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Review

Molecular Foundations of Heavy Metal Induced Neurotoxicity and the Neuroprotective Role of Rifampicin: A Review of Current Findings

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Abstract

The increasing intensity of environmental and industrial activities worldwide has led to elevated exposure to heavy metals, representing a significant environmental problem affecting human health. Heavy metals such as lead, mercury, arsenic, and cadmium can cross the blood brain barrier and accumulate in the central nervous system, where they induce neurotoxic effects. These effects are mediated by multiple interrelated mechanisms, including oxidative stress, mitochondrial dysfunction, disruption of calcium homeostasis, and sustained neuroinflammatory responses. Such mechanisms are known to contribute to the pathogenesis of several neurodegenerative disorders, particularly Alzheimer's and Parkinson's diseases.

The limited efficacy of current therapeutic approaches has increased interest in alternative and multi target treatment strategies. In this context, rifampicin (RIF), a well established antibiotic used in tuberculosis therapy, has been reported to exhibit antioxidant, anti inflammatory, and autophagy related effects beyond its antimicrobial activity. This review summarizes current knowledge on the molecular mechanisms of heavy metal induced neurotoxicity and discusses the potential neuroprotective role of RIF based on available experimental and clinical evidence.

1. Introduction

Industrial production, mining activities, agricultural practices, and rapid urbanization have led to a rapid increase in environmental heavy metal contamination in recent years. This increase poses significant long term risks to ecosystems and human health. Because heavy metals can accumulate in biological tissues, they represent a considerable source of toxicity in living organisms [1,2]. In particular, lead (Pb), mercury (Hg), arsenic (As), and cadmium (Cd) can cross the blood brain barrier (BBB), accumulate in the central nervous system (CNS), and thereby induce neurotoxic effects [3,4].

The nervous system plays a fundamental role in enabling an organism to respond to environmental stimuli and maintain internal homeostasis, which makes it particularly vulnerable to environmental toxins. Current epidemiological and experimental evidence indicates that heavy metal exposure associated with environmental pollution constitutes a significant risk factor for the development of neurodegenerative diseases [5,6]. The toxic effects of heavy metals on the CNS are not limited to a single mechanism; rather, they affect interconnected pathways, including oxidative stress, mitochondrial dysfunction, disruption of calcium (Ca^{2+}) homeostasis and neuroinflammation [7,8].

At the cellular level, one of the most prominent consequences of heavy metal exposure is the disruption of redox homeostasis. Excessive production of reactive oxygen species (ROS), combined with the suppression of antioxidant defense systems such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), leads to various cellular damages, including lipid peroxidation, protein oxidation, and DNA damage [9-11]. Furthermore, heavy metals can bind to thiol (–SH), histidyl (imidazole), and carboxyl ($–COO^-$) groups in proteins and low molecular weight compounds such as glutathione (GSH), thereby disrupting their structural and functional integrity. In addition, they can interfere with metabolic processes by displacing physiological metal ions from enzyme active sites [12,13].

Heavy metals exert neurotoxic effects that are not limited to oxidative stress. Inhibition of the mitochondrial electron transport system (ETS), decreased ATP production, and loss of mitochondrial membrane potential prevent neuronal cells from meeting their energy demands, thereby triggering apoptosis [7,8]. These toxic processes resemble the pathological features of neurodegenerative diseases such as Alzheimer's and Parkinson's. Studies have shown that heavy metal accumulation can accelerate neurodegenerative processes, including amyloid-β aggregation, tau hyperphosphorylation, and dopaminergic neuron loss [8]. In contrast, current treatment options, particularly chelation based approaches, provide limited benefits due to poor BBB penetration and adverse side effects [14]. This highlights the inadequacy of single target therapeutic strategies in neurodegenerative diseases.

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In recent years, drug repositioning studies for new indications have gained increasing attention in drug development [6,14-16]. Molecules capable of modulating multiple cellular pathways have emerged as particularly promising in neurodegenerative diseases with multifactorial pathogenesis. In this context RIF, long used for tuberculosis treatment, is known to possess antioxidant, anti inflammatory, and autophagy modulating properties beyond its antimicrobial effects [17].

Recent studies indicate that RIF can suppress neuroinflammation, modulate oxidative stress responses, and support neuronal integrity [18]. However, its potential effects in the context of heavy metal induced neurotoxicity have been addressed in only a limited number of studies. This underscores the need for a more comprehensive evaluation of RIF's potential role in heavy metal associated neurodegenerative processes.

This review aims to examine the molecular mechanisms underlying heavy metal-induced neurotoxicity using a systematic approach and to discuss the potential neuroprotective effects of RIF against these processes based on current literature. Accordingly, it seeks to identify existing knowledge gaps in heavy metal associated neurodegeneration and to provide a conceptual framework for future experimental and clinical studies.

2. Literature Search Strategy and Study Selection Criteria

This review aimed to provide a comprehensive assessment of the current literature on heavy metal induced neurotoxicity in the CNS and the potential neuroprotective effects of RIF. A systematic literature search was conducted across PubMed/MEDLINE, ScienceDirect (Elsevier), Web of Science, Scopus, Google Scholar, and SpringerLink databases. Search terms included combinations of "heavy metal neurotoxicity", "oxidative stress", "mitochondrial dysfunction", "neuroinflammation", "calcium homeostasis", "autophagy" and "neuroprotective effects of RIF." Studies published between 2010 and 2025, including both original research and review articles, were considered.

The literature screening process followed a systematic approach to ensure the selection of relevant studies which can bee seen in Figure 1. Initially, records retrieved from all databases were screened based on titles and abstracts. Duplicates were removed, and potentially relevant full texts were evaluated in detail. Inclusion criteria were:

1. Peer reviewed original research articles and review papers.
2. Studies investigating the neurotoxic effects of heavy metals (As, Cd, Pb, Hg) on the CNS.
3. Studies focusing on molecular and cellular mechanisms such as oxidative stress, mitochondrial dysfunction, neuroinflammation, and calcium imbalance.
4. Studies evaluating the neuroprotective mechanisms of RIF.

Exclusion criteria were:

1. Conference abstracts or reports without accessible full text.
2. Studies not directly related to neuronal or CNS mechanisms.
3. Duplicate publications across databases.
4. Studies lacking sufficient methodological or mechanistic detail.

Finally, studies meeting the predefined criteria were included in the qualitative synthesis of this review. Heavy metal-specific effects on oxidative stress, mitochondrial dysfunction, intracellular calcium imbalance, and neuroinflammation were analyzed, alongside the potential neuroprotective mechanisms of RIF, including modulation of antioxidant defense systems, autophagy, and inflammatory pathways.

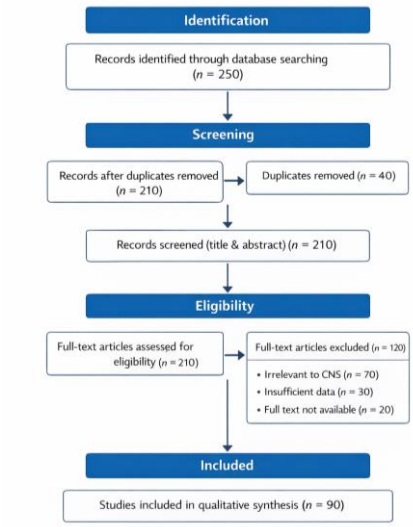

Figure 1. PRISMA flow diagram of the literature search and study selection process.

The figure illustrates the stepwise screening and selection of studies included in this review. Records identified through database searching were first screened for duplicates and relevance based on titles and abstracts. Full text articles were

then assessed against predefined inclusion and exclusion criteria, resulting in the final set of studies included in the qualitative synthesis.

3. Heavy Metal Neurotoxicity: Mechanisms and Neurodegenerative Relationships

Living organisms are influenced by both biotic and abiotic components of their environment. Heavy metals entering the human body due to environmental pollution and industrial waste can accumulate in biological systems (bioaccumulation), leading to neurotoxicity and thereby impairing neuronal structure and function [3,4,19]. The neurotoxic mechanisms of heavy metals in the CNS, their cellular consequences, and the associated neurodegenerative processes are summarized in Table 1.

Table 1. Neurotoxic Mechanisms of Heavy Metals in the CNS, Cellular Consequences, and Potential Links to Neurodegenerative Processes.

Heavy Metal	Primary Molecular Mechanism	Cellular Consequences	Potential Link to Neurodegenerative Processes (Conceptual)	References
Pb	Calcium mimicry (Ca^{2+}), mPTP opening.	Excitotoxicity, impaired synaptic transmission	May facilitate neuronal damage processes associated with synaptic dysfunction and excitotoxicity.	[3,4,11]
Cd	Inhibition of mitochondrial ETS complexes.	ATP depletion, activation of caspase dependent apoptosis	May increase neuronal vulnerability through mitochondrial energy failure.	[9]
As	Binding to protein thiol (-SH) groups.	Enzyme inhibition, impaired DNA repair mechanisms, epigenetic modifications	May support progressive damage by sustaining cellular stress responses and reducing repair capacity.	[7,13]
Hg	Microglial activation, pro inflammatory response.	Chronic neuroinflammation, BBB damage	May exacerbate secondary neuronal injury via persistent neuroinflammation and weakening of BBB integrity.	[3]
Common Effect	Oxidative Stress, ROS↑; GSH ↓	Lipid peroxidation, protein aggregation, cell death	May feed into mechanisms overlapping with neurodegenerative pathology through a common oxidative stress.	[8,10]

Table 1 comparatively summarizes the primary mechanisms by which As, Cd, Pb, and Hg induce neurotoxicity in the CNS, together with their cellular consequences. Although these metals initially affect different molecular targets, their effects often converge on common pathological pathways such as oxidative stress and mitochondrial dysfunction. In addition, disruption of Ca^{2+} homeostasis and activation of neuroinflammatory responses may further exacerbate neuronal damage [3,4,19]. Within this framework, heavy metal exposure is not limited to direct toxic effects but is also associated with neurodegenerative changes. The role of neuroinflammation in neurodegenerative processes has been widely discussed in the literature [19].

In the context of neurodegeneration, studies have reported that metal protein interactions may enhance protein aggregation and amplify cellular stress responses [20]. The pathogenesis of heavy metal induced neurotoxicity arises from the complex interplay of multiple cellular pathways and molecular targets. Cheng et al. (2025) summarized the primary toxic effects of heavy metals as increased oxidative stress caused by ROS overproduction, mitochondrial dysfunction, disruption of Ca^{2+} homeostasis, and neuroinflammation [21]. These cellular insults weaken synaptic connections and, through activation of apoptotic pathways, ultimately lead to neuronal cell death [19,20,22,23]. Therefore, the molecular alterations triggered by heavy metal exposure in the CNS may contribute to pathological processes that overlap with those observed in neurodegenerative disorders.

In summary, heavy metals such as As, Cd, Pb, and Hg can trigger multiple interconnected molecular mechanisms. These processes contribute to neuronal damage and may promote neurodegenerative changes. A schematic overview of the main mechanisms underlying heavy metal induced neurotoxicity is presented in Figure 2.

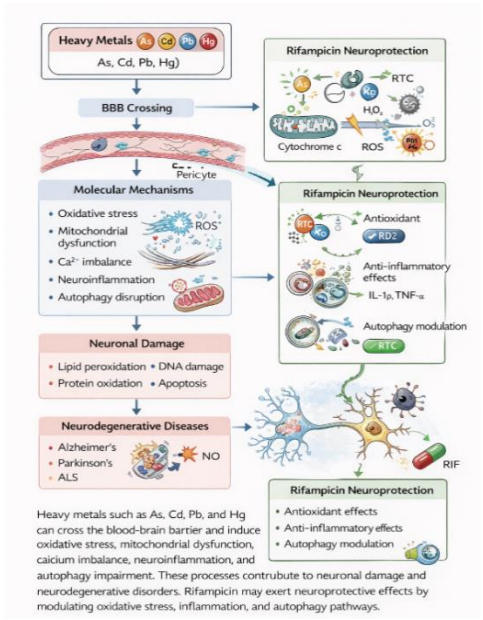

Figure 2. Heavy metal neurotoxicity mechanism.

3.1. Oxidative Stress in Heavy Metal Exposure

Oxidative stress is a key component of heavy metal induced neurotoxicity in the CNS. After entering cells, heavy metals disrupt redox homeostasis, exceeding the capacity of endogenous antioxidants [24,25]. Two main mechanisms drive this process: (1) overproduction of ROS, including superoxide anion ($O_2^{\bullet-}$), hydroxyl radical (•OH), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2); and (2) weakening of antioxidant defenses [23,26]. Key markers and outcomes of oxidative stress are summarized in Table 2.

Table 2. Markers and Outcomes of Oxidative Stress Mechanisms in Heavy Metal Exposure.

Process Component	Markers / Events	Cellular Consequence	References
Increased ROS	$O_2^{\bullet-}$, •OH, $^1O_2$, H_2O_2	Initiation of macromolecular oxidation.	[23,26]
Weakening of Antioxidant Defense	GSH↓, SOD/CAT activity ↓	Disruption of redox balance, exacerbation of oxidative stress.	[24,25]
Thiol (-SH) Targeting	GSH depletion, functional decline in glutathione dependent enzymes	Further reduction of antioxidant capacity.	[25,26]
Lipid Peroxidation	MDA↑, 4-hydroxy-2-nonenal (4-HNE) ↑	Impaired membrane integrity, loss of channel/receptor function.	[27]
Protein Oxidation	Carbonylation, tyrosine nitration	Loss of protein function, tendency for aggregation.	[28]
DNA Damage	Base modification, DNA protein cross links, strand breaks	Genotoxicity, p53 activation, tendency toward apoptosis.	[29]
Signaling Imbalance	Antioxidant response ↓, pro-inflammatory signaling ↑, Tumor Necrosis Factor alpha (TNF-α)↑, Interleukin-1 beta (IL-1β) ↑	Intensification of neuroinflammation, increased neuronal loss.	[7,13]

Table 2 indicates that oxidative stress in heavy metal exposure is not limited to increased ROS levels; rather, redox homeostasis is disrupted through multiple routes, including GSH depletion and suppression of antioxidant systems such as SOD and CAT. This imbalance creates a basis for the concurrent accumulation of lipid and protein oxidation and DNA damage, thereby deepening cellular functional impairment and increasing the tendency toward apoptosis. In addition, the progression of oxidative damage together with suppression of antioxidant responses and enhanced pro inflammatory signaling suggests that oxidative stress neuroinflammation crosstalk in the CNS is associated with increased neuronal loss [7,13].

Cellular thiol (–SH) groups are known to be one of the primary molecular targets in heavy metal toxicity. Cheng et al. (2025) demonstrated that heavy metals bind endogenous antioxidants such as GSH with high affinity, leading to depletion of cellular GSH stores and consequent suppression of key antioxidant enzyme activities, including SOD and CAT [25]. Reduced GSH levels not only weaken antioxidant protection but also contribute to decreased activity of glutathione dependent enzymes such as GPx and GST due to substrate insufficiency [25,26]. Moreover, displacement of essential

metal cofactors located in the active sites of enzymes such as SOD and CAT by heavy metals can lead to direct enzyme inhibition [30-32].

Oxidative stress often begins at the level of mitochondrial metabolism and, over time, spreads to different cellular components, thereby intensifying oxidative damage [33,34]. An increased oxidative stress burden triggers lipid peroxidation and leads to the formation of reactive aldehydes such as MDA and 4-hydroxy-2-nonenal (4-HNE). Covalent binding of these products to membrane proteins can impair the function of ion channels and receptors [27]. At the protein level, oxidative modifications such as carbonylation and tyrosine nitration can accumulate, contributing to loss of protein function and an increased propensity for aggregation [28]. At the nucleic acid level, reactive species have been reported to induce DNA base modifications, DNA protein cross links, and single and double strand breaks [29]. This genotoxic damage can activate the p53 pathway and increase susceptibility to apoptosis [35,36].

Oxidative damage may become more pronounced when accompanied by suppression of antioxidant responses and enhancement pro inflammatory signaling. This imbalance can enhance neuroinflammatory responses through increased levels of pro inflammatory cytokines such as TNF-α and IL-1β, and thereby lead to increased neuronal loss [7,13]. Overall, heavy metal induced neurotoxicity is considered a holistic process of cellular damage in which increased oxidative stress and weakened antioxidant defenses co occur and progress through thiol depletion, inhibition of antioxidant enzymes, macromolecular oxidation, and disruption of signaling pathways.

3.2. Mitochondrial Dysfunction in Heavy Metal Exposure

Mitochondria are key targets of heavy metal toxicity in the CNS. They maintain neuronal integrity by regulating energy metabolism, Ca^{2+} homeostasis, lipid biosynthesis, and apoptosis [37,38]. Their main function is ATP production through the ETS. Complexes I–IV in the inner mitochondrial membrane enable electron flow and proton translocation. Complex V (ATP synthase) uses the proton gradient to synthesize ATP [37,39]. Dependence on ETS activity makes mitochondria particularly vulnerable to heavy metal exposure [22,39].

During physiological ETS activity, a limited amount of reactive species is expected. However, $O_2^{\bullet -}$ formation and its conversion to H_2O_2 and other ROS can increase oxidative stress, leading to mitochondrial dysfunction [37,40]. Literature indicates that heavy metals can inhibit ETS function directly or indirectly. For example, Hg exposure may impair complexes I and III, causing loss of mitochondrial membrane potential ($\Delta\Psi m$) and reduced ATP production [41]. The primary stages of mitochondrial dysfunction during heavy metal exposure are summarized in Table 3.

Table 3. Primary Steps and Outcomes of Mitochondrial Dysfunction in Heavy Metal Exposure.

Mechanistic Stage	Marker / Event	Cellular Outcome	References
ETS dysfunction	Loss of function at complexes I/III	Electron leakage, increased oxidative stress	[37,40,41]
Reduced ATP production	ATP ↓	Energy failure; increased neuronal vulnerability	[22,39]
Loss of mitochondrial membrane potential	$\Delta\Psi m$ ↓	Impairment of mitochondrial structural integrity	[41]
Increased oxidative stress	$O_2^{\bullet -} \rightarrow H_2O_2$ / ROS ↑	Further impairment of mitochondrial functions	[37,40]
Activation of apoptotic pathways	Cytochrome c release; caspase activation	Programmed cell death; neuronal loss	[23]

Table 3 illustrates that mitochondrial damage during heavy metal exposure primarily begins with disruption of the ETS, resulting in loss of mitochondrial membrane potential ($\Delta\Psi m$) and decreased ATP production. Increased ROS associated with ETS impairment further compromises mitochondrial integrity, promoting cytochrome c release and activation of caspase dependent apoptotic pathways, leading to neuronal loss [23,37,40]. Some studies also report that opening of the mitochondrial permeability transition pore (mPTP) contributes to $\Delta\Psi m$ loss and enhanced pro apoptotic signaling [23,37,38]. Overall, heavy metal exposure represents an integrated pathophysiological process that amplifies programmed cell death mechanisms [37,38,40].

3.3. Neuroinflammation and Microglial Activation in Heavy Metal Exposure

The neurotoxic effects of heavy metals on the CNS are not limited to direct neuronal damage. Microglial activation and neuroinflammatory responses can further sustain the toxic process [17,42]. Under physiological conditions, microglia contribute to synaptic pruning and tissue homeostasis. However, during heavy metal exposure, elevated cellular damage signals and oxidative stress can shift microglia toward a pro inflammatory phenotype, exacerbating secondary neuronal injury in the CNS [22,42]. The underlying mechanisms of heavy metal associated microglial responses are summarized in Table 4.

Table 4. Primary Mechanisms and Outcomes of Microglial Activation and Neuroinflammation in Heavy Metal Exposure.

Mechanistic Stage	Marker / Event	Cellular Outcome	References
Microglial activation	Morphological activation; ionized calcium binding adaptor molecule 1 (Iba1) ↑ / cluster of differentiation 68(CD68) ↑	Shift toward a pro inflammatory phenotype; increased susceptibility to secondary injury	[17,42]
Pro inflammatory signaling	Activation of nuclear factor kappa B (NF-κB)	Enhanced cytokine response	[42]
Cytokine elevation	TNF-α ↑; IL-1β ↑; interleukin-6 (IL-6) ↑	Impaired synaptic function; neuronal stress	[42]
Oxidative/nitrosative burden	ROS ↑; inducible nitric oxide synthase (iNOS) / nitric oxide (NO) ↑	Exacerbation of neuronal damage	[22,42]
Inflammatory enzyme induction	Cyclooxygenase-2 (COX-2) ↑; iNOS ↑	Persistence of inflammation	[42]
Compromised BBB integrity	BBB permeability ↑	Increased impact of peripheral signals on the CNS	[3,4,19]

Table 4 shows that microglial activation during heavy metal exposure often occurs alongside oxidative stress. The neuroinflammatory response is amplified through the activation of signaling pathways such as NF-κB, leading to increased levels of pro inflammatory mediators, including TNF-α and IL-1β. This response negatively affects neuron glia interactions, disrupts synaptic integrity, and exacerbates neuronal loss [17,42].

Elevated pro inflammatory cytokines (TNF-α, IL-1β, IL-6), increased reactive nitrogen species, and induction of inflammatory enzymes (iNOS, COX-2) are key features of neuroinflammation [42]. During heavy metal exposure, these mechanisms can form a feedback loop with enhanced ROS production and mitochondrial stress. Thus, the inflammatory response is not merely a consequence but also a process that amplifies cellular damage [22,42].

Heavy metals can also compromise the BBB, facilitating the entry of peripheral inflammatory signals into the CNS and further promoting microglial activation [3,4,19].

In summary, microglial activation and neuroinflammation are critical components that exacerbate cellular damage in heavy metal neurotoxicity and reinforce pathological mechanisms shared with neurodegenerative processes. The nature and persistence of the inflammatory response represent key factors influencing the extent of neuronal loss in the CNS [17,42].

3.4. Disruption of Calcium Homeostasis in Heavy Metal Exposure

Mitochondria play a critical role in Ca^{2+} buffering within neurons. Literature indicates that heavy metals can disrupt mitochondrial regulation of Ca^{2+} homeostasis [43]. Elevated intracellular Ca^{2+} levels facilitate the opening of the mitochondrial permeability mPTP, compromising mitochondrial integrity [44]. This event can accelerate cytochrome c release and activate apoptotic molecular cascades [37,45]. Additionally, Pb's ability to mimic Ca^{2+} and promote mPTP opening suggests that the Ca^{2+} dysregulation mitochondrial stress axis may act as an early step in heavy metal neurotoxicity [3,4,11]. Key steps and cellular consequences of Ca^{2+} dysregulation during heavy metal exposure are summarized in Table 5.

Table 5. Primary Steps and Outcomes of Disrupted Ca^{2+} Homeostasis in Heavy Metal Exposure.

Mechanistic Stage	Marker / Event	Cellular Outcome	References
Pb mediated Ca^{2+} mimicry and mPTP propensity	Ca^{2+} mimicry; mPTP opening	Increased susceptibility to excitotoxicity; mitochondrial stress	[3,4,11]
Ca^{2+} loading	Intracellular Ca^{2+} ↑	Increased mitochondrial stress; disruption of energy balance	[37,45]
mPTP opening	Increased propensity for mPTP opening	Loss of $\Delta\Psi m$; impairment of mitochondrial integrity	[37,45]
Cytochrome c release	Cytochrome c ↑	Caspase activation; increased apoptotic tendency	[37,45]
Cd targeting of MAM	Disruption of ER mitochondria contacts (MAM)	Dysregulated Ca^{2+} flux; increased susceptibility to necroptosis/apoptosis	[12,46]
Cd-associated modulation of Ca^{2+} entry	Channel/receptor interaction (representative)	Reinforcement of Ca^{2+} dysregulation	[12,47]
Secondary outcomes	Oxidative stress ↑; $\Delta\Psi m$ ↓	Increased mitochondrial dysfunction; deepening of cellular damage	[12,47,48]

Table 5 shows that Pb exposure may induce Ca^{2+} mimicry and mPTP opening, while Cd exposure can disrupt ER mitochondrial Ca^{2+} flux via MAM impairment. Both processes contribute to sustained Ca^{2+} imbalance and enhance cell death pathways, including apoptosis and necroptosis [3,4,11,12,46]. Literature reports that Cd can impair mitochondrial associated ER membranes (MAMs), disrupting Ca^{2+} transfer and lipid metabolism, which promotes necroptosis and apoptosis through intracellular Ca^{2+} dysregulation [12,46]. Cd may also directly affect Ca^{2+} influx by interacting with cell surface receptors and ion channels. Consequently, disturbed Ca^{2+} homeostasis, together with $\Delta\Psi m$ loss, increased oxidative stress, and mitochondrial dysfunction, can drive apoptosis progression [12,47,48].

3.5. Epigenetic Alterations Emerging in Response to Heavy Metal Exposure

Interactions between genetic susceptibility and environmental factors, such as heavy metal exposure, may contribute to the initiation and progression of neurological and neurodegenerative diseases. Heavy metals can influence epigenetic processes that regulate chromatin organization and gene expression without directly altering the DNA sequence, thereby leading to various molecular disturbances in the CNS. The literature indicates that such epigenetic alterations may be associated with neurobehavioral, neurodevelopmental, and neurodegenerative outcomes. Therefore, heavy metal induced epigenetic disruptions may contribute to the development of persistent neurological and cognitive impairments over time [12].

In this context, prenatal exposure to As has been associated with reduced histone acetylation levels, which may impair long term spatial and episodic memory performance. Mechanistically, As can disrupt the activity of pyruvate dehydrogenase (PDH), the enzyme responsible for converting pyruvate to acetyl-CoA, a key precursor for histone acetylation. This disruption may limit the metabolic support required for proper histone acetylation [12,49]. Similarly, Cd exposure has been reported to increase the expression of histone deacetylase 2 (HDAC2), a regulator implicated in AD pathogenesis. Elevated HDAC2 levels may contribute to impairments in learning and memory processes [12,50].

In addition, Cd has been shown to inhibit vaccinia related kinases 1 and 2 (VRK1 and VRK2), which are involved in cell cycle regulation, DNA damage responses, and signal transduction. This inhibition may reduce histone H3 phosphorylation and consequently affect chromatin dynamics and transcriptional responses [12,51]. These epigenetic alterations and their potential functional consequences during heavy metal exposure are summarized in Table 6.

Table 6. Representative Mechanisms of Epigenetic Alterations in Heavy Metal Exposure and Their Potential Outcomes

Heavy Metal	Epigenetic Target / Step	Mechanism	Potential Functional Outcome	References
As	Histone acetylation	Impairment of pyruvate dehydrogenase complex (PDH) function → reduced acetyl-CoA supply → decreased histone acetylation	Associated with impaired spatial/episodic memory performance	[12,49]
Cd	Histone deacetylase 2 (HDAC2 regulation)	Increased HDAC2 expression	Learning and memory impairment; epigenetic profile associated with AD	[12,50]
Cd	Histone H3 phosphorylation	VRK1/VRK2 inhibition → decreased H3 phosphorylation	Altered chromatin dynamics; affected transcriptional responses	[12,51]

Table 6 indicates that heavy metals influence epigenetic regulation through multiple mechanisms rather than a single pathway. These effects may occur through the limitation of histone acetylation via metabolic intermediates (the As PDH acetyl-CoA axis) or through alterations in the expression and function of chromatin regulators, including Cd induced upregulation of HDAC2 and Cd mediated inhibition of VRK1/2 [12,49-51]. The association of these mechanisms with cognitive domains suggests that epigenetic dysregulation may provide an additional explanatory layer linking neurotoxicity to neurodegenerative processes. However, the magnitude of these effects and the degree of causality may vary depending on the experimental model and exposure conditions [12].

Overall, the ability of heavy metals to alter epigenetic regulation provides a mechanistic framework indicating that the neurological outcomes observed in the CNS are not limited to acute toxic effects but may also reflect long term alterations in gene expression programs [12]. Therefore, identifying epigenetic alterations associated with heavy metal exposure may represent a complementary approach to understanding the link between neurotoxicity and neurodegeneration. Nevertheless, a substantial portion of the current evidence is derived from experimental models. More comprehensive human studies that standardize exposure dose and duration while considering tissue specific epigenetic characteristics are needed to clarify the degree of causality.

3.6. Association of Heavy Metal Exposure with Neurodegenerative Diseases

Chronic toxicity caused by heavy metals in the CNS has been associated with several neurodegenerative disorders, including AD, PD, HD, amyotrophic lateral sclerosis (ALS), and various forms of dementia [21,52]. Metals that accumulate in the CNS due to industrial and environmental pollution may impair brain function and contribute to

mechanisms involved in neurodegenerative pathogenesis [53]. Moreover, heavy metal accumulation in individuals with existing neurodegenerative diseases may accelerate pathological processes and exacerbate clinical symptoms [54,55]. In disorders characterized by progressive motor and cognitive decline, such as ALS and HD, disruption of metal homeostasis may further increase neuronal loss and worsen the clinical condition [21]. Potential associations between heavy metal exposure and major neurodegenerative diseases, along with representative pathological links, are summarized in Table 7.

Table 7. Potential Associations and Mechanistic Links Between Heavy Metals and Neurodegenerative Diseases

Disease	Potentially Associated Metals	Proposed Mechanism	Disease-Specific Example Pathology (Representative)	References
AD	Pb (± others)	Oxidative stress; disruption of proteostasis	Processes associated with amyloid-β (Aβ) accumulation and tau hyperphosphorylation	[3,21,52]
PD	Cd (± others)	Mitochondrial dysfunction; oxidative stress	Findings associated with dopaminergic neuronal injury and motor symptoms	[3,52,53]
ALS	Various metals	Oxidative stress; disruption of metal homeostasis	Observations associated with neuronal loss and accelerated progression	[21,54,55]
HD	Various metals	Mitochondrial stress; redox dysregulation	Processes associated with aggravated motor/cognitive decline	[21,54,55]
Dementia subtypes	Pb, Cd (Ag in some studies)	Multiple mechanisms (increased ROS; mitochondrial dysfunction; impaired protein folding)	Findings associated with a tendency toward cognitive impairment	[52,53]

Table 7 indicates that heavy metal exposure consistently impacts shared mechanisms across various neurodegenerative phenotypes. At the disease specific level, certain metals may exacerbate processes related to pathological proteins. Thus, the literature suggests that metal exposure is associated not only with increased risk but also with progression of existing pathology [53-55]. For example, Pb exposure is linked to AD related pathological processes, including Aβ accumulation and tau hyperphosphorylation, with several experimental studies supporting this effect. Cd has been reported to exert toxic effects on dopaminergic neurons, contributing to PD like motor symptoms [3].

In this context, heavy metal exposure should be considered a risk factor that accelerates pathology in interaction with genetic predisposition and other environmental factors, rather than a sole cause of neurodegenerative disease [21,52,54,55]. However, due to limited human studies standardizing exposure dose and duration and accounting for inter individual variability, long term, well designed investigations are needed to clarify the causal relationship between metal exposure and neurodegeneration.

4. Neuroprotective Potential of RIF and Its Molecular Mechanisms of Action

RIF is a broad spectrum antibiotic used to treat tuberculosis (TB), leprosy, and other non tuberculous mycobacterial infections [56,57]. Recent studies indicate that beyond its classical antimicrobial activity, RIF can modulate key CNS pathways by enhancing antioxidant responses, suppressing pro inflammatory signaling, supporting autophagy, and regulating xenobiotic metabolism [57,58].

Vaezipour et al. (2025) reported that RIF use in the context of neurodegenerative diseases and CNS infections may be associated with reduced inflammatory responses and beneficial trends in select clinical and imaging outcomes [18]. However, clinical evidence varies depending on indication, dose, duration, and patient characteristics, highlighting the need for controlled studies to clarify RIF's neuroprotective potential. The most frequently discussed neuroprotective pathways and targets of RIF are summarized in Table 8.

Table 8 indicates that RIF's effects are not limited to a single target. RIF can enhance antioxidant defenses via Nrf2/ARE, suppress pro inflammatory responses through NF-κB, and promote cellular clearance via autophagy. Additionally, its potential to regulate detoxification responses through PXR and modulate P2X7 related signaling linked to microglial activation suggests that RIF exhibits a multi target profile in the CNS [57,58]. RIF exerts neuroprotective effects through multiple molecular pathways, including antioxidant activity, anti inflammatory effects, autophagy modulation, and detoxification of toxic compounds. These mechanisms are summarized in Figure 3.

Table 8. Potential Neuroprotective Pathways and Molecular Targets of RIF Reported in the Literature.

Target Pathway / Receptor	Reported Effect of RIF	Functional Outcome	Related Proteins	References
Nuclear factor erythroid 2 related factor 2 / antioxidant response element (Nrf2/ARE)	Activation / upregulation	Strengthening of antioxidant responses	SOD, CAT, heme oxygenase-1 (HO-1), NAD(P)H quinone oxidoreductase 1 (NQO1)	[57,58]
Nuclear factor kappa B (NF-κB)	Suppression / inhibition	Tendency toward reduced pro inflammatory mediators	TNF-α, interleukin-1 beta (IL-1β), iNOS	[57,58]
Autophagy	Induction / stimulation	Support of clearance of damaged organelles/proteins	LC3-II, sequestosome 1 (p62), Beclin-1	[57,58]
Pregnane X receptor (PXR)	Ligand-dependent activation	Regulation of detoxification/xenobiotic metabolism responses	cytochrome P450 3A4 (CYP3A4), multidrug resistance 1 (MDR1)	[57,58]
Purinergic P2X7 receptor (P2X7)	Antagonism / modulation (reported)	Tendency toward attenuated microglial inflammatory responses	caspase-1, interleukin-18 (IL-18)	[57,58]

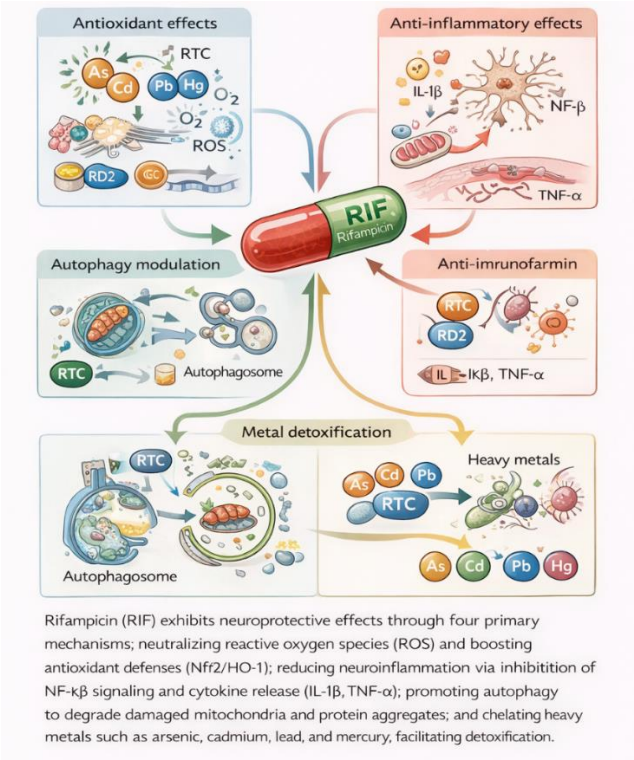

Figure 3. Neuroprotective partways of RIF.

4.1. Activation of the Nrf2 Signaling Pathway and Antioxidant Effects of RIF

RIF is a lipophilic antibiotic used in the treatment of TB and other mycobacterial infections. Its antimicrobial activity is primarily based on binding to the beta (β) subunit of bacterial RNA polymerase and inhibiting transcription [58]. However, an increasing number of studies indicate that the pharmacological actions of RIF are not limited to antimicrobial activity and that it may exert modulatory effects on defense axes related to oxidative stress in the CNS [58-60]. In this context, one of the most widely discussed mechanisms for RIF is its potential to support the Nrf2 signaling axis, a major regulator of cellular redox homeostasis.

Under physiological conditions, Nrf2 is sequestered in the cytoplasm by Kelch like ECH associated protein 1 (Keap1). With increased oxidative stress, Nrf2 dissociates from Keap1, translocates to the nucleus, binds to ARE regions, and initiates transcription of cytoprotective genes [61,62]. The literature indicates that the effect of RIF on the Nrf2 axis may, in some studies, be associated with activation of AMP-activated protein kinase alpha (AMPKα); this can facilitate dissociation of Nrf2 from the Keap1 complex and promote nuclear translocation, thereby increasing expression of target genes such as heme oxygenase-1 (HO-1) [59]. This Nrf2-mediated response is considered a central protective axis in limiting oxidative damage through induction of target genes such as HO-1, glutathione S-transferases (GSTs), and NQO1

[61,62]. Therefore, the ability of RIF to support the Nrf2/ARE axis may provide a potential biological rationale for reducing the oxidative stress burden that becomes prominent during heavy metal exposure [60,61]. The reported steps and representative outcomes of RIF action along the Nrf2/ARE axis are summarized in Table 9.

Table 9. Reported Steps and Representative Outcomes of RIF Action Along the Nrf2/ARE Axis.

Mechanistic Step	Event Associated with RIF	Functional Outcome	Marker/Outcome	References
Upstream signaling	AMPKα activation (reported)	Facilitated dissociation of Nrf2 from Keap1	p-AMPKα	[59]
Nrf2 mobilization	Dissociation from the Keap1–Nrf2 complex	Nuclear translocation of Nrf2	Increased nuclear Nrf2	[59,61]
ARE driven transcription	Binding of Nrf2 to ARE regions	Increased expression of cytoprotective genes	ARE target genes	[61,62]
Antioxidant/detoxification response	Increased HO-1, NQO1, and GSTs	Increased resistance to oxidative damage	HO-1, NQO1, GST	[59-62]

Table 9 indicates that the antioxidant potential of RIF can be explained not through a single target but via modulation of the Keap1 Nrf2 checkpoint through upstream signaling. This may create a multi target response window capable of supporting redox balance through coordinated induction of cytoprotective genes such as HO-1, NQO1 and GSTs [59-62].

4.2. RIF's NF-κB Inhibition and Anti-Inflammatory Mechanisms

Recent experimental studies indicate that RIF and some of its derivatives can modulate inflammatory responses in the CNS. Nuclear factor-κB (NF-κB), a key regulator of pro-inflammatory gene expression, plays a critical role in shaping cytokine responses associated with microglial activation, including TNF-α and IL-1β. Therefore, suppression of this signaling pathway is considered one of the principal mechanisms underlying the anti inflammatory effects of RIF. Microglia focused studies further suggest that RIF quinone (RIFQ), a derivative of RIF, can attenuate α-synuclein induced microglial inflammatory responses and may be associated with improved neuronal survival. In this context, Acuña et al. (2020) reported that pretreatment with RIFQ enhanced neuronal survival under conditions of α-synuclein induced microglial inflammation [43]. These findings support the view that RIF derivatives may reduce secondary neuronal damage by limiting microglia mediated inflammatory outputs.

In vivo evidence also provides a more direct framework for the involvement of NF-κB signaling. Bi et al. (2021) demonstrated that RIF administration in a lipopolysaccharide (LPS) induced mouse model of neuroinflammation was associated with inhibition of the Toll like receptor-4 / MyD88 / NF-κB (TLR4–MyD88–NF-κB) signaling pathway, accompanied by improvements in cognitive and motor functions [63]. These results suggest that RIF may act as a modulator capable of suppressing proinflammatory transcriptional responses, particularly under inflammatory conditions triggered by infection or tissue injury.

Overall, the reported anti inflammatory effects of RIF and RIFQ are mainly associated with the attenuation of microglial inflammation and, in certain experimental models, the suppression of the TLR4–MyD88–NF-κB signaling axis. The current evidence supporting these mechanisms is summarized in Table 10.

Table 10. Outcomes of Anti-Inflammatory Effects of RIF/RIFQ Related to the NF-κB Axis.

Model / Stimulus	Intervention	Target Axis	Marker/Event	Functional Outcome	References
Microglia α-synuclein induced inflammation	RIFQ (pretreatment)	Modulation of microglial inflammatory responses	Findings consistent with attenuation of the inflammatory response	Trend toward improved neuronal survival	[43]
Mouse LPS induced neuroinflammatin	RIF	TLR4/MyD88/NF-κB	Inhibition within the NF-κB pathway	Trend toward improved cognitive and motor impairments	[63]

Table 10 indicates that the anti inflammatory effects associated with RIF and RIFQ are primarily characterized by suppression of the NF-κB signaling axis, accompanied by a reduction in pro-inflammatory cytokine levels. However, these findings should be interpreted in light of the experimental context, as the observed outcomes are largely model

dependent. Accordingly, the magnitude of the effect and its translational relevance may vary considerably depending on the type of stimulus, exposure conditions, and dose duration parameters.

4.3. Negative Crosstalk Between RIF-Induced Pregnan X Receptor and NF-κB

RIF is one of the best known non endogenous agonists of the human pregnane X receptor (hPXR). Activation of hPXR plays a central role in regulating xenobiotic sensing and detoxification responses, particularly in tissues such as the liver and intestine. This regulatory network extends beyond drug metabolism and also influences gene networks involved in lipid and glucose metabolism [64]. Upon hPXR activation, increased expression of detoxification enzymes, most notably cytochrome P450 3A4 (CYP3A4) is observed. This induction process is largely mediated by enhanced interactions between the receptor and coactivators such as SRC-1 [64,65].

However, hPXR activation is not limited to gene induction, as repression of certain gene sets has also been reported. This effect may arise from competition with other nuclear receptors, such as HNF4α, for a limited pool of coactivators, as well as from direct protein protein interactions. In this context, suppression of genes involved in bile acid synthesis such as cytochrome P450 7A1 (CYP7A1) and cytochrome P450 8B1 (CYP8B1) and downregulation of certain drug transporters, including organic cation transporter1 (OCT1/SLC22A1), have been linked to hPXR dependent coactivator dynamics. In addition, RIF has been reported to enhance the interaction between hPXR and the small heterodimer partner (SHP), thereby altering transcriptional balance and introducing an additional regulatory layer that may influence CYP3A4 induction [64-68].

The metabolic consequences of hPXR activation in glucose regulation appear to be context dependent. Some studies suggest that hPXR can suppress cyclic adenosine monophosphate (cAMP) mediated activation of the glucose-6-phosphatase (G6Pase) gene through cross talk with the cAMP response element binding protein (CREB). Conversely, under different molecular conditions, activation of the serum/glucocorticoid regulated kinase 2 (SGK2) axis may promote gluconeogenic signaling [69,70]. These divergent findings indicate that the metabolic outcomes of hPXR activation are highly sensitive to cellular context and signaling environment.

Another important aspect is that hPXR exhibits negative cross talk with nuclear factor-κB (NF-κB), a key regulator of inflammatory transcription. The literature emphasizes that this receptor cross talk is largely shaped by the shared use of transcriptional coactivators and reciprocal protein interactions. Consequently, the regulatory impact of hPXR involves both gene activation and transcriptional repression [64]. Within this framework, hPXR activation may indirectly attenuate NF-κB mediated pro inflammatory gene expression through coactivator competition and transcriptional interference. Taken together, the hPXR mediated actions of RIF particularly when considered alongside NF-κB suppression may represent an important mechanistic component of its proposed anti inflammatory and neuroprotective profile [64]. The principal induction and repression targets of hPXR activation and its negative interaction with NF-κB are summarized in Table 11.

Table 11. Targets and Mechanisms of RIF–hPXR Activation and Negative Crosstalk with NF-κB.

Effect Type	Target/Pathway	Possible Mechanism (Summary)	Functional Outcome	References
Induction	CYP3A4	Strengthened interaction with coactivators (e.g., SRC-1)	Increased detoxification response	[64,65]
Repression	CYP7A1 / CYP8B1	Coactivator competition and protein protein interactions	Repression of bile acid synthesis genes	[66-68]
Repression	Organic cation transporter 1 (OCT1; SLC22A1)	Competition for coactivators (e.g., SRC-1)	Trend toward decreased drug transporter expression	[67]
Context dependent effect	CREB G6Pase	Crosstalk with CREB; suppression of cAMP driven response	Trend toward decreased G6Pase expression	[69]
Context dependent effect	SGK2 axis	SGK2 mediated signaling	Trend toward increased gluconeogenesis	[70]
Negative crosstalk	NF-κB driven transcription	Shared coactivator usage/competition; transrepression framework	Trend toward attenuated pro inflammatory gene responses	[64]

Table 11 demonstrates that the hPXR activation response induced by RIF is bidirectional: (1) marked induction of detoxification related genes, such as CYP3A4, and (2) repression of specific metabolic and transporter genes through coactivator competition and protein protein interactions. Similar coactivator dynamics also provide a mechanistically consistent framework for explaining the negative cross talk between hPXR and NF-κB signaling pathways [64-68].

4.4. RIF's Modulation of Protein Aggregation and Microglial Inflammation via Autophagy

In neurodegenerative diseases, pathological protein accumulation and neuroinflammation are interrelated processes that can mutually exacerbate each other. Within this axis, increased microglial activation may amplify neuronal damage [42]. Notably, microglial responses triggered by α-syn fibrils have been linked to neurodegenerative outcomes and can be pharmacologically modulated [17]. Consequently, the autophagy lysosome system is considered a critical regulatory mechanism for both reducing toxic protein load and modulating inflammatory responses.

Experimental evidence indicates that RIF can suppress microglial inflammation and enhance neuronal survival [71]. A key proposed mechanism involves RIF mediated enhancement of autophagic processes, which limits inflammatory outputs. In a rotenone induced microglial inflammation model, RIF treatment reduced inflammatory responses, and this effect was abolished in the presence of autophagy inhibitors, supporting an autophagy dependent mechanism [72].

RIF's effects are not limited to autophagy induction. It has also been shown to support lysosomal functions necessary for autophagic flux completion. In rotenone treated microglia, RIF enhanced lysosomal function, reducing inflammation, with lysosomal acidification and autophagosome lysosome fusion facilitated via V-ATPase components such as ATP6V0A1 [73].

Enhanced autophagic flux may attenuate microglial pro inflammatory responses by promoting more efficient clearance of inflammatory signaling components and damaged organelles [73]. Regarding protein aggregation, RIF has been reported to reduce amyloid-β (Aβ) accumulation in the hippocampus by increasing autophagy in an LPS induced cognitive impairment model [74]. The concept that increased autophagic flux facilitates lysosomal degradation of misfolded proteins such as Aβ and α-syn aligns with studies emphasizing the importance of autophagy in PD pathogenesis [75]. Furthermore, considering that heavy metals can disrupt the autophagy lysosome axis and contribute to aging and neurodegenerative processes, RIF mediated enhancement of this pathway may provide a mechanistic framework for limiting heavy metal induced protein damage and aggregation [12]. Autophagy lysosome related regulatory steps and representative outcomes associated with RIF are summarized in Table 12.

Table 12. RIF's Modulation of Protein Aggregation and Microglial Inflammation via the Autophagy Lysosome Axis.

Model/Context	Proposed Target Axis	Effect Associated with RIF	Outcome	References
Neuroinflammation (general framework)	Microglial activation	Microglial inflammation contributes to neuronal injury	Pro inflammatory responses are associated with pathology	[42]
Microglia neuron interaction	Microglial inflammation	Suppression of inflammation and support of neuronal survival with RIF	Trend toward improved neuronal survival	[71]
Rotenone model	Autophagy induction	Reduced inflammation with increased autophagy; loss of effect with autophagy inhibitors	Reduced microglial inflammation	[72]
Rotenone treated microglia	Lysosomal function	Improved lysosomal function (including V-ATPase/ATP6V0A1)	Enhanced autophagic flux	[73]
LPS mouse model	Increased autophagy	Suppression of Aβ burden via autophagy	Trend toward reduced Aβ accumulation	[74]
PD context	Autophagy protein clearance	Autophagy is linked to α-syn burden (general)	Importance of proteostasis	[75]
Heavy metal context	Autophagy lysosome impairment	Heavy metals impair the autophagy lysosome axis	Increased risk of aggregation/damage burden	[12]

Table 12 indicates that RIF's effects on mitigating microglial inflammation and limiting pathological protein accumulation may be mediated through complementary mechanisms, including the induction of autophagy and enhancement of lysosomal function [71-75].

5. In Vitro Neuronal Models and Evaluation Methods

In vitro neuronal models are widely used experimental platforms for elucidating the molecular mechanisms of heavy metal induced neurotoxicity and for the preliminary evaluation of potential neuroprotective agents. Human neuroblastoma (SH-SY5Y), mouse hippocampal (HT22), and rat pheochromocytoma (PC12) cell lines are frequently employed due to their retention of key neuronal characteristics, high reproducibility, and relatively fewer ethical constraints [76-78]. The primary applications and recommended assessment parameters for these models are summarized in Table 13.

Table 13. Use Focus and Recommended Evaluation Readouts in *In Vitro* Neuronal Models.

Model	Origin / Key Features	Use Focus in This Review	Recommended Core Readouts (Examples)	References
SH-SY5Y	Human neuroblastoma; dopaminergic like phenotype	Metal related stress responses; PD relevant axes	Viability (MTT/CCK-8), LDH; ROS (DCFDA); ΔΨm (JC-1); apoptosis (Annexin V/PI, caspase-3); PI3K/Akt, BDNF/TrkB	[76-80]
HT22	Mouse hippocampal cell line	Glutamate driven oxidative stress; metal + glutamate synergy	ROS; GSH/GSSG; lipid peroxidation (MDA); cell death/apoptosis markers	[76-78,81]
PC12	Rat pheochromocytoma; NGF induced neuron like differentiation	Metal toxicity; autophagy/aggregate clearance (e.g., RIF)	Neurite outgrowth/morphology; ROS; apoptosis; autophagy (LC3-II/p62); Akt/mTOR phosphorylation	[76-78,82,83]

SH-SY5Y cells, due to their dopaminergic like phenotype, are frequently employed to investigate PD related processes and heavy metal induced cellular stress responses, including ROS production, mitochondrial dysfunction and apoptosis. For instance, ellagic acid has been reported to exert protective effects against Cd, Hg, and Pb toxicity in SH-SY5Y cells by reducing ROS levels and enhancing antioxidant responses [79]. Similarly, Cd exposure disrupts the brain derived neurotrophic factor/tropomyosin receptor kinase B (BDNF/TrkB) and phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) pathways, whereas stilbene derivatives such as tetrahydroxystilbene glucoside (THSG) can support these pathways to elicit a neuroprotective response [80].

The HT22 cell line is widely used to model glutamate-induced oxidative stress and hippocampal damage. Concurrent exposure to heavy metals and glutamate has been shown to exacerbate synergistic neurotoxicity in HT22 cells, intensifying oxidative stress-mediated cellular injury [81]. In this model, integrating cell viability/death metrics with redox balance indicators (ROS, GSH) and oxidative damage markers enables more robust mechanistic insights.

PC12 cells, which can differentiate into neuron like phenotypes upon nerve growth factor (NGF) stimulation, are commonly used to evaluate processes related to oxidative stress, apoptosis, and synaptic function. Li et al. (2025) reported that pea peptides (PP4) increased calsyntenin-1 (CLSTN1) levels in PC12 cells, conferring neuroprotective effects against heavy metal toxicity [77]. Similarly, Banaeeyeh et al. (2025) demonstrated that natural compounds such as morin reduced As and Cd induced ROS production, thereby decreasing cell death [82]. Furthermore, the PC12 model provides an appropriate experimental framework to assess the potential of RIF to modulate protein load and aggregation dynamics via autophagy related pathways, including the Akt/mTOR axis [83].

Collectively, these three neuronal cell models allow for comparative evaluation of the oxidative stress mitochondria inflammation axis involved in heavy metal exposure and the autophagy/lysosome based modulatory potential of RIF within a unified experimental platform.

5.1. Cytotoxicity and Cell Viability Assays in *In Vitro* Neurobiological Studies

In vitro studies serve as fundamental reference frameworks for cell viability and cytotoxicity assessments. These assays quantitatively evaluate the effects of metal exposure and pharmacological interventions on cellular metabolic activity and membrane integrity. Among the most commonly employed methods are the MTT assay, which reflects metabolic activity, and the LDH release assay, which indicates membrane integrity. The measured biological parameters and key considerations for their interpretation are summarized in Table 14.

According to Table 14, interpreting cell viability and cytotoxicity measurements in isolation can be misleading. The MTT assay reflects cellular reducing capacity, and under conditions of slowed metabolic activity, it may underestimate actual cell viability. In contrast, the LDH assay provides a more direct measure of membrane damage. Therefore, when a decrease is observed in MTT, it is critical to distinguish whether this reflects true cell death or merely metabolic suppression. Similarly, evaluating increases in LDH release across multiple time points helps clarify whether the observed damage occurs early or later during exposure. In this context, a dual validation strategy serves as a practical standard that enhances the reliability of interpreting "protective" effects in heavy metal and RIF combination studies [84-86].

Among metabolic activity based assays, MTT is widely employed, providing a quantitative measure of cell viability through assessment of reducing capacity. For example, in studies examining the cytotoxicity profile of RIF, cell viability remained high at 24 hours under low to moderate doses, whereas higher doses and prolonged exposure (e.g., 200 μg/mL for 48–72 hours) resulted in marked reductions [84]. Similarly, MTT and analogous assays have been frequently used to evaluate heavy metal toxicity, with metals such as As and Cd producing significant, dose and time dependent decreases in viability [85]. Thus, accurate interpretation of neuroprotective effects requires consideration of both dose response and time response relationships for the metal and RIF.

Table 14. Biological Parameters Used for Cell Viability and Cytotoxicity Analyses in Neurobiological *In Vitro* Studies

Test / Approach	Primary Measurement	Indicate	Strength	Points to Consider When Interpreting Results	References
MTT (metabolic activity based)	Cellular reducing capacity	Trend in viability / metabolic status	Widely used, quantitative, comparable	Dose time window is critical; metabolic alterations may not always map one to one onto "cell death"	[84,85]
LDH release (membrane integrity based)	LDH released into the medium	Membrane damage / cytotoxicity	Complements viability assays	Multiple time points are recommended to distinguish early vs late injury; serum/medium conditions can influence baseline LDH	[86]
Combined reporting (MTT + LDH)	Metabolism + membrane damage	More robust cytotoxicity interpretation	More reliable than a single assay	If readouts diverge (e.g., preserved MTT with increased LDH), mechanistic interpretation is required	[84-86]

The LDH release assay complements metabolic viability measurements by providing a direct readout of membrane integrity. When membrane damage occurs, cytoplasmic LDH is released into the culture medium, serving as a direct indicator of cytotoxicity. For instance, Pb and Cd exposure has been reported to reduce metabolic activity while simultaneously increasing LDH release, consistent with compromised membrane integrity [86]. This dual validation approach mitigates the limitations of single assay interpretations and provides a more robust framework for assessing RIF's "protective" effects under heavy metal exposure [84-86].

In conclusion, in vitro neurotoxicity studies are recommended to include at least one metabolic viability assay (e.g., MTT) alongside a membrane integrity/cytotoxicity assay (e.g., LDH), with measurements conducted at multiple time points to ensure reliable and comprehensive assessment [84-86].

5.2. Assessment of Oxidative Stress, Antioxidant Defense, and Biochemical Parameters

Following heavy metal exposure, measurement of ROS levels is one of the most commonly employed approaches to capture early stages of oxidative stress. In this context, SH-SY5Y cells serve as a practical and reliable in vitro model due to their neuronal characteristics and widespread use in experiments focusing on PD related processes [87]. A frequently used probe for ROS detection, chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA), is converted to its active form by intracellular esterases upon entry into the cell. Under oxidative conditions, it is oxidized to fluorescent 2′,7′-dichlorofluorescein (DCF), and the resulting fluorescence intensity is considered a quantitative indicator of intracellular oxidant load [88].

However, reducing heavy metal toxicity to a single "ROS increase" readout is insufficient. ROS measurements should be interpreted alongside complementary endpoints, including lipid peroxidation markers (e.g., MDA), antioxidant defenses (e.g., SOD, GPx), and other relevant cellular processes [31]. Indeed, heavy metals have been reported to deplete GSH pools, suppress antioxidant enzymes such as SOD and CAT, and impair enzymatic activity through metal cofactor interactions [25,28,29]. For agents like RIF, documenting protective effects should not rely solely on reductions in ROS levels but also incorporate improvements in MDA levels, SOD/GPx activity, and regulation of antioxidant related genes and proteins. Evidence that RIF can enhance protective responses against oxidative stress via activation of the AMPK/Nrf2 axis provides a coherent rationale for the combined use of multiple endpoints [59]. A summary of the markers and methodologies employed is provided in Table 15.

Table 15 demonstrates that heavy metal induced oxidative stress is not limited to increases in ROS; rather, a more coherent assessment framework emerges when changes in MDA and antioxidant defense capacity (SOD/GPx, GSH/GSSG) are also considered. Therefore, instead of relying solely on a single ROS measurement, it is recommended to evaluate both damage markers and defense parameters concurrently. Additionally, monitoring targets such as *Sod1/Gpx* and *Bax/Bcl-2* via RT-qPCR can strengthen the correlation between biochemical findings and the associated cellular processes.

6. Conclusion and Future Perspectives

The evidence reviewed in this study indicates that although heavy metals such as As, Cd, Pb, and Hg target distinct initial regions within the central CNS, they ultimately converge on shared pathological axes. These axes prominently include oxidative stress (increased ROS and compromised antioxidant defenses), mitochondrial dysfunction, disrupted Ca^{2+} homeostasis, and chronic neuroinflammation. Collectively, these processes compromise neuronal integrity and facilitate the activation of programmed cell death pathways [3,7,12,22,23,31,37,41,45-48]. Furthermore, the literature emphasizes that over time, these mechanisms can interact with protein aggregation and epigenetic modifications, establishing a common pathological framework for neurodegenerative processes [20,49-51,52-55].

Within this pathological context, RIF can be considered a multi target (pleiotropic) candidate beyond its antimicrobial role. On the antioxidant front, RIF's ability to activate the AMPK/Nrf2 axis and enhance protective responses such as

HO-1 provides a robust biological rationale for limiting the elevated oxidative burden induced by heavy metal exposure [59-61]. In the inflammatory domain, suppression of NF-κB signaling networks and modulation of microglial responses contribute to counteracting the chronic neuroinflammatory dimension of metal toxicity [42,63].

Additionally, RIF enhances autophagy lysosomal flux, facilitating the clearance of damaged proteins and organelles as well as inflammatory components. This mechanism contributes to neuroprotection by reducing toxic protein load and modulating microglial activation [72-75]. Conversely, while PXR activation is critical for detoxification networks, potential drug drug interactions warrant careful consideration in clinical settings [64]. The balance between these advantages and limitations should be evaluated in the context of the clinical framework summarized in Table 16 [18,56,64].

Table 15. Oxidative Stress, Antioxidant Defense, and Evaluation of Biochemical Parameters.

Component	Marker / Event	Primary Interpretation	References
ROS burden	CM-H_2DCFDA DCF fluorescence	An early indicator of increased intracellular oxidant load; a decrease following intervention supports attenuation of oxidative stress.	[88]
Lipid peroxidation	MDA (e.g., TBARS)	Biochemical evidence of oxidative damage to membrane lipids; an increase suggests progression of injury.	[31]
Enzymatic antioxidant defense	SOD activity	Reflects depletion/recovery of antioxidant capacity; a decrease is expected under metal exposure, whereas recovery is anticipated with protective interventions.	[25,28,29]
Enzymatic antioxidant defense	GPx activity	Indicates the status of the GSH dependent defense line; a decrease may be consistent with an increased oxidative stress.	[25,29]
Glutathione balance	GSH/GSSG ratio	A more direct indicator of cellular redox balance; a reduced ratio supports redox disequilibrium.	[25]
Transcriptional antioxidant response	sod1, gpx (RT-qPCR)	Gene level support for biochemical findings; indicates whether antioxidant defense programs are regulated.	[25]
Cell fate balance	bax/bcl-2 (RT-qPCR)	Supports a shift in the apoptosis survival balance; bax↑/bcl-2↓ indicates a pro apoptotic tendency.	[25]
RIF associated antioxidant axis	Nrf2 targets (e.g., HO-1/NQO1)	Mechanistically supports RIF's potential to strengthen antioxidant responses.	[59]
Panel approach	Combined ROS + MDA + SOD/GPx + RT-qPCR	Multi endpoint validation rather than a single readout; increases confidence in interpreting a "protective effect."	[31]

Table 16. Advantages and Clinical Limitations of RIF in Neuroprotection.

Parameter	Advantages	Limitations	References
Clinical use	Longstanding clinical experience; generic and accessible	Clinical evidence for CNS indications remains limited and heterogeneous	[18,56]
Safety	Established monitoring framework	Risk of adverse effects with prolonged use high doses; requires monitoring	[56]
CNS access	Good absorption and systemic distribution	BBB penetration and target tissue exposure remain uncertain; dose optimization is needed	[14,56,75]
Multi target profile	Engages Nrf2, NF-κB, and autophagy lysosome related axes	Biomarker standardization is needed to align clinical outcomes with mechanisms	[59-63,72-75,18,88]
Drug interactions	May influence detoxification networks	PXR mediated enzyme induction → increased interaction risk	[64]
Human evidence	Positive signals reported in some areas	Lack of robust randomized designs and long term outcomes	[18]

In conclusion, RIF's multi target profile encompassing antioxidant, anti inflammatory, and autophagy lysosomal pathways provides substantial advantages at the preclinical level. Its clinical applicability, however, depends on critical factors including adequate CNS tissue exposure, effective management of drug drug interactions, and the use of standardized endpoints and biomarkers for outcome assessment [14,18,64,75,88]. Taken together, RIF's multifaceted modulatory capacity positions it as a promising and strategic approach for mitigating heavy metal induced neurotoxicity and associated neurodegenerative processes [89,90].

Future Perspective

Current evidence highlights several priorities for accurately assessing the therapeutic potential of RIF in the context of heavy metal induced neurotoxicity. First, to strengthen mechanistic interpretations and reliably evaluate protective effects, assessments should not be limited to a single ROS measurement or a single viability assay. Instead, cell viability/cytotoxicity data (MTT-LDH) should be reported alongside oxidative stress markers (e.g., ROS, MDA, SOD/GPx) and relevant gene/protein panels (e.g., Nrf2 targets, *bax*/*bcl-2* balance) to provide a more comprehensive mechanistic insight [84-86,88]. Second, standardized experimental designs are necessary to minimize variability arising from differences in cell lines (e.g., SH-SY5Y, PC12), metal types, dose time regimens, and co exposure scenarios (including metal mixtures), thereby enhancing comparability across studies [12,78,85]. Third, pharmacokinetic studies are essential to determine CNS distribution and achieve adequate target tissue exposure while maintaining safety. Overcoming the BBB remains a critical challenge, highlighting the need for optimized administration and formulation strategies [14,75]. Finally, although existing human data provide some encouraging signals, heterogeneity and design variability limit definitive conclusions, emphasizing the importance of well designed clinical trials and biomarker based monitoring strategies [18].

In conclusion, heavy metal induced neurotoxicity is complex and multifactorial, and cannot be explained by a single mechanism. Accordingly, protective strategies targeting multiple biological pathways simultaneously are likely to be more effective [3,7,12,22,23]. Current literature indicates that RIF exhibits a multitarget profile through modulation of Nrf2, NF-κB, and autophagy related pathways. Successful clinical translation will depend on effective BBB penetration, careful management of potential drug–drug interactions, and stepwise validation using standardized endpoints and biomarkers [14,18,56,59-64,72-75,84-86,88]. Within this framework, elucidating RIF's role in heavy metal associated neurodegenerative processes requires a standardized, biomarker guided research trajectory extending from experimental models to clinical studies [18,84-86].

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