly presented for ubiquitination by Nedd4 proteins. The list of known Ndfip1 targets remains small; apart from DMT1, Ndfip1 is linked to the ubiquitination of JunB (23). The use of adaptors such as Ndfip1 can endow greater versatility to the system; by adding a higher level of selectivity and access to targets that may otherwise be inaccessible to Nedd4. Since Ndfip1 can also be ubiquitinated by Nedd4 (22), the process may be rapidly turned off by degradation of Ndfip1.

In conclusion, we have shown that human neurons in culture are susceptible to metal toxicity in cellular assays involving increasing concentrations of environmental Fe and Co. This serves as an in vitro paradigm of brain injury paralleling stroke and brain trauma where transient levels of metals are known to flood the injury site. We have identified DMT1 as the key player in the entry of metal ions in the brain, and this process may be reversed by DMT1 degradation. Regulation of DMT1 levels is achieved by ubiquitination, via binding of DMT1 to the Nedd4–adaptor, Ndfip1. We demonstrate that increasing Ndfip1 in neurons provides effective defense against high concentrations of metal. In addition to these acute assays, we also show that Ndfip1 is critical for the longer-term regulation of metal in the brain. In mice lacking Ndfip1, Fe is abnormally concentrated in cortical neurons suggesting a housekeeping role for Ndfip1 in metal homeostasis.

Materials and Methods

Human Embryonic Cortical Neuron Cultures. Human cortices from 18-week-old embryos were obtained from NSW Fetal Tissue Consortium. Brain tissue was collected in HEPES buffered MEM and all procedures were conducted under sterile conditions. Tissue was dissociated and plated in neurobasal media supplemented with 1 × B27 (Invitrogen) and 0.5 mM L-glutamate. Neurons were seeded at 4 × 10⁴ cells per plate, and cultured with 5% CO₂ at 37 °C. Transfection of neurons was performed using Amaxa according to the manufacturers protocol. Inducible Ndfip1 plasmids (2 μg total) were added and cells electroporated using the mouse hippocampal neuron protocol.

Cell Culture and Lentiviral Infection. See SI Materials and Methods.

Metal Toxicity and Apoptosis Quantification by Flow Cytometry. See SI Materials and Methods.

Cobalt Uptake and Staining of SH-SY5Y Cells. See SI Materials and Methods.

Western Blots and Immunoprecipitation Assays. See SI Materials and Methods.

Immunohistochemistry. See SI Materials and Methods.

Histology and Brain Fe Measurements. See SI Materials and Methods.

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Supporting Information

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SI Materials and Methods

Cell Culture and Lentiviral Infection. SH-SY5Y cells were cultured in RPMI media supplemented with 15% FCS, 2 mM L-glutamate, and 50 µg/mL PenStrep. Lentiviral infection of SH-SY5Y cells was used to make stable cell lines of inducible Ndfip1-Flag (see Fig. 2A) and shRNAi Ndfip1 knockdown cells (Open Biosystems clone V2LHS.99041). To generate lentiviral particles, wild-type HEK293T cells with packaging constructs pCMV 6R8.2 and VSVg and the relevant lentiviral plasmid using Effectene reagent according to the manufactures protocol (Qiagen). For inducible Ndfip1-Flag, two separate viruses were made, one containing the Ndfip1-Flag construct and the other containing the GEV16 Super construct, infection with both viruses created the inducible Ndfip1-Flag cells. The virus containing supernatants were harvested at 100,000 g for 10 min. Target cells were infected with virus supernatant for 24 h. Successful infection was selected for with puromycin (2 mg/mL, Sigma) or hygromycin B (100 µg/mL, Sigma). From this selection stable cell lines for inducible Ndfip1-Flag and shRNAi Ndfip1 were produced.

Metal Toxicity and Apoptosis Quantification by Flow Cytometry. Assays to determine Ndfip1’s regulation in response to metals were conducted by treating cells with varying concentrations of CoCl2 or FeCl2 for 18 h before analysis by western blot. Rabbit polyclonal anti-N terminus Ndfip1 antibody (polyclonal Ndfip1) was used as the primary antibody to detect Ndfip1 levels. Apoptosis was assessed quantitatively by flow cytometry using double labeling with propidium iodide and annexin-V-FLUOS (Roche) according to the manufactures protocol. Briefly 2 × 105 cells were grown in 6-well plates, Ndfip1 was induced by the addition of 100 nM 4-hydroxy tamoxifen 15 h before addition of CoCl2 or FeCl2. Control cells contained no 4-hydroxy tamoxifen. Cells were incubated for 18 h after addition of metals. All cells (adherent and in suspension) were collected by trypsinization and centrifugation before being washed with binding buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, and 2.5 mM CaCl2), and labeled with annexin-V-FLUOS, 10 µg/mL PI for 15 min at RT. At least 40,000 cells from each sample were analyzed by FAC-Scaliber flow cytometer (Becton Dickinson). Positive staining of the plasma membrane with annexin V in the absence of concomitant staining of nuclei with PI indicates apoptosis at an early stage (quadrant 2 in Fig. 2B). Double-positive staining with both annexin V and PI suggests apoptosis at later stages (quadrant 3 in Fig. 2B). Control cells of wild type SH-SY5Y treated with 4-hydroxy tamoxifen showed no toxicity assay. For Ebselen experiments, wild-type SH-SY5Y cells were treated with Ebselen (1 µM) for 30 min before the addition of FeCl2 for 18 h. Cell death was measured by flow cytometry as outlined above.

Iron Uptake and Staining of SH-SY5Y Cells. Inducible SH-SY5Y cells were treated with 150 and 300 µM FeCl2 for 18 h. Cells were washed three times in TBS and 0.12% ammonium sulfide was added for 5 min. Cells were washed and then fixed in 4% PFA for 5 min. Elemental Fe was then stained using a silver enhancer kit as described in the manufacturer’s protocol (Sigma-Aldrich).

Western Blots and Immunoprecipitation Assays. Human cortical neuron cultures and SH-SY5Y cells were lysed in RIPA buffer containing complete protease inhibitors (Roche Applied Science). For ubiquitin assays N-ethyl maleimide (NEM) was added to the lysis buffer to prevent loss of ubiquitin tags. Lysates were incubated with appropriate antibody; polyclonal Ndfip1 (1:2,000), purified rabbit polyclonal Nedd4-2 (1:2,000), rabbit polyclonal DMT1 (1:2,000), mouse monoclonal Itch (1:2,000; BD Transduction Laboratories), for 1 h followed by 30 min incubation with Protein A agarose (Zymed). Beads were washed five times with RIPA buffer and separated on a 10% SDS/PAGE, before transfer to nitrocellulose membrane. Western blots were then probed with the appropriate primary antibody [polyclonal Ndfip1, monoclonal Flag M2 (Sigma), polyclonal DMT1, monoclonal multibiquitin clone FK2 (MBL), and polyclonal Nedd4-2, monoclonal Itch] followed by anti-rabbit or mouse HRP. Blots were detected using Amersham ECL reagent (GE Healthcare). All blots are representative of at least three different experiments.

Immunohistochemistry. Immunohistochemistry was performed on 18-week-old human embryonic brain tissue fixed in 4% paraformaldehyde for 24 h. Tissue was sectioned into 14 µm slices and blocked in 10% normal horse serum in 0.1 M PB with 0.2% Triton X-100, and then incubated overnight in primary antibodies. Primary antibodies used were rat monoclonal Ndfip1 (1:200) and mouse monoclonal human DMT1 (1:500; Abnova). Secondary antibodies were Alexa Fluor 594-conjugated goat anti-rat IgG (1:500; Invitrogen) and Alex 488-conjugated goat anti-mouse IgG (1:500; Invitrogen). Images were taken on an inverted Zeiss Axiore 200-LSM 5-Pa confocal microscope.

Histology and Brain Fe Measurements. Brains were dissected out of Ndfip1+/− and Ndfip1−/− mice at P3. Tissue was sectioned into 1-mm slices and cultured for 18 h in the presence of 200 µM FeCl3. Sections were placed in 20% sucrose for 2 h then fresh frozen in OCT. Tissue was then cut into 20-µm sections before fixing in 4% PFA for 20 min. Iron accumulation was stained using Perls Prussian blue and counter stained with 2% neutral red. Ndfip1+/− and Ndfip1−/− animals at 3 weeks of age were fed a low Fe diet (15 mg/kg) or high Fe diet (20 g/kg) for 3 weeks (standard rodent diet contains 180 mg/kg Fe). Brain tissue was measured by weight and ICP mass spectroscopy was used to analyze the levels of Fe within the cortex of both Ndfip1+/− and Ndfip1−/− mice fed on the low or high Fe diet. For the analysis of DMT1 protein levels lysates were prepared from brain tissue from three animals per genotype for each of the diets. Western blots were performed and probed with anti-DMT1 rabbit antibody (Abnova, 4EC).
Fig. S1. The increase in Ndfip1 protein in metal treated cells is due to transcriptional upregulation. SH-SYSY cells treated with CoCl₂ upregulate Ndfip1 (two left lanes). However, treatment with cycloheximide (CHX) for 6 h prevents the increased expression of Ndfip1 in response to CoCl₂ (two right lanes).
Fig. S2. Transferrin receptor (TfR) protein levels are lower in Ndfip1<sup>-/-</sup> mice brains. Transferrin receptor is a major pathway for Fe entry into cells. Ndfip1<sup>-/-</sup> mice brains (P7) were probed with TfR antibody (Invitrogen) and were found to have lower levels of TfR when compared with heterozygous or wild-type littermates (two separate litters shown).