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Mapping the future of oxidative RNA damage in neurodegeneration: Rethinking the status quo with new tools

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Over two decades ago, increased levels of RNA oxidation were reported in postmortem patients with ALS, Alzheimer's, Parkinson's, and other neurodegenerative diseases. Interestingly, not all cell types and transcripts were equally oxidized. Furthermore, it was shown that RNA oxidation is an early phenomenon, altogether indicating that oxidative RNA damage could be a driver, and not a consequence, of disease. Despite all these exciting observations, the field appears to have stagnated since then. We argue that this is a consequence of the shortcomings of technologies to model these diseases, limiting our understanding of which transcripts are being oxidized, which RNA-binding proteins are interacting with these RNAs, what their implications are in RNA processing, and as a result, what their potential role is in disease onset and progression. Here, we discuss the limits of previous technologies and propose ways by which advancements in iPSC-derived disease modeling, proteomics, and sequencing technologies can be combined and leveraged to answer new and decades-old questions.

oxidative RNA damage | neurodegeneration | RNA-binding proteins | oxidative stress | induced pluripotent stem cells

RNA Oxidation in Neurodegenerative Disease

Neurodegeneration and Oxidative Stress. The rise of age-dependent neurodegenerative diseases—such as Alzheimer's (AD), Parkinson's (PD), and amyotrophic lateral sclerosis (ALS)—poses a major threat worldwide. In the United States, an estimated 6 million people currently suffer from AD, 1 million from PD, and 25,000 from ALS (1–3). As the number of people aged 60 or older is expected to double by 2050, the incidence of neurodegenerative disease is expected to increase along with it (4). Decades of research have condensed the basic hallmarks of neurodegenerative disease pathology into eight categories: pathological protein aggregation, synaptic and neuronal network defects, aberrant proteostasis, cytoskeletal abnormalities, altered energy homeostasis, DNA and RNA defects, inflammation, and neuronal cell death (5) (Fig. 1). The overlap between these features has furthered investigation efforts and generated hypotheses regarding how they could be related.

Examining mitochondrial damage during aging gave rise to the Mitochondrial Cascade Hypothesis, highlighting mitochondria's capacity to initiate or exacerbate pathological hallmarks of AD as the main intracellular producer of reactive oxygen species (ROS) (6, 7). Age-dependent mitochondrial decline often drives reduction in the electron transport chain activity and overproduction of free radicals (7). Similar phenotypes have been observed in postmortem AD (8), motor neurons of sporadic and familial ALS (9), and postmortem PD (6). However, the origin of this accelerated mitochondrial dysfunction in disease requires further evaluation. Complementarily, the Neuroimmunomodulation Hypothesis proposes a role for the innate immune system and neuroinflammation in neurodegeneration (10). First proposed to converge risk factors and early onset AD-specific phenotypes, it has since been revised to encompass a range of neurodegenerative diseases including ALS and PD, as researchers found activated microglia, the brain-resident immune cells, and proinflammatory profiles in patients (10–13). This theory suggests that damage signals generated in response to tissue distress activate microglia and cause them to secrete proinflammatory cytokines and cytotoxic reactive oxygen and nitrogen species (RONS) (11, 14).

While the hypotheses above chronicle different features implicated in disease pathology, they both highlight generators of RONS. Mitochondria continuously generate primary ROS, such as hydrogen peroxide (H_2O_2) and superoxide (O^-_2) as a by-product of oxidative phosphorylation, which in turn can form hydroxyl radicals (OH^-). Activated microglia trigger inducible nitric oxide synthase (iNOS) and NADPH oxidase (NOX), which are capable of generating both oxygen and nitrogen species, like H_2O_2, O^-_2, peroxynitrite ($ONOO^-$), nitric oxide (NO), and nitrogen dioxide (NO_2) (15, 16). Furthermore, these cells produce myeloperoxidase (MPO) which can catalyze the formation of hypochlorous acid (HOCl) from H_2O_2 and chloride (4). While this enzyme is rarely expressed in the brain, an increased abundance of MPO has been described in microglia and astrocytes in AD (17–19) and PD (20). The way by which these cells are activated in disease is difficult to ascertain as there are many possible exogenous (i.e., repeated injury, toxin exposure) and endogenous sources (i.e., disease-associated molecular patterns (DAMPs), gut

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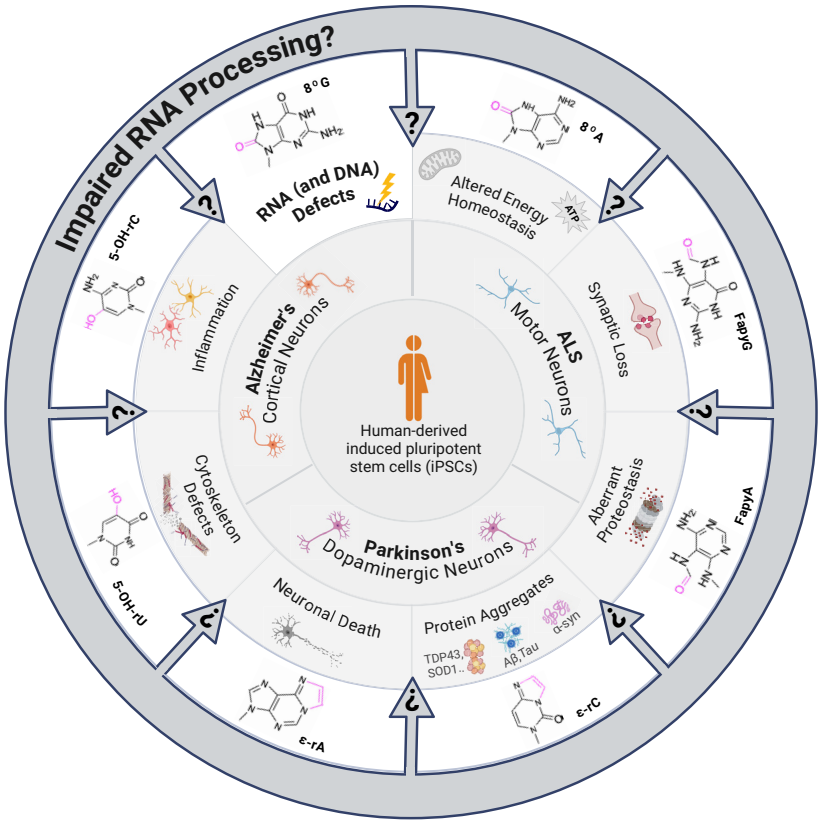

Fig. 1. Proposed link between oxidative RNA lesion-driven defects in RNA processing and other hallmarks of neurodegenerative disease. Donor fibroblasts or PBMCs can be reprogrammed to iPSCs and subsequently differentiated into disease-relevant cell types. iPSC-derived neuronal systems have been shown to closely recapitulate the eight hallmarks of neurodegenerative disease (5) and can be leveraged to elucidate disease-associated mechanisms. The interplay between oxidative RNA damage, impaired RNA processing, and the manifestation of one or more of the shared neurodegenerative phenotypes has yet to be explored. A deeper understanding of converging phenotypes will aid in determining the role of RNA oxidation on dysregulated processes and neurodegenerative disease. Created with BioRender.com.

microbial metabolites, genetic susceptibility) (12, 13, 15, 21). It is believed that repetitive activation by one or more of these sources may prime the microglia for an intensified secondary response, resulting in chronic RONS production and neuroinflammation (22).

In healthy states, the continuous generation of reactive species is balanced by the activity of antioxidant pathways. In excess, these control mechanisms are bypassed, and RONS can overwhelm the cell, giving rise to oxidative stress (23). Overwhelming formation of RONS can then lead to lesions on nucleic acids, lipids, and proteins by either direct oxidation, nitration, halogenation, or indirectly by interacting with lipid peroxidation products (24–26). The increased presence of oxidative damage has been well established as a pathological signature in postmortem patient samples with AD (27, 28), PD (29, 30), and ALS (31, 32).

Nucleic Acid Oxidation in Disease. Historically, DNA oxidation and its relationship with neurodegeneration have been at the forefront of nucleic acid investigation, while its counterpart—RNA—was broadly considered a temporary intermediate. The general understanding was that damaged RNAs were targeted for degradation and de novo synthesized, while DNA strand breaks and DNA nucleotide damage were repaired to maintain genomic information (33). For this reason, characterizing the growing list of RONS-derived base modifications on DNA and their products has been at the forefront of investigation (34, 35). However, as knowledge on RNA processing continued to expand, its relationship with oxidative stress was revisited, especially after RNA oxidation was found to be more prevalent than DNA oxidation in AD and PD (30, 36).

RNA oxidation may occur directly on an RNA molecule or through the incorporation of oxidized NTPs from the nucleotide pool by RNA polymerases. While much is still unknown about RNA oxidation, some lesions derived from $ONOO^-$ and OH^- (NO and H_2O_2-generated) have been described (37). Yanagawa and colleagues identified four primary OH^--derived lesions in yeast: 8-oxo-7,8-dihydroguanosine (8^OG), 8-oxo-7,8-dihydroadenosine (8^OA), 5-hydroxyuridine (5-HO-rU), and 5-hydroxycytidine (5-HO-rC) (25, 26). Similar to DNA, oxidized purine products—8^OG and 8^OA—have low redox potential and are highly vulnerable to further oxidation, forming 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyrG) and 4,6-diamino-5-formamidopyrimidine nucleosides (FapyrA), as well as imidazolone, oxazolone, guanidinohydantoin (Gh), and spiroiminodihydantoin (Sp) derivatives (34, 37). Furthermore, ROS can result in RNA depurination (38), while 40% of direct OH^- reactions with RNA have been proposed to lead to strand scission (39). Significantly, $ONOO^-$ can also form 8^OG as well as 8-nitroguanosine (8-nitro-rG) (40). Finally, lipid peroxidation products can result in the formation of N^6-ethenoadenine (εA) and 3,N^4-ethenocytosine (εC) (41) (Fig. 1).

Guanine is the most redox-sensitive nucleotide and is highly vulnerable to oxidative damage. Therefore, 8^OG (and 8oxodG in DNA) is the most abundant lesion under oxidative

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stress and, thus, is commonly used as a metric for assessing oxidative phenotypes (42). While 8oxodG and its repair mechanisms have gained the most attention, $8^{O}G$ was shown to be 14-25-fold higher than 8oxodG in H_2O_2-challenged human A549 lung epithelial cells and threefold higher in 2-nitropropane-treated rats (42, 43). With no known repair mechanisms for oxidized RNA (besides $8^{O}GTP$ clearance by MTH1) (44), these phenomena have the potential to impact base pairing during transcription, translation, secondary structure formation, and protein interactions.

In disease pathology, RNA oxidation has been shown to be region-specific to vulnerable neuronal subtypes characteristically affected in each neurodegenerative disease, namely cortical, dopaminergic, and motor neurons in AD, PD, and ALS, respectively (8, 45–47). Nunomura and colleagues were the first to suggest this after observing, in postmortem AD samples, a higher abundance of oxidized RNAs in pathologically vulnerable neurons, but not other cell types (36). Concurrently, Zhang and colleagues showed comparable patterns in PD patient samples. Specifically, RNase-sensitive RNA oxidation was observed in neurons of the substantia nigra of the midbrain but not in the cerebral cortex or cerebellum of PD patients (30). Furthermore, studies have postulated that within these vulnerable neuronal subtypes, damage is not entirely random, as some mRNA transcripts were found to be more susceptible to damage than others, independent of transcript abundance or disease progression (36, 45–51). Originally, damage abundance was thought to be dependent on disease progression, however, a growing number of studies suggest that RNA oxidation could serve as an early marker for disease, as it may precede widespread neuronal death (51), motor defects (45), and AD-mediated cognitive impairments (46).

Neurodegenerative Disease Model Systems. The technologies required to appropriately investigate disease pathology and oxidative damage often surpass the capabilities of available model systems. Many groundbreaking studies have utilized postmortem samples to characterize terminal disease pathology in ALS (31, 32), AD (27, 28), and PD (30, 52). The primary limitation in characterizing terminal samples is that early markers of disease are often indistinguishable from advanced pathology, diminishing critical details that may lead to disease progression. Primary cell culture systems obtained directly from live patient biopsies have been used as an alternative approach to bypass these limitations. However, the inherent risks of an invasive procedure, combined with the challenges of culturing primary cells, have led researchers to innovate new disease models (53).

In the early 2000s, Takahashi and Yamanaka pioneered a breakthrough in the advancement of cellular reprogramming by generating induced pluripotent stem cells (iPSCs) (54, 55). These reprogrammed terminally differentiated-derived cells can be used to generate neuronal 2D (monolayer and mixed) and 3D culture systems (56). This advancement has proven widely beneficial as many neurodegenerative disease phenotypes are recapitulated in iPSC models (Fig. 1).

AD. AD predominately arises from familial or sporadic genetic mutations in the *APP*, *PS1*, or *PS2*. These mutations lead to the accumulation of amyloid-beta (Aβ) plaques and neurofibrillary tangles (NFTs) made up of hyperphosphorylated tau, which in turn drive glial activation, synaptic loss, neuronal death, and cognitive decline (57–60). The dynamic interaction between these two neuropathologies has proven challenging to study in vivo, as primary transgenic models are effective in replicating Aβ- or tauopathy-associated phenotypes, but not both (61, 62). However, iPSC-derived models show both Aβ plaques and NFTs in a single system (63, 64). This allows for a comprehensive analysis of how oxidative stress and mitochondrial damage may contribute to specific disease-related neuropathies. Thus far, AD iPSC-derived neurons and astrocytes studies have reported heightened susceptibility to ROS production and oxidative stress, increased oxidative DNA damage, and abnormal oxidative phosphorylation before the onset of Aβ aggregation and tauopathies (65–67).

PD. PD is characterized by the accumulation of misfolded α-synuclein aggregates and the formation of Lewy Bodies, accompanied by mitochondrial dysfunction, oxidative stress, and ultimately, the loss of dopaminergic neurons in the substantia nigra (53, 68). The slow deterioration of dopaminergic neurons is the primary hallmark of PD and remains challenging to translate to disease model systems. Traditional models, like MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) toxin-treated system, is an example of this as it reflects some PD neuropathies but fails to mimic the disease's progression accurately (69). The neurotoxin treatment drives dramatic depletion of dopaminergic neurons in a short period of time, restricting disease progression analysis (69, 70). iPSC-derived dopaminergic neuronal systems with genetic mutations in *LRRK2, SNCA, PINK1, GBA, VPS35,* and *PARK2* have revolutionized PD research. These models have enabled researchers to observe early disease phenotypes that precede the formation of Lewy Bodies, including elevated oxidative stress and impaired mitochondrial morphology and function (71). iPSC-derived glial monocultures and cocultures have broadened these investigations by correlating PD-associated neuronal dysfunction with impaired immune responses (72–74).

ALS. ALS is characterized by a progressive loss of upper and lower motor neurons in the brain, brainstem, and spinal cord, resulting in paralysis and followed by death. It is associated with mutations in *SOD1*, *FUS*, *TARDBP*, *TBK1*, and *C9ORF72* (75). While the pathological cellular phenotypes are diverse (mitochondrial dysfunction, impaired RNA metabolism, autophagy, nuclear accumulation of toxic RNA foci, and cytoskeleton defects), they converge in the form of mislocalization and aggregation of RNA-binding proteins (RBPs) (76, 77). Current transgenic models are integral in exploring ALS; however, they often fail to mimic the mislocalization of affected RBPs (53).

iPSC technology has helped advance ALS research by enabling the study of patient-derived cells, disease-associated mutations, and their effects on RBP localization (78). Using iPSC-derived motor neurons, Harley and Patani investigated aberrant localization patterns of various RBPs (including TDP43, FUS, SFPQ, hnRNPK, and hnRNPA1) tied to neurodegenerative pathology. The results exhibit RBP-specific nuclear and cytoplasmic redistribution in response to oxidative stress (79). In addition to dysregulated RBPs, patient-derived iPSC lines have been utilized to characterize additional phenotypes associated with ALS, such as nuclear aggregates of RNA foci, TDP43 mislocalization, production of

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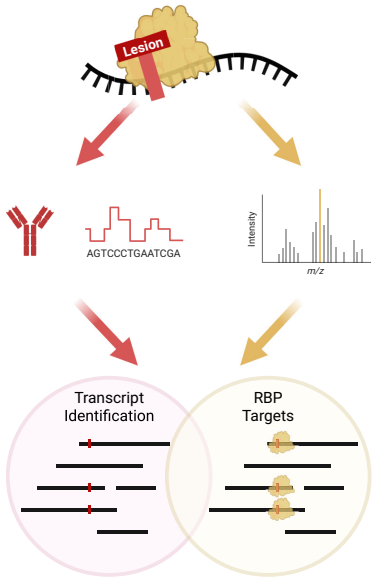
RAN translation products, impaired mitochondrial activity, and ROS generation (80–82).

Organoid modeling. 3D modeling of neurodegenerative disease serves as an alternative approach to traditional monolayer cultures, capturing complex microenvironments and multicellular interactions (83). This has proven advantageous as they more closely recapitulate physiological conditions compared to 2D systems (84). Park and colleagues incorporated a 3D human AD triculture system of iPSC-derived neurons, astrocytes, and microglia to model neurodegenerative and neuroinflammatory phenotypes. Using this system, they recapitulated key AD features, such as Aβ plaques, phosphorylated tau accumulation, and neuroinflammatory profiles, alongside less well-characterized features, like microglial recruitment and RONS release (85). A separate study utilized AD patient–derived cerebral organoids, cocultured with astrocytes and microglia, to study genomic alterations resulting from an *APOE4* risk variant in each cell type. Transcriptomic profiling revealed differentially expressed genes involved in synaptic activity in neurons, lipid metabolism in astrocytes, and immune responses in microglia-like cells (86). "BrainSphere" organoid structures composed of PD iPSC-derived neurons, astrocytes, and oligodendrocytes were generated to study selective impairment of dopaminergic neurons and mitochondrial dysfunction using neurotoxin-induced RONS accumulation (87). Moreover, they have been shown to overcome 2D limitations by displaying robust natural PD-associated disease phenotypes (i.e., increased cell death, reduced differentiation, branching, and altered mitochondrial morphology) without the use of stressors (88). Furthermore, more advanced 3D approaches, such as iPSC-derived "organ-on-a-chip" technology, have been revolutionary in elucidating multicellular organ and tissue-specific function in disease (89, 90). Osaki and colleagues incorporated this approach, namely "ALS-on-a-chip", to better understand ALS-related atrophy (91). In conclusion, while 3D modeling systems are still under development and come with their own set of complications, they present an exciting alternative to 2D monocultures to explore the implications of RNA oxidation in neurodegenerative diseases.

Detection of RNA Lesions and Their Effects on RNA Processing

RNA processing involves a series of intricate steps coordinated by RBPs. RNA–RBP interactions occur cotranscriptionally (for all RNA polymerases) and conclude upon RNA degradation, encompassing splicing, polyadenylation, editing, transport, translation, and turnover events. The repercussions of oxidative stress on RNA components involved in these processes include impaired translation (41), miRNA targeting (92), and potentially, posttranscriptional modifications of tRNA (93) and rRNA (94). More recently, impaired RBP recognition in the presence of oxidized RNA has been suggested as well (95). These findings are significant as many studies have explored aberrant RBP function in neurodegeneration (96), but only a few have assessed how oxidative RNA lesions may perturb RBP recognition and activity by modifying essential RNA–RBP interactions (95). To elucidate modified RNA–RBP interactions amid oxidative stress and disease pathology, it will be essential to merge past and novel methods designed to assess each component within these interactions (Fig. 2).

Detection of Proteins that Recognize Oxidized RNAs and Their Effects. An important step toward understanding the effects of oxidative RNA lesions on RNA processing is identifying the RBPs that recognize them. Predominantly, most of these interactions have been elucidated in HeLa cell extracts via in vitro pulldown using an oxidized RNA bait followed by western blotting or mass spectrometry. Curiously, only 8^{O}G baits, and not any other oxidative RNA lesions, have



	1.0	2.0
Antibody Isolation	8^{O}G IP-seq	o^{8}G-miSeq Lesion Antibodies
Transcript Identification	Microarray RT-PCR	Sequencing Reverse transcription signatures
RBP Identification	Bait pulldown	OOPS XRNAX LEAP-RBP
RBP Targets	RIP-seq	eCLIP TRIBE STAMP REMORA

Fig. 2. Novel detection methodologies for identification of RNA oxidation and their protein interacting partners. Oxidative RNA lesions (in red) can be recognized by RBPs (in dark yellow). To determine the identity of damaged transcripts two approaches can be used. Immunoprecipitation (IP) with anti-lesion antibodies followed by sequencing, or alternatively sequencing (without IP) with a focus on damage signatures introduced during reverse transcription. Lesion-binding RBPs can be identified by novel interactome phase separation methods (OOPS, XRNAX, and LEAP-RBP) via LC–MS. Their RNA targets can be further determined via eCLIP or other recent interactome technologies such as TRIBE, STAMP, and REMORA. The table on the right shows a comparison of previous (1.0) and novel (2.0) methods used for antibody isolation of damaged RNAs, identification of these transcripts, and identification of their interacting RBPs as well as these RBPs' targets. Created with BioRender.com.

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Table 1. RBPs that bind to (or are repelled by) $8^{O}G$

RBP	Model	Reference	Method	Proposed $8^{O}G$ function
YBX1	Recombinant human protein in *Escherichia coli*	(95)	RNase protection	ROS resistance
PNPase	Hela cells	(94)	$8^{O}G$ level reduction upon overexpression	ROS resistance and reduce $8^{O}G$ levels in RNA
DAZAP1	HeLa S3 extracts	(96)	Bait pulldown	Unknown
SF3B4	HeLa S3 extracts	(96)	Bait pulldown	Unknown
HNRNPD	HeLa S3 extracts	(96, 97)	Bait pulldown	Promotes RNA decay
HNRNPC	HeLa S3 extracts	(96)	Bait pulldown	Unknown
PCBP1	HeLa S3 extracts	(100)	Bait pulldown	Promotes apoptosis
XRN1	Yeast and HEK293	(101)	$8^{O}G$ accumulation upon knockdown	Degrades translationally stalled mRNA
PCBP2	HeLa S3 extracts	(99)	Bait pulldown	Promotes cell survival
IGF2BP1	HeLa S3 extracts	(92)	Bait pulldown	Unknown
IGF2BP2	HeLa S3 extracts	(92)	Bait pulldown	Unknown
HNRNPDL	HeLa S3 extracts	(92)	Bait pulldown	Unknown
RBM4	HeLa S3 extracts	(92)	Bait pulldown	Repelled by $8^{O}G$

been used. These have yielded twelve RBPs that bind to $8^{O}G$ (Table 1). Among them, PNPase degrades $8^{O}G$ and confers ROS resistance upon overexpression in HeLa cells (97). YBX1 was shown to protect $8^{O}G$ baits from nuclease digestion where its role is thought to mediate oxidized RNA sequestration and decay (98). DAZAP1, SF3B4, and HNRNPC bind $8^{O}G$ and are also implicated in pre-mRNA processing, nevertheless, their specific function following $8^{O}G$ binding has not been fully investigated (99). HNRNPD plays a role in the degradation of $8^{O}G$–RNAs (100) and assists APE1 in resolving R-loops (101). PCBP1 promotes apoptosis upon sensing heavily oxidized RNA while PCBP2 prevents it (102, 103). XRN1, a 5′-3′ exoribonuclease, degrades $8^{O}G$-RNAs stalled during translation (104). Recently, $8^{O}G$-bait cross-linking enabled by a photoactivatable 5DzU allowed for the identification of $8^{O}G$-RBPs by mass spectrometry, identifying again HNRNPD as well as members of the IGF2BP1 family (95). Additionally, $8^{O}G$ was shown to interfere with the binding of RBM4 to its canonical motif, highlighting another mechanism by which RNA oxidation can affect RNA–RBP interactions.

RBPs and their RNA cargos are also active components of many membraneless organelles that can be directly affected by oxidative stress (105). These organelles include stress granules to modulate translation, P-bodies for degradation, and nuclear speckles for splicing and processing in a direct, RNA-mediated, or RNA-shielded manner (106). In consequence, oxidative RNA lesions have the potential to impact their dynamics. Interestingly, the formation of $8^{O}G$-rich granules in response to ROS was recently described (107). These oxidized RNA bodies (ORBs) are different from stress granules and p-bodies and are hypothesized to serve as quality control centers.

Detection of Oxidative RNA Lesions Across RNA Species and Their Consequences. Oxidative RNA lesions have been detected and quantified by a variety of methods. These include liquid chromatography and mass spectrometry (LC–MS), antibody immunostaining or pulldown, and sequencing (previously microarray technologies, now high-throughput sequencing) (Fig. 2). Tissue, cell, and RNA isolation techniques combined with these detection methods allow for the quantification of lesions in specific RNA subtypes, including mRNA, rRNA, tRNA, and miRNAs. Antibody-based methods, if used for immunostaining, can provide cell type and subcellular resolution, when coupled with the right cell or organelle markers. Furthermore, when used for pulldown followed by sequencing, antibodies have enabled the identification of damaged transcripts, but have missed oxidative RNA lesions at base resolution.

Currently, the only method that allows the identification of all known oxidative RNA lesions is LC–MS (33). In contrast, only $8^{O}G$ antibodies have been widely established and used. Interestingly, an antibody against $8^{O}A$ was recently developed (108) but no antibodies against any other oxidative RNA lesions are available. As a consequence, studies that have investigated oxidative RNA damage in neurodegeneration are mostly focused on $8^{O}G$ detection or IP (i.e., AD (36), PD (30), and ALS (45)). Remarkably, Seok and collaborators iterated and improved the IP procedure to more efficiently isolate and sequence $8^{O}G$-containing RNAs (109).

The potential to sequence RNA lesions depends on their ability to create and detect a signature upon reverse transcription. A characteristic feature of $8^{O}G$ is its capability to adopt a *syn*- or *anti*-conformation and pair with cytosine or adenine, creating a G-to-T signature (109). Eom and collaborators leveraged this signature to locate $8^{O}G$ sites in miRNA sequences from The Cancer Genome Atlas and further validated the functional effects of these $8^{O}G$ sites in vivo (110). Other oxidative RNA lesions can result in different signatures, as was the case following reverse transcription with three different enzymes across $8^{O}G$, $8^{O}A$, 5-OHrC, ε-rA, ε-rC, abasic site, Gh, and Sp. Importantly, for some lesions, different transcriptases produced different signatures, including the inability to bypass them (37, 111, 112). Outstandingly, $8^{O}G$-miSeq combines antibody pulldown and reverse transcription signatures to identify oxidized miRNAs (109).

The use of one or more of the aforementioned methods has provided valuable information on the prevalence and effects of various oxidative RNA lesions across RNA subtypes. For example, in vitro studies have shown that 5-OHrU, 5-OHrC, $8^{O}G$, and $8^{O}A$ significantly slow down translation when present in mRNA while ε-rA, ε-rC, and abasic sites fully abolish it (41). Importantly, $8^{O}G$-containing mRNAs can still associate with

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polysomes but result in decreased or truncated protein in vitro (104, 113). Furthermore, mRNA oxidation in cultured neurons is transcript-specific, regardless of abundance, leading to a significant and specific decrease in translation of damaged transcripts such as *SOD1* (51). Similar observations have been replicated in different oxidative stress models. For example, exposing bronchial cells to either formaldehyde or air pollutants leads to oxidation of yet a different set of mRNAs, reducing their protein levels (48, 49). Similarly, a murine model of multiple sclerosis found, ROS-dependent, reduced translation of the trans-mitochondrial protein NAT8L, driven by its oxidized mRNA (50). Significantly, mRNA oxidation is an early characteristic of several neurodegenerative diseases like ALS (45), AD (114), and PD (30), and is restricted to the affected neuronal subtypes characteristic of each of these diseases. Notably, the mechanisms behind transcript-specific oxidation remain unknown, as does residue specificity within oxidized transcripts.

Increasing evidence indicates that oxidative stress can also damage tRNA and rRNA, and contribute to aberrant protein abundance in disease (94, 115). ROS can alter many redox-sensitive posttranscriptional modifications required for tRNAs. For example, uridine in the wobble position is commonly modified. Oxidation-induced desulfuration of thiouridine residues in tRNAs reduced ribosome binding (116). Interestingly, 5-OHrU is a precursor of uridine-5-oxyacetic acid (cm^5rU), an important modification for the reading of wobble rG, but the enzyme responsible for producing 5-OHrU has yet to be identified (117). Alternatively, ROS can produce tRNA fragments directly or by activating angiogenin (118) that subsequently promotes cell death (119), stress granule formation, or suppression of translation (120). Similarly, ribosomes are oxidized in AD leading to decreased translation efficiency (115), which precedes neuronal death (94, 121). Studies in *E. coli* show that, depending on the oxidized rRNA residue, it can have different consequences. For example, 8^{O}A2451 slows peptide bond formation, 5-OHrC2063 increases translation in polyU translation assays, while 5-OHrU2506, 8^{O}A2602, and 8^{O}G2447 do not affect translation even though they were placed in the peptidyl transferase center (122).

Oxidative RNA lesions can also occur in miRNAs. Specific residues in miRNA have been shown to trigger broad changes in gene expression. miRNAs typically recognize targets through their seed sequence in positions 2 to 8; guanine oxidation in these spots is favored due to RNA's lower redox potential toward the 5′ end (123). This phenomenon has been observed particularly for miRNA-bearing 8^{O}G, where 8^{O}G-A base pairing occurs between miRNAs and their targets. For instance, highly oxidized miR-184 gains the ability to bind to the 3′UTR of antiapoptotic genes like *Bcl-w* and *Bcl-xL*, hindering their translation and increasing susceptibility to heart injury (92). Subsequent studies have shown other examples of site-specific 8^{O}Gs in miRNAs leading to various outcomes such as cardiomyopathy, hepatocellular carcinoma progression, and increased survival rates in lower-grade gliomas (109, 110).

Combining Advancements in Disease Models, RBP, RNA, and Interactome Technologies to Investigate Oxidative RNA Lesions in Neurodegeneration

While groundbreaking work on RNA oxidation and neurodegeneration was evident in the late 1990s and early 2000s, the intersection between these fields has remained underexplored (Fig. 3), in great part due to the lack of advancements in RNA and disease-modeling methodologies. In fact, in recent years, a resurgence in interest in RNA oxidation, and RNA damage in general, is evident in recent reviews (39, 78, 93, 124–126) as well as primary literature (48, 49, 95, 102, 104, 109, 110). Here, we propose that novel iPSC, differentiation, RNA sequencing, and RNA-interactome technologies can be leveraged to better assess the role of RNA oxidation and its interacting partners in a variety of neurodegenerative diseases, including but not restricted to AD, ALS, and PD. With neuronal and organoid

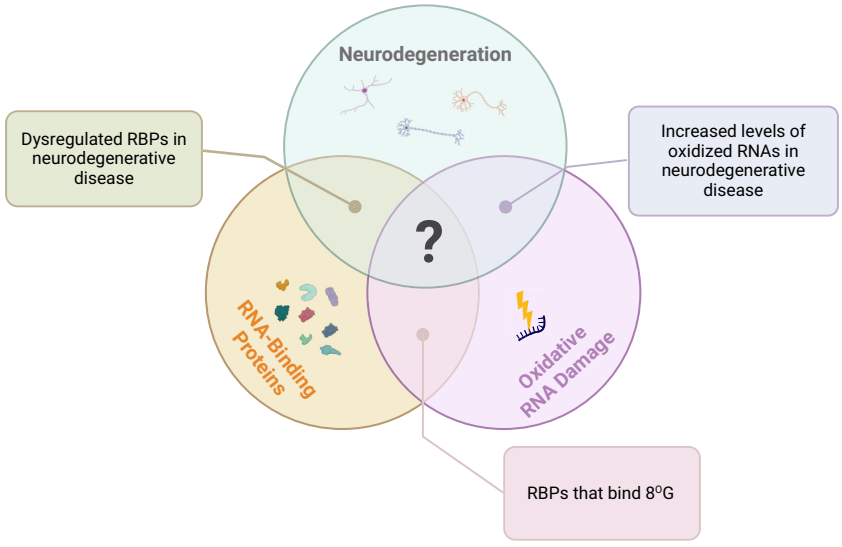

Fig. 3. Knowledge gap in the interactions between neurodegeneration, RNA oxidation, and RBPs. To date, research in the three shown disciplines has focused either on a single discipline or the interactions between two of them. The nature and main takeaways from these interactions are highlighted for each of the three combinations. This work postulates the idea that all three disciplines must be combined, together with novel technological advancements, to explore the potential role that oxidative RNA damage plays in neurodegeneration through RNA processing. Created with BioRender.com.

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iPSC-derived models at the forefront, we are technologically poised to i) identify RNA targets of oxidation at base resolution, ii) map the RBP–RNA interactome and the consequences of oxidative stress, iii) examine the effect of oxidative RNA lesions on RNA processing, and iv) explore commonalities across neurodegenerative diseases characterized by increased oxidative stress (Fig. 2).

To achieve these goals, it will be of the utmost importance to incorporate patient lines that represent different ethnic backgrounds and genders. This is made possible by the growing number of disease-specific iPSC repositories. The benefits of such an effort are exemplified by Workman and colleagues who leveraged over 400 control and ALS patient lines hosted by the AnswerALS Consortium (127). They performed immunohistochemistry and gene expression analysis of iPSC-derived motor neurons and demonstrated that the strongest drivers of expression variation were gender and cell composition. Other studies have taken advantage of iPSC-derived motor neuron proteomics datasets hosted by AnswerALS and identified potential pathways and biomarkers linked to disease progression (128).

Assessing Transcript-Specific Vulnerabilities to Oxidative Stress. The identification of oxidized transcripts is essential when exploring disease-specific repercussions of oxidative damage. To date, oxidized transcript identification has heavily relied on antibody pulldown followed by sequencing. While recent work has improved the process by 3,500-fold, antibodies against RNA modifications have produced conflicting results (129). Future sequencing efforts should avoid antibodies and instead leverage the base-pairing properties of oxidative lesions or novel chemical reactions that convert damaged moieties to a sequence signature. Moreover, novel direct long-read RNA sequencing technologies, such as Oxford Nanopore, will enable isoform resolution of the transcripts and damaged sites. One of the main challenges with these technologies is the identification of sequencing signatures characteristic of the modified base of interest. Leveraging knowledge from short-read sequencing efforts will help address these challenges (130) (Fig. 2). Moreover, a fully sequencing-based approach would allow for future single-cell and spatial transcriptomics analyses of both iPSC-derived organoids and patient samples. These are of particular relevance given the evidence that RNA oxidation and its potential effects are cell-type specific (30, 45, 51, 114).

Characterization of Oxidized RNA–RBP Interactions in Disease Models. The dynamic interactions between RBPs and oxidized RNAs have yet to be fully evaluated in more disease-relevant models, as previous efforts have relied on immortalized cell lines, mainly HeLa cells (Table 1). Furthermore, RBP–RNA interactions were predominately probed using RNA oligo baits containing one or more lesions in HeLa cell extracts. While the baits approach has resulted in the discovery of several RBPs that interact with (or are repelled by) $8^{O}G$, it is constrained in at least three ways. First, the sequences of the oligos might be biased for the recognition of a subset of RBPs. Second, these sequences might result in secondary structures that can vary in the presence or absence of damaged bases, which can also be biased for a subset of RBPs and confound differential binding assessment. Finally, cell extracts might not recapitulate relevant in vivo conditions that some RBPs might require to recognize lesions. Recently developed RNA-interactome methodologies overcome many of these limitations (Fig. 2). For example, organic phase separation methods (i.e., OOPS, XRNAX, LEAP-RBP), where UV-cross-linked RNA-bound RBPs are isolated from the interphase of acidic guanidinium thiocyanate-phenol-chloroform (AGPC) biphasic extractions, enables unbiased RBP identification (131–133). While each of these methods has its strengths and caveats, they all allow for a small cell number input (1% of previous methods), are not mRNA biased, and capture interactions occurring inside cells when UV-cross-linked. Importantly, these methodologies are fully amenable to models or treatments that can recapitulate oxidative stress conditions such as disease-associated mutations or oxidation reagents, such as hydrogen peroxide and sodium arsenite, commonly used in the study of ALS and stress granule formation.

Elucidation of novel interactions between RBPs and damaged RNAs in neuronal and neurodegeneration models, together with RBPs found in HeLa cell extracts with baits, will logically lead to the investigation of these RBPs' RNA targets. One of the most used methods to probe RBPs' targets is enhanced cross-linking and immunoprecipitation (eCLIP). eCLIP, which has widely substituted RIP-seq methods, is characterized by using 254 nm UV light to cross-link RBPs to their interacting RNA partners. Importantly, this approach is highly amenable to different oxidative stress models or conditions, including iPSC-derived cultures (134). Moreover, RBP-binding sites and damage sites could be further cross-referenced with eCLIP ENCODE datasets for known RBP targets, and the generation of novel hypotheses. Nevertheless, eCLIP and other pulldown-based methodologies demand high-quality antibodies against each RBP of interest. Furthermore, while large starting cell number constraints have been improved, millions of cells are still necessary to obtain high-quality results. This can be challenging when handling patient samples or iPSC-derived models. Novel technologies such as TRIBE (135), STAMP (136), and REMORA (137) overcome some of these limitations by fusing RBPs of interest to base editors. As an added benefit, these methods are compatible with single-cell transcriptomics as well as long-read sequencing platforms (Fig. 2).

Repercussions of Oxidative RNA Damage on RNA Processing. We expect that the identification of novel RBPs as oxidative RNA lesions interactors, as well as novel lesion sites, will inform investigation into their effects on RNA processing and potentially neurodegenerative disease onset. If iPSC-derived models mirror RNA oxidation features in patient samples, we expect several damaged RNA transcripts to be directly associated with disease. Moreover, we foresee the discovery of new mechanisms by which oxidative RNA lesions can perturb RBP interactions besides changing the motif or secondary structure that an RBP recognizes. For example, oxidized RNAs could act as "sponges" and sequester RBPs that then would be unavailable to interact with their targets, similar to the effects of repeat expansions in myotonic dystrophy and other neurological diseases (96). Finally, the fact that many RBPs can be oxidized themselves, added to the

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potential oxidation of their targets, might result in interaction dysregulation at multiple levels. These could include but are not limited to changes in RBP and RNA localization and decay.

Other Considerations. Only once we begin to understand the role of RNA oxidation at a molecular level and its implications in neurodegenerative disease onset, will we be able to utilize this knowledge to our advantage. In the future, we foresee researchers harnessing damaged-RNA-RBP recognition to create function-specific effectors. For example, RNA-targeting CRISPR technologies can be leveraged to specifically target deleterious damaged transcripts by considering altered base-pairing properties of damaged RNAs. Similarly, RBP effectors can be targeted to relevant damaged transcripts to elicit defined molecular responses. It does not escape our attention that while there are currently no known oxidative RNA lesion repair mechanisms, one might be discovered in the future with some of the methodologies proposed here. Similarly, directed evolution of known RNA oxidation interacting proteins could aid in the development of effectors, including potential repair mechanisms. This idea is not novel as PNPase has been computationally evolved to better recognize $8^{O}G$ and, as a result, increase cell tolerance to H_2O_2 (138).

While this manuscript mostly focuses on intracellular oxidative RNA damage, oxidative RNA lesions have been reported in bodily fluids such as cerebrospinal fluid, urine, and serum (33). Levels of oxidative RNA lesions in these compartments could be altered in neurodegenerative diseases. Therefore, future work should also revolve around the search for mechanisms to connect these biomarkers and molecular readouts, such as the ones proposed here. These approaches could result in the development of algorithms to predict disease predisposition or stage, and potentially an avenue for early prevention and treatment strategies. Nevertheless, any future strategies for the downregulation of RONS should be mapped out carefully, as RONS are also known to play essential roles in neuronal development, differentiation, polarization, synapse maturation, neuropeptide release, and neurotransmitter transport (23).

An outstanding advantage of iPSCs is that they can be genome-engineered, enabling the introduction of disease-relevant mutations or complementations. As demonstrated by the Neurodegenerative Disease Initiative (iNDI) which aims to create a panel of isogenic iPSC and daughter lines with relevant knockouts, and knockins of AD, PD, and ALS-related dementias (139). One of the primary limitations of iPSC models is their inability to recapitulate all age-related phenotypes. This is substantiated by research showing, in iPSCs, an increase in telomere length (140), mitochondrial fitness (141), and loss of senescence markers (142), supporting the concept of reversal of age-related properties in these cells. Nevertheless, as shown here, several of these models recapitulate many important disease phenotypes (Fig. 1). Excitingly, a recent technological advancement that can help overcome this limitation is the direct differentiation of fibroblasts to desired neuronal populations (143, 144). Moreover, organoid models, while also recapitulating many fetal developmental hallmarks, lack some important cell types. For example, this is the case for microglia and vasculature in cerebral organoids. One novel strategy to address some of these barriers is by the use of assembloids, where two or more organoids of different makeups are cultured together, resembling interactions between different tissues (145).

In summary, the emergence of iPSC and direct differentiation methods will continue to advance neurodegenerative disease research by providing sophisticated and translational disease model systems. Importantly, they provide an opportunity to further "personalize" patient care to find approaches that may alleviate patient symptoms or prevent disease. The use of 3D iPSC technology, such as organ-on-a-chip, is an exceptional candidate for this goal as it generates a comprehensive system composed of many cell types to evaluate pathological phenotypes (91). Similar approaches have already been employed by institutions, like NASA, to identify astronaut-specific vulnerabilities to space ("tissue-chips") and for ALS research (ALS-on-a-chip) (91). Furthermore, we believe that these disease models should be combined with novel sequencing and RNA–RBP interactome technologies. This approach will be instrumental in addressing the gap in our understanding of the role oxidative RNA lesions play in RNA processing, through RBP interactions, in neurodegeneration (Fig. 3).

Data, Materials, and Software Availability. There are no data underlying this work.

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