PROTEIN DEGRADATION, AGGREGATION, MEMBRANE TRAFFICKING AND EXOSOMES IN NEURONAL HEALTH AND DISEASE

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EDITORIAL
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Editorial: Protein Degradation, Aggregation, Membrane Trafficking and Exosomes in Neuronal Health and Disease

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Keywords: degradation, neurodegeneration, exosomes, disease, membrane, neuron

Editorial on the Research Topic

Protein Degradation, Aggregation, Membrane Trafficking and Exosomes in Neuronal Health and Disease

Neurons are highly specialized and polarized cells. Comprising usually of a single long axon and multiple shorter dendrites, their unique geometry creates challenges for cellular homeostasis. For example, how and where cellular components are expressed or removed at the right time and place, or if any abnormality in neurons or other neural-system cells may compromise their function and initiate disease pathology are questions that remain largely unanswered. This Research Topic aims to cover the beforementioned questions with new discoveries but also revising the preexisting literature. We as editors, and the authors of the articles tackle main processes of neuronal cell biology homeostasis, such as protein degradation, membrane trafficking, or exosomes and explore some of their normal and abnormal roles, therefore this topic is covering fundamental aspects of neuronal cell biology, which are related to disease.

One important pathway for protein degradation is the ubiquitin proteasome system (UPS), which plays key roles in different physiological processes, such as fear conditioning, a type of memory implicated in several brain diseases. Despite key roles for the UPS in fear conditioning and the possibility it could underlie post-traumatic stress disorder (PTSD), a systematic screening for substrates of this pathway had not been performed and proteasome targets had remained unknown. Here, Farrell et al., *via* proteomics and liquid chromatography/mass spectrometry (LC/MS) describe proteasome targets in the amygdala following fear conditioning, and find evidence that these targets can vary depending on the sex. The authors raise the possibility that despite the targets being different between sexes [they have previously shown differences in a role for the UPS in fear conditioning between male and female mice (Martin et al., 2021)], the underlying cellular expression of the fear conditioning, such as structural remodeling of neurons could be similar between the sexes.

As we learn more about neurological diseases, more complexity is unearthed and interactions between cellular systems are revealed. Initial studies identified alterations in proteostasis including protein aggregation underlying disorders such as Alzheimer's disease (AD) and others. However, clinical trials with agents that attempt to remove aggregated proteins have unfortunately largely failed to provide a significant delay in disease progression or improve symptoms. While there can be many reasons for these failures, one could be the failure of several cellular systems interacting together. Therefore, effective therapies may be needed which target multiple cellular pathways. Recently, the identification of mediators of inflammation in neurological diseases provides a system which may interact with proteostasis.

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Ebstein et al., review the importance of UPS substrates in neurodevelopmental disorders. In their survey they found that many of these protein substrates have roles in the innate immune response, establishing the possible involvement of chronic inflammation in these disorders. In a similar direction Hulse and Bhaskar, discuss the crosstalk between the aggregation prone proteins normally involved in Alzheimer's and Parkinson's diseases (AD/PD) and the multi-protein inflammasome complex (ASC). Instead of being a byproduct of protein aggregation, inflammation mediated by the NLRP3 inflammasome and ASC can act as a driver stabilizing a feedback loop that promotes neurodegeneration. Release of ASC and cytokines extracellularly by microglia and subsequent uptake of ASC by other microglia propagates the spreading of protein aggregation in the brain.

Autophagosomes and endosomes transport proteins from the synaptic connections to the soma for its degradation, this is an incredibly demanding event, thus being susceptible to be involved in pathological conditions, Hu et al., review the critical role of the neuronal endolysosomal trafficking pathway in ischemic brain injury. Another type of vesicle, the extracellular vesicles (EVs) are critical for cell-to-cell communication, their cargo is highly varied including proteins, nucleic acids and mitochondria, Amari and Germain carefully reviewed and discussed recent evidence suggesting that mitochondria transferred through EVs have the capacity to alter metabolic and inflammatory responses in the recipient cells. Besides the capacity of EVs to elicit a response in the target cells, EVs can also accelerate the spreading of protein aggregation, and this capacity is closely related to lipid alterations. Estes et al., examined how these two pathways accelerate the progression of both AD and PD. A better understanding of EVs, and Lipid metabolic pathways and how they contribute to the spread of protein aggregation could lead to the discovery of new therapeutic targets to slow the spread down. One known cargo of EVs is a-Synuclein, which is related to PD. Paralleled to motor dysfunction there is an incremental increase in exosomal a-synuclein levels in plasma. This increase could be used to study PD progression or even help its diagnosis, for this reason it is important to understand how a-synuclein reaches the exosomes. Sepulveda et al., evaluate the relationship between the autophagy-lysosomal pathway and its contribution to the secretion of a-synuclein by exosomes. Some neurodegenerative disorders such as Charcot-Marie-Tooth (CMT) or distal hereditary motor neuropathy arise as consequences of mutations in the heat shock protein 27 (HSP27). However, the complete structure of this protein remains to be fully understood, Holguin et al., review recent insights providing the required structural context to explain the relationship between mutations in HSP27 and the resulting loss or gain of function that leads to disease. The discovery of new genes related to brain diseases is crucial to understanding the pathological processes and to developing new treatments. Chuvakova et al., studied groups of genes related to Audiogenic Epilepsy (AE). Comparing transcriptomes from the *corpora quadrigemina* in the midbrain zone-a region crucial for AE development-between three rat strains with different propensities to suffer AE. The Krushinky-Moldodkina strain, most prone to AE showed increased expression of genes involved in the positive regulation of the MAPK signaling cascade and genes involved in the positive regulation of apoptotic processes, among others. These data show the complex multigenic nature of AE inheritance in rodents. The maintenance of proteostasis is critical to the physiological functioning of neurons, abnormalities in which can play roles from neurodegeneration to addiction. Addiction propensity or risk is related to changes in proteostasis *via* endoplasmic-reticulum-associated degradation (ERAD), with micro RNA (miRNA) regulation playing an important role. In this topic Wang et al. describe that miR-181a may be indirectly responsible for methamphetamine addiction by down-regulating GABAAa1 through the regulation of ERAD, rather than through direct regulation of GABAAa1 itself. Furthermore, low-dose methamphetamine appears to be neuroprotective in other conditions where changes in proteostasis and ERAD occur, such as Alzheimer's amyloid pathology via the down regulation of miR-181a. Further studies could implicate miR-181a as an additional strategy for the treatment of addiction and neurodegenerative disorders.

AUTHOR CONTRIBUTIONS

DSC and BA-C wrote the manuscript. MD-H revised the manuscript. All authors contributed to the article and approved the submitted version.

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MicroRNA-181a Is Involved in Methamphetamine Addiction Through the ERAD Pathway

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The regulation of microRNA (miRNA) is closely related to methamphetamine (METH) addiction. Past studies have reported that miR-181a is associated with METH addiction, but the mechanism pathways remain elusive. On the basis of our past studies, which reported the endoplasmic reticulum-associated protein degradation (ERAD) mediated ubiquitin protein degradation of GABAAα1, which was involved in METH addiction. The present study, using qRT-PCR and bioinformatics analysis, further revealed that miR-181a may be indirectly responsible for the METH addiction and downregulation of GABAAα1 through the regulation of ERAD.

Keywords: methamphetamine, miR-181a, endoplasmic reticulum-associated protein degradation, GABAAα1, addiction

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INTRODUCTION

Drug abuse has been widely recognized as a debilitating chronic disease of the brain, and it is one of the most dangerous diseases to human physical and mental health in modern society.

Methamphetamine (METH), one of the commonly used drugs, is addictive and neurotoxic. Long-term use of methamphetamine can lead to addiction, causing varying degrees of physical and psychological harm to the addicts (Anna and Patrick, 2017), and methamphetamine (MA) abuse is considered a serious global public health problem. Despite the severity of the consequences of drug abuse, there is still no fully effective treatment for methamphetamine addiction. Therefore, research into the mechanisms of methamphetamine addiction is critical.

Traditionally, the main mechanism of ATS addiction is the increased activity of the midlimbic dopamine system. However, the neurobiology of METH is more complex than the traditional view of it as a monoaminergic modulator. It may include oxidative stress, excitatory neurotoxicity, endoplasmic reticulum stress, and neuroinflammation (Paulus and Stewart, 2020). More complex regulatory mechanisms may also be involved in the addictive process of METH.

MicroRNAs (miRNAs) are small (21–25-nucleotides long), non-coding RNA molecules that are responsible for the suppression of protein expression by targeting the mRNA of protein-coding genes through translational repression (Reuben et al., 2012). MicroRNAs act as regulators of gene expression and play a key regulatory role in different drug addiction. Some studies showed that miRNAs have been implicated as mediating the effects of cocaine (Hollander et al., 2010; Bali and Kenny, 2013), alcohol (Torres et al., 2018; Kyzar et al., 2019), nicotine (Qin et al., 2017;

Du et al., 2019), and several other classes of drugs (Rodríguez, 2012; Pinson and Miranda, 2019; Zhang et al., 2019). A growing body of evidence suggests that miRNAs and their processing machinery is involved in in METH addiction (Zhu et al., 2015a; Sim et al., 2017). Previous studies have reported the involvement of miR-181a in METH addiction (Reuben et al., 2012; Kai et al., 2016; Zhao et al., 2016). However, the mechanisms involved need to be further elucidated. Our earlier study revealed that the GABAAα1 expression was downregulated in the dorsal striatum (Dstr) of METH-addicted rats, which was caused by protein ubiquitin degradation and mediated by endoplasmic reticulum-associated protein degradation (ERAD) (Jiao et al., 2016, 2017). Taking into account the role of miR-181a in direct regulation of GABAAα1 (Zhao et al., 2012; Sengupta et al., 2013), it was assumed that miR-181a plays some role in the downregulation of GABAAα1. On the basis of the experimental study and bioinformatics analysis, it was found that miR-181a may be involved in the downregulation of GABAAα1 through the regulation of the ERAD pathway and not through direct regulation of the expression of GABAAα1, thus influencing METH addiction.

EXPERIMENTAL METHODS

Animals and Drug

Sprague–Dawley male rats weighting 220–300 g (aging 50–60 days) were obtained from the Laboratory Animal Center, Bengbu Medical College (Bengbu, Anhui, China). Rats were housed 2–3 per cage and maintained on a 12-h light/dark cycle with access to food and water *ad libitum*. All experimental procedures in this manuscript were in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996) and approved by the Institutional Animal Care and Use Committee of Bengbu Medical College (Bengbu, Anhui, China). METH was provided by China Academy of Military Medical Science.

Conditioned Place Preference (CPP)

The CPP apparatus (Zhenghua Software and Instruments, Anhui, China) was divided into two equal-sized compartments [40 cm (length) × 40 cm (width) × 60 cm (height)] separated by a removable board (10 × 10 cm), which allowed rats free access to each compartment. Two compartments were distinguished by visual and tactile cues: one was a black and white horizontal striped wall with an iron wire floor, whereas the other was a black and white vertical striped wall with a steel bar floor. These distinctive tactile and visual stimuli served as the conditioning.

CPP model follows the experimental reported in our previous article (Jiao et al., 2016, 2017). The place conditioning procedure used in this experiment included four phases: habituation, preconditioning, conditioning, and testing. In the habituation phase, the rats were allowed to freely explore the entire apparatus for 30 min. In the preconditioning phase, the rats were allowed to freely explore the entire apparatus for 15 min. The time spent in each compartment was recorded in the preconditioning phase, and rats showing a strong unconditioned aversion (one compartment >720 s) for either compartment were eliminated from the study. Conditioning occurred over the next 8 days. On the first day of the conditioning phase, the rats were injected with either METH (1 mg/kg, i.p.) or saline (1 ml/kg, i.p.) and then confined to the non-preferred compartment in a counterbalanced manner for 45 min. This compartment will be referred to as the "drug treatment-paired compartment." On the second day, the rats were injected with saline (1 ml/kg, i.p.) and then confined to the opposite compartment from the first day for 45 min. This procedure was repeated four times in the conditioning phase. The testing phase occurred 24 h after the conditioning trial, and all rats were allowed to freely explore the entire apparatus for 15 min; the amount of time spent in each compartment was recorded. The CPP score represents the time in the drug treatment-paired compartment during the testing phase minus the time in that compartment during the preconditioning phase.

Quantitative Reverse Transcriptase PCR (qRT-PCR)

Rats were sacrificed and the brains were removed immediately after CPP conditioning. The brain was dissected into coronal slices (1 mm thick) using a rat brain slicer (Braintree Scientific), and the dorsal striatum was punched by a blunt end, 17-gauge syringe needle. Total RNA was isolated from rat Dstr using a commercially available kit (RNeasy Plus Mini Kit, Qiagen). QRT-PCR was performed on a ABI7500 Real-Time PCR system (ABI) using SYBRr Premix Ex TaqTM kit (TaKaRa Bio Group, Japan). A typical reaction of a total volume of 20 μl consisted of 2 μl Template DNA, 10 μl 2 × SYBR Green Reaction Mix, 0.4 μl PCR Forward Primer (10 mM), 0.4 μl PCR Reverse Primer (10 μM), 0.4 μl ROX Reference Dye II and 6.8 μl DEPC treated water. PCR amplification was done with an initial incubation at 95℃ for 30 s, and then followed by 40 cycles of 95℃ for 5 s, 60℃ for 34 s, and final melting curve from 95℃ for 5 s, 60℃ for 60 s. Primer specificity was confirmed by melting curve analysis.

Primers utilized were as follows: rno-miR-181a-5p, MIMAT0000858: 5′AACAUUCAACGCUGUCGGUGAGU; rno-miR-181b-5p, MIMAT0000859: 5′AACAUUCAUUGCU GUCGGUGGGU; rno-miR-181c-5p, MIMAT0000857: 5′AAC AUUCAACCUGUCGGUGAGU; rno-miR-181d-5p, MIMA T0005299: 5′AACAUUCAUUGUUGUCGGUGGGU; Hs_RNU 6-2_11, GeneGlobe Id: MS00033740. Above primers were purchased from Qiagen Company.

UBE2D3: Fdw-5′CTATGGCGCTGAAACGGATT, Rev-5′G GGCTGTCATTAGGTCCCAT; RNF169: Fdw-5′TCCATTCCA GCAAGGAGAGG, Rev-5′GGAATGGCAGGTTTCCAGTG; FB XO33: Fdw-5′CTCAGCATCCGGAACAACAG, Rev-5′ATAA ACCCACAGGACAGCCA; RNF145: Fdw-5′TCTCCAGGTTC TGGGAACAC, Rev-5′GCGGTAAGTGCCATTCACAT; RAD 23B: Fdw-5′TCTGAACCTGCACCTACTGG, Rev-5′AGACTG

ACCTGTCACAAGGG; NEURL1B: Fdw-5′CTGGTCACACG ACCTGGATA, Rev-5′TGGTTCGCCATCATTGACAC; PCN P: Fdw-5′GCGATCAGCTGAAGACGAAG, Rev-5′; KLHL 15: Fdw-5′ATAGACGACGGTGGAGACAC, Rev-5′CAACAA GGGTTGCTGGTGAA; KLHL5: Fdw-5′GCTCCCACATCCAA CTTGAC, Rev-5′CACTGCAGTCCACATGTCTG; TULP4: F dw-5′GAGCATGGACCTCTGCTTTG, Rev-5′GTGGCCAACC ATCCTTCTTC; RNF34: Fdw-5′AAGGAAATCCTGGCTCG GAA, Rev-5′TCCAGCAGGACACAGTCAAT; KLHL2: Fdw-5′GAGTACTTGGTGCAGAGGGT, Rev-5′AGGTTCATGGGTG TCCTCAG; CAND1: Fdw-5′TGCCAGAAGCTCAGTGGTTA, Rev-5′CAGTGACGGCTTGTTATGGG; HSP90B1: Fdw-5′A CTGCATTCAGGCTCTTCCT, Rev-5′TCTCTGTTGCTTCC CGACTT; DERL1: Fdw-5′GCCATGGATATGCAGTTGCT, Rev-5′TTGATGACCGAGCCTCCAAT; HSPA5: Fdw-5′GGTGGGCAAACCAAGACATT, Rev-5′TCAGTCCAGCAA TAGTGCCA; GAPDH:Fwd-AACTTTGGCATTGTGGAAGG, REV-5′ACACATTGGGGGTAGGAACA.

Above primers were purchased from Genewiz in China.

Bioinformatic Analysis

The details regarding the base sequence and species conservation of miR-181a-5p were retrieved from the online database miRbase[1]. Using Venn plot, the target gene of miR-181a-5p was screened by the intersection of TargetScan[2], miRDB[3], and miRanda[4] online databases. Functional enrichment analysis was performed using the DAVID (Database for Annotation, Visualization, and Integrated Discovery)[5] online database, which included gene function GO (Gene Ontology) analysis (such as biological process, cellular component, molecular function) and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analysis. STRING (Search Tool for the Retrieval of Interaction Gene)[6], an online software, was used to analyze the interaction between target gene-encoded proteins and to visualize the protein interaction networks. RNA high-throughput sequencing data of animal brain tissues treated with METH were retrieved from the EMBL-EBI (European Molecular Biology Laboratory's European Bioinformatics Institute)[7] (Zhu et al., 2015b) online database, and cluster analysis was performed where $P < 0.05$ was considered to be statistically significant.

qRT-PCR Statistical Analysis

U6 or GAPDH was used as an endogenous control for the qRT-PCR. Fold changes in expression were calculated with a log 2 transform. Independent-sample *t*-tests were used to test for significant differences (SPSS v23.0, SPSS Inc., United States). Differences in the values with $p < 0.05$ were considered to be statistically significant. These results were presented as mean ± standard deviation (SD).

[1]http://www.mirbase.org/

[2]http://www.targetscan.org/vert_72/

[3]http://mirdb.org/

[4]http://cbio.mskcc.org/microrna_data/miRanda-aug2010.tar.gz

[5]https://david.ncifcrf.gov/

[6]https://string-db.org/

[7]https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-2843/samples/

RESULTS

Decrease in miR-181a-5p and miR-181b-5p Expressions in METH-Induced CPP Rat

The expression levels of miR-181a-5p, miR-181b-5p, miR-181c-5p, and miR-181d-5p in the dorsal striatum of METH-induced CPP rats were investigated (**Figure 1**). The results indicated that the expression of miR-181a-5p ($n = 8$, $^{**}p < 0.01$) and miR-181b-5p ($n = 8$, $^{*}p < 0.05$) were downregulated in the METH-induced CPP group when compared to the control group, while the expression of miR-181c-5p and miR-181d-5p did not show any significant change ($n = 8$, $^{*}P > 0.05$). Keeping in mind the findings from several studies (Kai et al., 2016; Zhao et al., 2016) reporting significant decrease in the expression of miR-181a-5p, bioinformatics analysis was performed on miR-181a-5p.

Conservation Analysis of miR-181a-5p

The mature sequences of miR-181a-5p of 10 species including human (hsa), rat (rno), chicken (gga), and frog (xtr) were retrieved from online data base, miRbase[1]. The mature sequences of each of the gene family of miR-181a-5p gene cluster in different

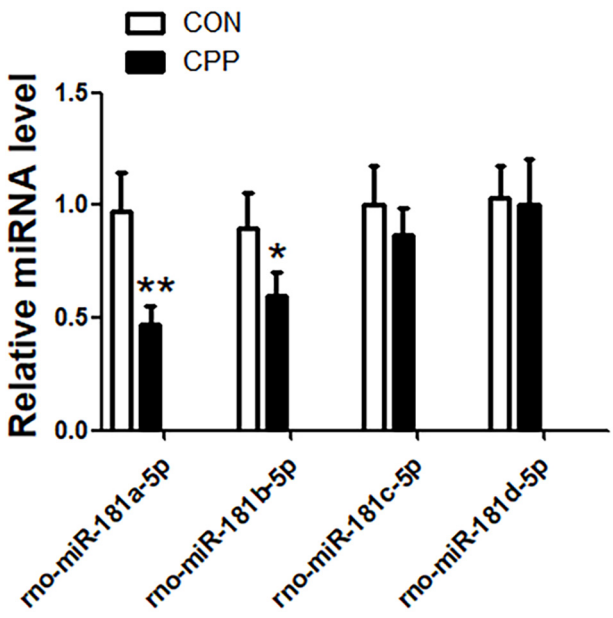

FIGURE 1 | The expression of miR-181a-5p and miR-181b-5p were downregulated in the dorsal striatum of METH-induced CPP rats group, but the expression of miR-181c-5p and miR-181d-5p were not changed. mRNA was determined by qRT-PCR, and the values were normalized to the geometric mean of a control gene U6. Data represent the mean ± SD, $n = 8$ in each group. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with control group, two tailed Student's *t*-test.

Serial number	Species	Name	Conserved sequence																									
MIMAT0000256	human	hsa-miR-181a-5p	39–	A	A	C	A	U	U	C	A	A	C	G	C	U	G	U	C	G	G	U	G	A	G	U–61
MIMAT0001168	chicken	gga-miR-181a-5p	20–	A	A	C	A	U	U	C	A	A	C	G	C	U	G	U	C	G	G	U	G	A	G	U–42
MIMAT0004154	opossum	mdo-miR-181a-5p	3–	A	A	C	A	U	U	C	A	A	C	G	C	U	G	U	C	G	G	U	G	A	G	U–25
MIMAT0003625	frog	xtr-miR-181a-5p	14–	A	A	C	A	U	U	C	A	A	C	G	C	U	G	U	C	G	G	U	G	A	G	U–36
MIMAT0000858	rat	rno-miR-181a-5p	39–	A	A	C	A	U	U	C	A	A	C	G	C	U	G	U	C	G	G	U	G	A	G	U–61
MIMAT0002505	Chimpanzee	ptr-miR-181a-5p	39–	A	A	C	A	U	U	C	A	A	C	G	C	U	G	U	C	G	G	U	G	A	G	U–61
MIMAT0000210	mouse	mmu-miR-181a-5p	7–	A	A	C	A	U	U	C	A	A	C	G	C	U	G	U	C	G	G	U	G	A	G	U–29
MIMAT0002506	monkey	mml-miR-181a-5p	39–	A	A	C	A	U	U	C	A	A	C	G	C	U	G	U	C	G	G	U	G	A	G	U–61
MIMAT0003543	cattle	bta-miR-181a-5p	39–	A	A	C	A	U	U	C	A	A	C	G	C	U	G	U	C	G	G	U	G	A	G	UU–62
MIMAT0006707	dog	cfa-miR-181a-5p	1–	A	A	C	A	U	U	C	A	A	C	G	C	U	G	U	C	G	G	U	G	A	G	–22

FIGURE 2 | miR-181a-5p are conserved in at least 10 vertebrate species including human (Homo sapiens; hsa), mouse (Mus musculus; mmu), chicken (Gallus gallus; gga), opossum (onodelphis domestica; mdo), frog (Xenopus tropicalis; xtr), rat (Rattus norvegicus; rno), Chimpanzee (Pan troglodytes; ptr), monkey (Macaca mulatta; mml), cattle (Bos taurus; bta), and dog (Canis familiaris; cfa).

species were compared and revealed that the sequence of miR-181a-5p gene cluster AACAUUCAACGCUGUCGGUGAG was highly conserved in the vertebrates (**Figure 2**).

Establishment of miR-181a-5p Target Gene Set

The number of target genes predicted by the three online databases (TargetScan, miRDB, and miRanda) were 835, 1408, and 4503, respectively. After the target genes were screened by the intersection, a total of 402 unreplicated target genes were obtained as the total gene set for subsequent analyses (**Figure 3**).

GO and KEGG Enrichment Analyses of miR-181a-5p Target Gene

A total of 402 target genes were analyzed by the DAVID software, and the results of GO analysis and KEGG analysis have been presented in **Figure 4**. Among them, 36 genes related to the endoplasmic reticulum and ubiquitin degradation are shown in **Table 1**. **Table 1** indicated that (1) for biological processes (BP), twenty-two genes (KLHL29, RNF34, UBE2B, KLHL15, CUL3, MYCBP2, UBE2D3, TULP4, MED8, KLHL2, TNFAIP1, FBXO33, MID2, BACH2, PCNP, NEURL1B, RNF169, CAND1, KLHL5, TRIM2, RNF182, BIRC6) are particularly enriched in protein ubiquitination and 10 genes (ABTB2, PCNP, RNF145, RNF34, UBE2B, CUL3, BTBD3, UBE2D3, TNFAIP1, RAD23B) are particularly enriched in proteasome-mediated ubiquitin-dependent protein catabolic process. (2) For cell component (CC), seven genes (KLHL29, KLHL5, KLHL15, CUL3, KLHL2, TNFAIP1, BACH2) are particularly enriched in Cul3-RING ubiquitin ligase complex and three genes (HSPA5, HYOU1, HSP90B1) are particularly enriched in endoplasmic reticulum chaperone complex. (3) For molecular function (MF), seventeen genes (KLHL29, TRIM71, RNF34, UBE2B, UBE3C, KLHL15, CUL3, UBE2D3, KLHL2, CBLB, TNFAIP1, BACH2, KLHL5, HECW2, TRIM2, RNF182, BIRC6) are particularly enriched in ubiquitin-protein transferase activity. (4) KEGG analysis results were shown in **Table 1**, which demonstrated that eight genes (HSPA5, SEL1L, UBE2D3, DERL1, HYOU1, SEC24C, RAD23B, HSP90B1) were particularly enriched in protein processing in endoplasmic reticulum.

The specific molecular pathway of the endoplasmic reticulum chaperone complex involved in the ubiquitin-dependent protein catabolic process has been shown in **Figure 5**.

Interaction Analysis of miR-181a Target Gene With SYVN1 and GABAAα1

Thirty-six target genes obtained from GO and KEGG analyses were analyzed by the STRING software for studying the construction of the protein–protein interaction (PPI) network (**Figure 6A**). The study conducted by the investigators earlier found that SYVN1 was involved in ERAD-mediated ubiquitination of GABAAα1. Therefore, SYVN1, GABAAα1, and 36 other target genes were used for the purpose of analysis

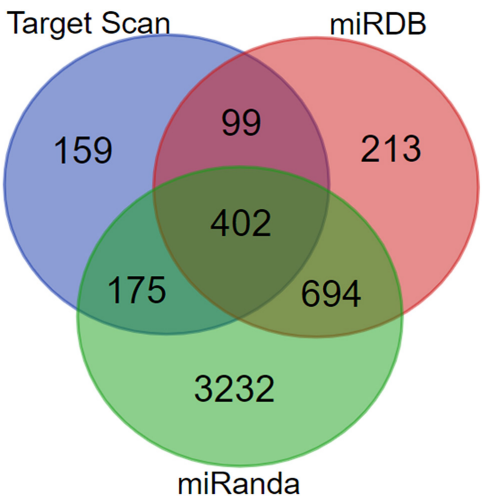

FIGURE 3 | Construction of miR-181a-5p target gene set.

TABLE 1 | 36 genes are particularly enriched in protein ubiquitination and endoplasmic reticulum chaperone complex involved in ubiquitin-dependent protein catabolic process.

Category	Term	Count	PValue	Genes	Benjamini
BP	GO:0016567~protein ubiquitination	22	7.39E-05	KLHL29, RNF34, UBE2B, KLHL15, CUL3, MYCBP2, UBE2D3, TULP4, MED8, KLHL2, TNFAIP1, FBXO33, MID2, BACH2, PCNP, NEURL1B, RNF169, CAND1, KLHL5, TRIM2, RNF182, BIRC6	0.028745
BP	GO:0043161~proteasome-mediated ubiquitin-dependent protein catabolic process	10	0.041985	ABTB2, PCNP, RNF145, RNF34, UBE2B, CUL3, BTBD3, UBE2D3, TNFAIP1, RAD23B	1
CC	GO:0031463~Cul3-RING ubiquitin ligase complex	7	0.00281	KLHL29, KLHL5, KLHL15, CUL3, KLHL2, TNFAIP1, BACH2	0.134878
CC	GO:0034663~endoplasmic reticulum chaperone complex	3	0.021374	HSPA5, HYOU1, HSP90B1	0.598462
MF	GO:0004842~ubiquitin-protein transferase activity	17	0.002424	KLHL29, TRIM71, RNF34, UBE2B, UBE3C, KLHL15, CUL3, UBE2D3, KLHL2, CBLB, TNFAIP1, BACH2, KLHL5, HECW2, TRIM2, RNF182, BIRC6	0.20809
KEGG_PATHWAY	hsa04141:Protein processing in endoplasmic reticulum	8	0.046659	HSPA5, SEL1L, UBE2D3, DERL1, HYOU1, SEC24C, RAD23B, HSP90B1	0.286044

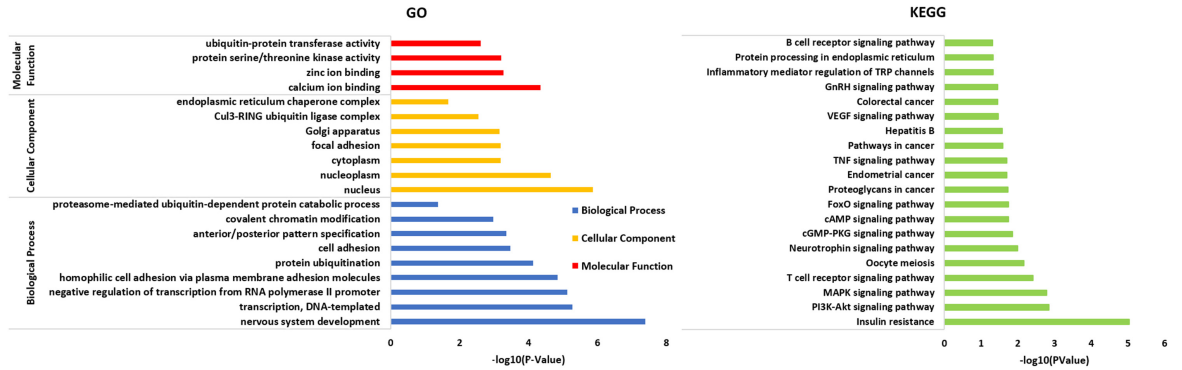

FIGURE 4 | All 402 target genes analyzed by DAVID software and the results of GO analysis and KEGG analysis ($P < 0.05$) shown in the figure above. The results of GO analysis were classified into three functional groups, including biological process (BP), cellular component (CC) and molecular function (MF). On the basis of GO item classification, the KEGG analysis of the target gene set was mainly enriched in multiple biological pathways ($P < 0.05$), for example, MAPK signaling pathway, cAMP signaling pathway, TNF signaling pathway and the specific molecular pathway of the endoplasmic reticulum chaperone complex involved in the ubiquitin-dependent protein catabolic process.

by STRING analysis. **Figure 6B** represents the interaction among these genes, it indicated that the 36 target genes and SYVN1 synergistically were involved in endoplasmic reticulum-related GABAAα1 protein degradation.

METH Increased the Expression of Some Target Genes Regulated by miR-181a-5p in RNA High-Throughput Sequencing Data

To investigate whether the expression of target genes mentioned above increased after METH treatment, RNA high-throughput sequencing data obtained from animals treated with the approximate dose as in this experiment (2 mg/kg) was retrieved from the EMBL-EBI website and cluster analysis was conducted. The level of significance was set at $P < 0.05$, and it was found that the expression levels of 7,046 genes altered after treatment with METH, out of which 3,872 genes exhibited increase in their expression level while 3,174 genes showed a decrease. After screening the results of the intersection of the abovementioned 36 target genes, the results revealed that METH enhanced the expression of 16 target genes, suppressed the expression of 9 target genes, while the expression of 11 target genes remained unchanged (**Figure 7**), indicating that the expression of some target genes regulated by miR-181a-5p changed on receiving METH treatment. Thus, we demonstrated that the ERAD process was activated after METH treatment.

The Expression of 16 Target Genes in the Dorsal Striatum of METH-Induced CPP Rats Was Verified by qRT-PCR

To further verify whether the 16 elevated target genes (UBE2D3, RNF169, FBXO33, RAD23B, NEURL1B, PCNP, TULP4, RNF34, DERL1, HSP90B1, RNF145, KLHL15, KLHL5, CAND1, KLHL2,

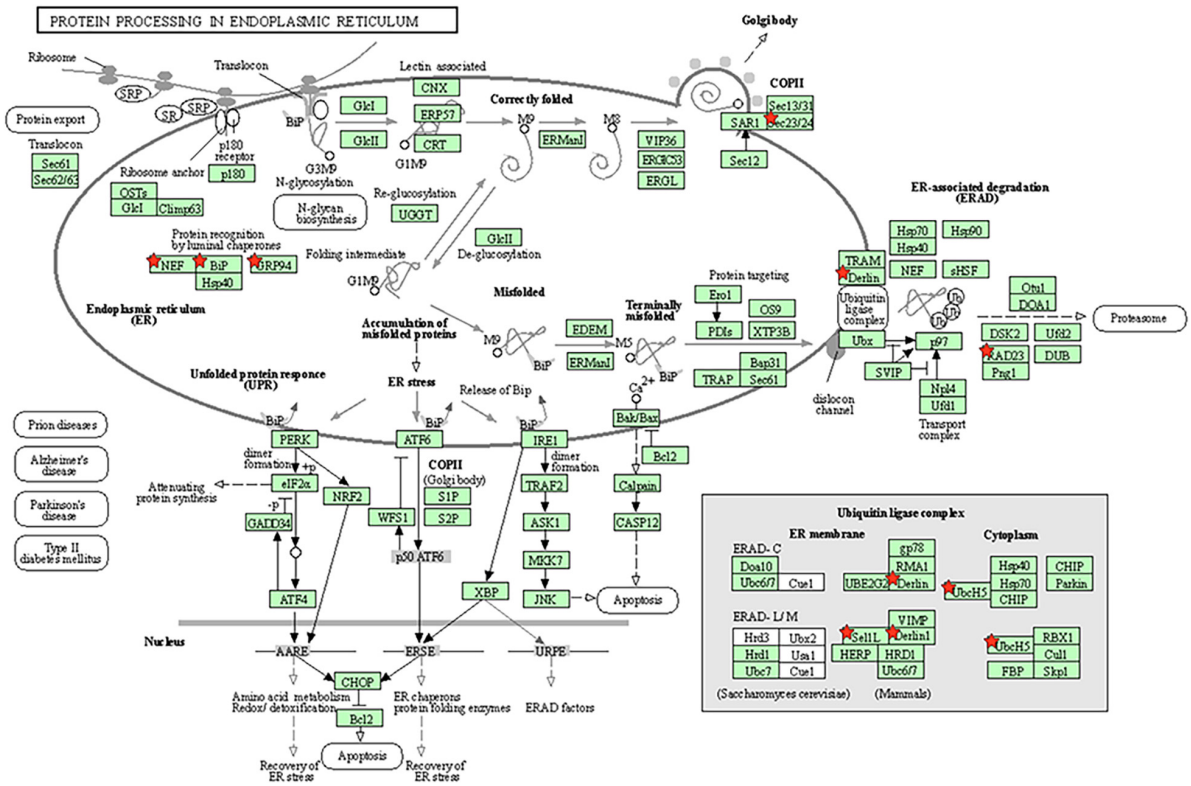

FIGURE 5 | Molecular pathway of endoplasmic reticulum chaperone complex involved in ubiquitin-dependent protein catabolic process. Genes marked the little red pentagram were genes regulated by miR-181a. Figure was cited from DAVID online database.

HSPA5) in **Figure 7** are also increased in the dorsal striatum of METH-induced CPP rats. The expression levels of 16 target genes in the dorsal striatum of METH-induced CPP rats were investigated by qRT-PCR. The results showed that the expression levels of 11 target genes, UBE2D3, RNF169, FBXO33, RAD23B, NEURL1B, PCNP, TULP4, KLHL15, RNF34, DERL1, HSP90B1, were upregulated in the METH-induced CPP group when compared to the control group ($n = 8$, $P < 0.05$), while the expression of 5 target genes, RNF145, KLHL5, CAND1, KLHL2, HSPA5, did not change significantly ($n = 8$, $P > 0.05$) (**Figure 8**).

DISCUSSION

Our earlier study revealed that the expression of GABAAα1 in the dorsal striatum of METH-addicted rats decreased, which was associated with the protein ubiquitin degradation, mediated by ERAD (Jiao et al., 2017). Past studies indicated miR-181a directly regulates the expression of GABAAα1 (Zhao et al., 2012; Sengupta et al., 2013). Keeping this aspect in mind, we attempted to ascertain the expression levels of miR-181a in the dorsal striatum of METH-addicted rats in order to determine whether the decrease of GABAAα1 expression was regulated by miR-181a. However, it was found out that the expression of miR-181a was downregulated. As an inhibitor of the target gene, miRNA showed the potential to inhibit the translation of mRNA or direct degradation of specific mRNA by pairing with 3′-UTR base of mRNA molecules of a specific target gene. The results of the study revealed that the expression of miR-181a decreased, which is contradictory to the findings from past studies, which reported miR-181a directly regulates the expression of GABAAα1. Studies conducted earlier have also reported that METH downregulates the expression of miR-181a (Kai et al., 2016; Zhao et al., 2016; Sim et al., 2017); this finding is consistent with the results of the present study. The obtained results suggest that miR-181a may not be directly involved in the downregulation of GABAAα1, but probably regulates the expression of GABAAα1 through other pathways.

In this study the results of bioinformatics analysis suggested that the regulation of target genes and molecular pathways by miR-181a involved proteasome-mediated ubiquitin-dependent protein catabolic process and endoplasmic reticulum chaperone complex. Taking into consideration the findings from the past studies, it was found that ubiquitin E3 ligase SYVN1 was involved in the downregulation of GABAAα1 in the dorsal striatum of METH-addicted animals through ERAD (Jiao et al., 2017). It was speculated that miR-181a was possibly related to METH addiction and the downregulation of GABAAα1 through the regulation of ERAD.

GABAAα1 folds and assembles correctly in the endoplasmic reticulum and gets exported to cell membrane for physiological

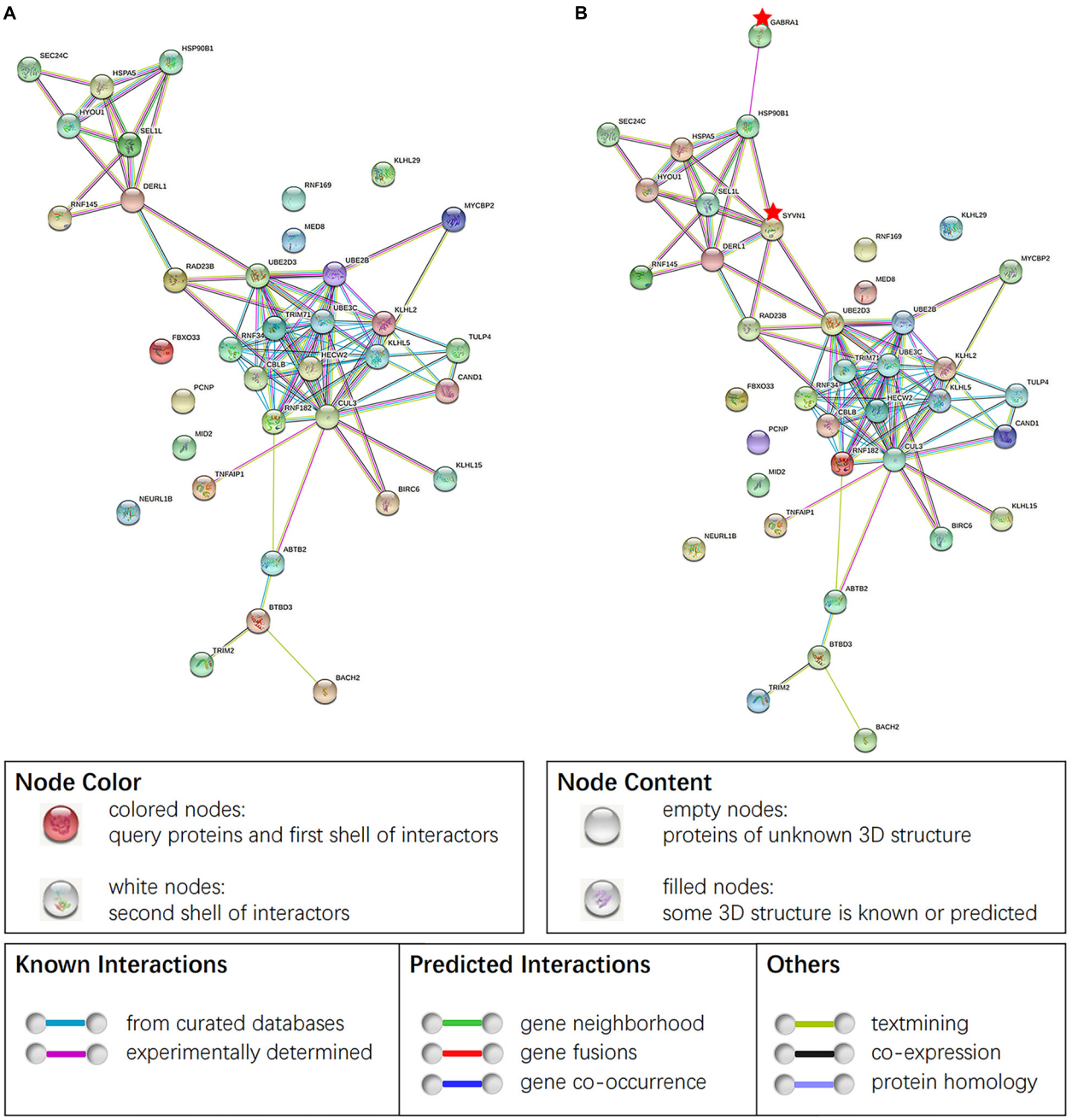

FIGURE 6 | (A) PPI of 36 target genes of miR-181a-5p. **(B)** PPI obtained by taking SYVN1 and GABAAα1 together with 36 genes in STING analysis. Network nodes represent proteins, proteins splice isoforms or post-translational modifications are collapsed, i.e., each node represents all the proteins produced by a single, protein-coding gene locus. Edges represent protein-protein associations, associations are meant to be specific and meaningful, i.e., proteins jointly contribute to a shared function. Genes marked the little red pentagram were SYVN1 and GABAAα1. Figure was cited from STRING online database.

function. Previous studies have found that METH caused dysfunction of the endoplasmic reticulum because of unfolded or misfolded proteins. The proteins get accumulated in the endoplasmic reticulum and are followed by the endoplasmic reticulum stress (Jayanthi et al., 2009; Wongprayoon and Govitrapong, 2017). METH may also induce the unfolded or misfolded GABAAα1. Misfolded GABAAα1 participates in the ubiquitin-degradation process through ERAD and finally gets degraded by proteasome, resulting in the downregulated expression of GABAAα1. This process involves the activation of the ubiquitination system and the endoplasmic reticulum chaperone system.

The results of GO and KEGG analyses by using the DAVID software revealed that 36 target genes and molecular pathways,

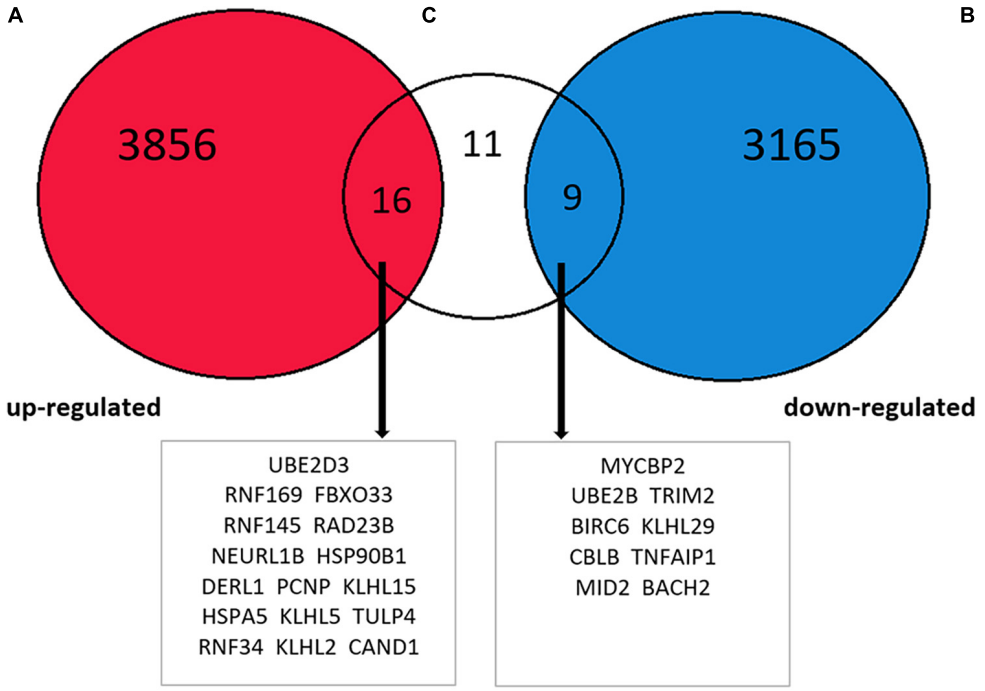

FIGURE 7 | Intersection between EMBL-EBI website METH processing animal brain tissue RNA high-throughput sequencing data analysis results and 36 target genes. The increased expression of 16 target genes, decreased expression of 9 target genes, and 11 target genes without any changes.

which were regulated by miR-181a, involved ubiquitin-mediated protein degradation and endoplasmic reticulum chaperone. The results of further analysis conducted using the STRING software provided results that support the interaction of these 36 target genes with SYVN1 in association with GABAAα1. The high-throughput RNA sequencing database of animal brain tissue treated with METH available on the EMBL-EBI website were retrieved for the purpose of cluster analysis. After intersecting the results of cluster analysis with the 36 target genes, it was found that 16 of the target genes regulated by miR-181a experienced upregulation after induction with METH. In order to verify whether the expression levels of 16 elevated target genes in **Figure 7** are also increased in the dorsal striatum of METH-induced CPP rats, expression levels of 16 genes in the dorsal striatum of METH-induced CPP rats were detected by qRT-PCR. The results showed that 11 of the 16 target genes were up-regulated. Among the 11 target genes, 9 genes (UBE2D3, RNF169, FBXO33, RAD23B, NEURL1B, PCNP, KLHL15, TULP4, RNF34) were responsible for protein ubiquitination, ubiquitin-protein transferase activity and protein-mediated ubiquitin-dependent protein catalytic process, and 2 genes (DERL1, HSP90B1) were responsible for processing of the proteins in the endoplasmic reticulum. It is further confirmed that METH may activate the ERAD pathway through miR-181 and may induce the ubiquitination degradation of GABAAα1, which is involved in the formation of addiction.

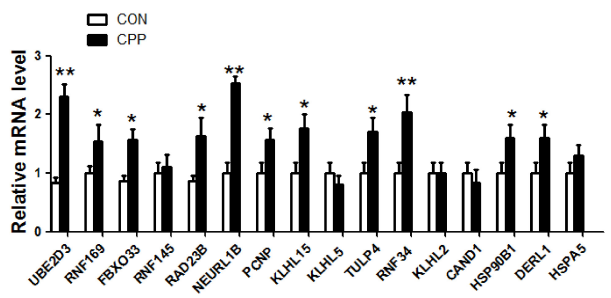

FIGURE 8 | The expression of 16 target genes in the dorsal striatum of METH-induced CPP rats was verified by qRT-PCR. The results showed that mRNA expression of 11 target genes was increased, and 5 target genes had no change. The values were normalized to the geometric mean of a control gene GAPDH (Glyceraldehyde-3-Phosphate Dehydrogenase). Data represent the mean ± SD, $n = 8$ in each group. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with control group, two tailed Student's *t*-test.

The results of the present study revealed that the expression of miR-181a decreased after receiving methamphetamine treatment. This finding was consistent with the findings of past studies (Kai et al., 2016; Zhao et al., 2016; Sim et al., 2017). However, some studies found that the expression of miR-181a increased in the peripheral blood of animals (Reuben et al., 2012) or cocaine addicts (Viola et al., 2019) treated with other excitatory drugs such as cocaine and amphetamines, which can be attributed to the differential effects of drugs on miRNA production.

In addition, it has been speculated that the downregulated expression of miR-181a in the nervous system may also plays

an endogenous protective role. The possible reason is that the enhanced ERAD process helped decrease the accumulation of misfolded proteins and, consequently, decreased endoplasmic reticulum stress and apoptosis (Hwang and Qi, 2018). ERAD can also regulate Alzheimer's amyloid pathology and memory function by modulating degradation of Aβ protein (Zhu et al., 2017). Previous studies have reported that low-dose METH pre-treatment has a protective effect. For example, studies have found that the early use of low-dose METH pre-treatment could lower the neurological injury score of patients with craniocerebral injury (Duong et al., 2018; El Hayek et al., 2020). Low-dose METH pretreatment improved the cognitive functions of Alzheimer's patients (Shukla et al., 2019; Shukla and Vincent, 2020) and reduced the neurotoxic effects of high dose of METH (Lu et al., 2019). MiR-181a also plays an essential role in cell proliferation, apoptosis, differentiation, immunity, and tumor progression (Chang et al., 2014). Studies have also found that the high expression of miR-181a inhibits cell growth (Zhang et al., 2013) and promotes cell apoptosis (Zhu et al., 2012). Therefore, the downregulation of miR-181a induced by METH may play a protective role in promoting nerve cell growth and in inhibiting nerve cell apoptosis. This finding calls for further research on miR-181a, which may provide a useful reference for further exploring the protective effects of low-dose METH pre-treatment.

CONCLUSION

The results of the bioinformatics analysis revealed that miR-181a can cause ubiquitination of GABAAα1 and induce METH addiction through the regulation of ERAD. Further experimental studies need to be conducted in order to probe deeper into the role of miR-181a in METH addiction. This study provides preliminary findings on the role of miR-181a in drug addiction, which may provide a new intervention target for the treatment of methamphetamine addiction. Further studies need to be conducted to determine the role of miR-181a in neuroprotective effects of low-dose METH treatment.

DATA AVAILABILITY STATEMENT

The data used to support the findings of this study are available from the corresponding author upon request.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of Bengbu Medical College (Bengbu, Anhui, China).

AUTHOR CONTRIBUTIONS

YuW and TW wrote the first draft of the manuscript. DJ and WZ provided critical revision of the manuscript for important intellectual content. All authors gave input to the manuscript text, approved the final version of the manuscript, and materially participated in the manuscript preparation.

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REVIEW
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Neurodevelopmental Disorders (NDD) Caused by Genomic Alterations of the Ubiquitin-Proteasome System (UPS): the Possible Contribution of Immune Dysregulation to Disease Pathogenesis

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Over thirty years have passed since the first description of ubiquitin-positive structures in the brain of patients suffering from Alzheimer's disease. Meanwhile, the intracellular accumulation of ubiquitin-modified insoluble protein aggregates has become an indisputable hallmark of neurodegeneration. However, the role of ubiquitin and a fortiori the ubiquitin-proteasome system (UPS) in the pathogenesis of neurodevelopmental disorders (NDD) is much less described. In this article, we review all reported monogenic forms of NDD caused by lesions in genes coding for any component of the UPS including ubiquitin-activating (E1), -conjugating (E2) enzymes, ubiquitin ligases (E3), ubiquitin hydrolases, and ubiquitin-like modifiers as well as proteasome subunits. Strikingly, our analysis revealed that a vast majority of these proteins have a described function in the negative regulation of the innate immune response. In this work, we hypothesize a possible involvement of autoinflammation in NDD pathogenesis. Herein, we discuss the parallels between immune dysregulation and neurodevelopment with the aim at improving our understanding the biology of NDD and providing knowledge required for the design of novel therapeutic strategies.

Keywords: ubiquitin, proteasome, autoinflammation, neurodevelopmental disorders, protein aggregation

INTRODUCTION

Neurodevelopmental disorders (NDD) are a broad spectrum of early onset syndromes affecting the development of the central nervous system (CNS) with a prevalence in children that exceeds 15% worldwide (Romero-Ayuso, 2021). Formerly referred to as "mental retardation" NDD are typically characterized by deficits in cognitive function and adaptive behavior (Micai et al., 2020; Hanly et al., 2021). They traditionally encompass a wide range of different neurologic diseases ranging from mild to severe that include intellectual disability (ID), developmental delay (DD), autism

spectrum disorder (ASD), cerebral palsy (CP), attention deficit/hyperactivity disorder (ADHD), Down syndrome (DS), bipolar disorders (BP), and epilepsy and schizophrenia (Ismail and Shapiro, 2019). One usually discriminates between NDD and neurodegenerative diseases (ND), the latter being a heterogeneous group of late-onset disorders marked by the intracellular accumulation of insoluble protein aggregates perturbing CNS function (Johnson, 2000; Koziorowski et al., 2021). Prominent ND include Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), and Lewy body dementia (LBD) which all mostly affect elderly individuals (Popa-Wagner et al., 2020; Tittelmeier et al., 2020). The NDD/ND dichotomy is, however, not strict since neurodegeneration may in some cases accompany neurodevelopmental anomalies and vice versa (Thibaut, 2018).

Brain pathologies such as NDD and/or ND are complex disorders which are caused for the most part by genetic and/or environmental factors (Cardoso et al., 2019; Dunn et al., 2019). For instance, unquestionable risk factors for the development of NDD include prenatal asphyxia (Adhikari and Rao, 2017) as well as exposure to ethanol (Sokol et al., 2003), heavy metals (Ijomone et al., 2020), and/or organic pollutants (Mesnil et al., 2020). The genetic components of a large fraction of these psychiatric disorders are difficult to unravel since most of them are not necessarily Mendelian (dominant, recessive, or X-linked) and involve the participation of allelic variants in several genes (Au et al., 2020; Savatt and Myers, 2021). However, it is estimated that approximately 40% of NDD are monogenic conditions predominantly due to lesions of a single gene (Deciphering Developmental, and Disorders, 2017; Brunet et al., 2021), and this figure even rises to about 50% in the case of ID (Kaufman et al., 2010; Karam et al., 2015; Reichenberg et al., 2016; Vissers et al., 2016). Because many of these vulnerable genes do not necessarily encode proteins specifically expressed in the brain with documented functions in neurodevelopment, our current understanding of disease pathogenesis remains extremely limited.

Hence, since their initial descriptions, increasing efforts have been made to better understand how NDD/ND emerge from deteriorated genes. One major breakthrough in this field was made by identification of ubiquitin-positive inclusion bodies in the brain of patients with AD (Mori et al., 1987), which led to the assumption that dysfunctions of the ubiquitin-proteasome system (UPS) may contribute to neurodegeneration. This notion was confirmed 1 year later by a work from the same group showing that Lewy bodies in the brain of six cases with LBD and PD were enriched with ubiquitin (Kuzuhara et al., 1988). Shortly afterward, it became evident that the accumulation of ubiquitin aggregates was not necessarily a histological hallmark restricted to neurodegeneration, but could also be found in the brain of children suffering from various NDD (Del Bigio et al., 1997). Meanwhile, the constantly increasing number of genomic alterations in genes encoding components of the UPS identified in patients with neurological phenotypes unambiguously points to its participation in the pathogenesis of psychiatric disorders. Nevertheless, the extreme versatility of the UPS makes it difficult to fully pinpoint its precise implication in disease pathogenesis, as discussed below.

THE UBIQUITIN-PROTEASOME SYSTEM (UPS)

The UPS is a highly conserved pathway across eukaryotic species which ensures the rapid elimination of ubiquitin-tagged proteins by the 26S proteasome (Cetin et al., 2021). The ability of the UPS to remove virtually any type of protein substrate makes it indispensable for almost –if not all– basic cellular processes such as cell division, gene expression and signal transduction (Ebstein et al., 2012). A prerequisite for protein breakdown by 26S proteasomes is the covalent modification of intracellular targets with ubiquitin molecules (Wilkinson et al., 1980; Pickart, 2001, 2004; Pickart and Eddins, 2004). In this process, also referred to as ubiquitination (or ubiquitylation), three enzymes (i.e., E1, E2, and E3) catalyze the coordinated transfer of ubiquitin moieties to acceptor residues of proteins destined for degradation (Haas and Siepmann, 1997). Ubiquitination requires the activation of ubiquitin by a E1 ubiquitin-activating enzyme in an ATP-dependent reaction prior to its subsequent transfer onto a E2 ubiquitin-conjugating enzyme. With the help of E3 ubiquitin ligases, the charged E2-ubiquitin transfer ensures the ubiquitination of protein substrates on lysine, cysteine, serine or threonine residues (Tait et al., 2007; McDowell et al., 2010; Golnik et al., 2016; Swatek and Komander, 2016). Depending on their mode of ubiquitin transfer, E3 ubiquitin ligases can be divided into RING-, HECT- and RBR-type E3 ubiquitin ligases (Petroski and Deshaies, 2005; Metzger et al., 2012; Metzger et al., 2014). In contrast to ligases containing the RING (Really Interesting New Gene) finger domain which catalyze the ubiquitin transfer directly from the E2 to substrate proteins, HECT (homologous to E6AP C-terminus)-type E3 ligases first receive ubiquitin from the E2 on a cysteine residue and then transfer it to substrate proteins (Metzger et al., 2012). Among the RING-type ligases, Cullin-RING-type ligases (CRL) are multi-subunit ligases whose major component is a specific cullin (CUL) molecule which itself binds simultaneously to a RING-box protein (Rbx1 or Rbx2) and a substrate receptor (via an adaptor subunit in some cases) at its C- and N-terminus, respectively, (Harper and Schulman, 2021). Because RING-box proteins recruit conjugated E2, CUL are widely regarded as scaffold molecules bridging E2 to substrate proteins. Typical substrate receptors include F-BOX proteins, BTB domain-containing proteins and DCAF proteins which are ligands for CUL1/7, CUL3, and CUL4, respectively, (Harper and Schulman, 2021). Finally, the RBR (RING-in -between RING)-type E3 ubiquitin ligase family includes members that transfer ubiquitin to substrates in a non-canonical manner via a RING/HECT combined process (Uchida and Kitagawa, 2016).

The tagging of intracellular proteins with one ubiquitin moiety is referred to as mono-ubiquitination and is widely viewed as a post-translational process regulating subcellular localization (Sigismund et al., 2004) and gene expression (Marsh et al., 2020). Multiple mono-ubiquitination, namely the addition of one ubiquitin molecule on multiple sites of the same substrate

occurs as well and signals either endocytosis, protein trafficking and lysosomal degradation or proteasome-mediated degradation (Livneh et al., 2017).

Most importantly, the ubiquitin molecule itself may be subjected to ubiquitin modification on either one of its eight acceptor sites (K6, K11, K27, K29, K33, K48, K63, and Met-1), thereby generating poly ubiquitin chains carrying distinct ubiquitin linkages. The linkage type determines both the topology of the poly ubiquitin chain and the outcome of the modified substrate. It is well established that poly ubiquitin chains bearing K48-linkages typically deliver the modified protein for subsequent degradation by 26S proteasomes (Pickart and Fushman, 2004). The 26S proteasome is a multi-subunit complex consisting of a 19S regulatory particle and a barrel-shaped 20S core particle (Dahlmann, 2005; Tanaka et al., 2012). While the 19S regulatory particle is specialized in ubiquitin recognition and removing as well as substrate unfolding, the 20S core particle ensures protein breakdown into short peptides via its catalytic β-subunits (Finley et al., 2016; Bard et al., 2018). Substrates modified with K48-linked are rapidly recognized by the ubiquitin receptors PSMD4 and ADRM1 on the 19S regulatory particle which facilitate their translocation into the 20S core particle (Deveraux et al., 1995; Husnjak et al., 2008). The binding of ubiquitin-modified proteins with 26S proteasomes is usually strengthened with the help of protein shuttles which are capable of interacting with both ubiquitin chains and proteasomes via their UBA and UBL domains, respectively, (Chen et al., 2016).

The degradation signal exemplified by K48-linked ubiquitin chains represents just the tip of the iceberg of the ubiquitin code, as the other seven ubiquitination sites of ubiquitin may be used either singly or in combination to generate homotypic or mixed poly ubiquitin chains, respectively, that convey multiple cellular functions including lysosomal targeting and DNA repair to name a few (Akutsu et al., 2016; Grumati and Dikic, 2018). Complexity to the UPS pathway arises further with the existence of ubiquitin-like modifiers which, via a conjugation process similar to that of ubiquitin, are capable of modifying cellular targets in a covalent manner. Ubiquitin-like proteins encompass the ISG15, FAT10, NEDD8, URFM1, UFM1, and ATG12 modifiers as well as those of the ATG8 and SUMO families (Cappadocia and Lima, 2018). Thanks to their ability to tag intracellular substrates, ubiquitin-like modifiers generate an extreme variety of signals including proteolytic and non-proteolytic ones (StreichJr., and Lima, 2014). Strikingly, SUMO, and to a lesser extent NEDD8 and ISG15, may themselves be subjected to ubiquitination at various lysine residues, thereby giving rise to hybrid chains whose biological functions, however, have not been fully elucidated (Perez Berrocal et al., 2019; Mulder et al., 2020).

Importantly, both ubiquitin and ubiquitin-like modifications are reversible processes which can be counteracted anytime by ubiquitin hydrolases (also called deubiquitinating enzymes, DUB). Up to now, an approximate number of 100 DUB have been identified, whereby the largest families are represented by the ubiquitin-specific proteases (USP), the ovarian tumor proteases (OTU), and ubiquitin C-terminal hydrolases (UCH) (Clague et al., 2019). Many DUB and E3 ubiquitin ligases regulate fundamental cellular pathways including cell division or death, genomic integrity, epigenetic control, developmental, and differentiation pathways as well as cellular homeostasis (Morgan and Crawford, 2021).

As alluded to earlier, the UPS pathway is frequently damaged in several forms of NDD by genomic alterations that may affect either one of the many genes encoding its various components. Because virtually any gene seems vulnerable, any stage of this process may be impaired from ubiquitin transfer to ubiquitin removal and/or proteasome-mediated breakdown of ubiquitin-modified proteins (**Figure 1**). These observations clearly point to a cause-and-effect relationship between perturbed UPS function and NDD onset, as discussed below.

E3 UBIQUITIN LIGASES IN NDD

Ubiquitin ligases are by far the largest group of UPS genes identified as NDD causing-genes. The first identified member of this constantly growing family is the *UBE3A* gene encoding the E6-AP HECT-type E3 ubiquitin ligase and whose loss-of-function has been shown to cause Angelman syndrome more than twenty years ago (Kishino et al., 1997; Matsuura et al., 1997; Sutcliffe et al., 1997). Because *UBE3A* is exclusively expressed from the maternal allele in neurons, any deletion or point mutations affecting the maternal chromosome leads to loss of E6-AP expression in these cells and result in the acquisition of a neuronal phenotype mostly characterized by absent speech, intellectual disability and happy demeanor with unusually frequent laughing smiling (Maranga et al., 2020).

Since the original reports associating *UBE3A* with Angelman syndrome in 1997, approximately forty-five further genes coding for E3 ubiquitin ligases or CRL substrate receptors have been identified as causative genes for forty-eight different forms of NDD (**Tables 1**, **2**). Clinical features commonly observed in all these syndromes include developmental delay, cognitive deficits, dysmorphic facial features, hypotonia and seizures. However, given the large variety of signals generated by E3 ubiquitin ligases, the phenotypic spectrum of NDD subjects with loss-of-function in E3 genes may vary to a large degree. Herein, limb anomalies such as brachydactyly, polydactyly, or camptodactyly are frequently detected in patients carrying genomic alterations in the *HUWE1*, *TRAF7*, *UBE3B*, *ITCH,* or *FBXW11* genes (Buntinx and Majewski, 1990; Lohr et al., 2010; Moortgat et al., 2018; Tokita et al., 2018; Holt et al., 2019), while gonadal dysfunction seems to be restricted to a subset of NDD cases carrying variants of the *RNF216*, *STUB1*, *TRIM37,* or *KLHL15* genes (Seminara et al., 2002; Jagiello et al., 2003; Heimdal et al., 2014; Mignon-Ravix et al., 2014).

E3 UBIQUITIN LIGASES AND PROTEIN AGGREGATION IN NDD

Over the last two decades, many attempts have been made to unravel the molecular pathogenesis of NDD caused by ubiquitin ligase dysfunction. One straightforward route to address this point consists of identifying downstream target substrates of

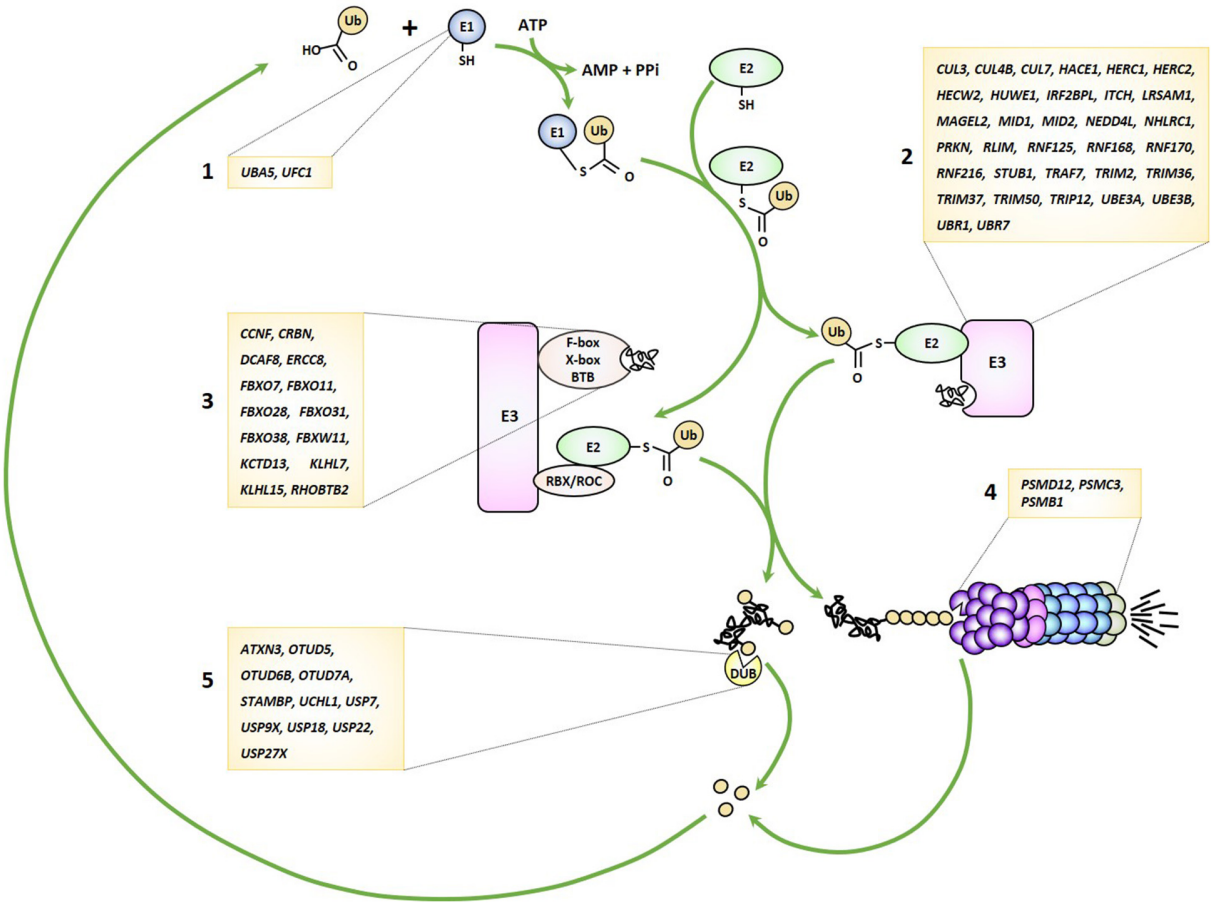

FIGURE 1 | Genetic lesions identified in patients with NDD may affect any stage of the UPS pathway. UPS-related genes disrupted in NDD encode various components of the ubiquitin-conjugation system including E1 ubiquitin-activating enzymes (1), E3 ubiquitin ligases (2), Cullin-RING-type E3 ubiquitin ligases (3), proteasome subunits (4) and deubiquitinating enzymes (5), as indicated.

the affected ligases by proteomic-based methods (Rayner et al., 2019). This strategy is nevertheless hampered by the fact that E3 ubiquitin ligases may have multiple substrates which themselves may fulfill many different functions. One prime example of such ligases is CHIP encoded by the *STUB1* gene and whose genomic alterations cause spinocerebellar ataxia (Shi et al., 2013). Thanks to its ability to bind to cellular chaperones such as HSP70 and HSP90, CHIP mediates the ubiquitination of misfolded proteins, thereby targeting them for subsequent degradation by 26S proteasomes (Edkins, 2015; Wang et al., 2020). Such misfolded proteins typically encompass defective ribosomal products (DRIPS) which are generated during protein biosynthesis as a consequence of ribosomal mistranslation (Yewdell et al., 1996). Pioneering work of J. Yewdell and colleagues has estimated that DRIPS may account for 25% of the total pool of newly synthetized proteins in eukaryotic cells (Schubert et al., 2000; Princiotta et al., 2003; Qian et al., 2006). This implies that virtually any intracellular protein may become a target of CHIP, making it impossible to associate CHIP defects with one particular cellular pathway and/or function. The extremely broad substrate specificity of CHIP also presupposes that *STUB1* loss-of-function results in the accumulation of various misfolded and/or damaged proteins that fail to undergo ubiquitination. Whether these protein aggregates are toxic as a whole and contribute to the pathogenesis of spinocerebellar ataxia is unclear. A fortiori, these inclusions would be devoid of ubiquitin molecules and, as such, not reminiscent of those typically accumulating during neurodegeneration. Herein, this assumption would underline a major distinction between NDD and ND, as it would preclude that the perturbations of protein homeostasis associated with NDD are not due to proteolytic dysfunction. Besides DRIPS, one cannot exclude that NDD due to *STUB1* loss-of-function mutations may occur as a consequence of the inability of the cells to remove specific full-length mature proteins, which would then drive the disease by perturbing specific cellular pathways.

Like CHIP, UBR1, and UBR7, whose deficiencies reportedly cause the Johanson-Blizzard (Zenker et al., 2005) and Li-Campeau syndromes (Li et al., 2021), respectively, are E3 ubiquitin ligases with multiple potential substrates. As members of the N-end rule pathway, UBR1 and UBR7 target

TABLE 1 | NDD-causing genes encoding E3 ubiquitin ligases and associated syndromes.

Gene	OMIM	Syndrome	References	Described regulator of:	References
CUL3	619239	NEURODEVELOPMENTAL DISORDER WITH OR WITHOUT AUTISM OR SEIZURES	Nakashima et al. (2020)	T-cell function	Mathew et al. (2012)
CUL4B	300354	MENTAL RETARDATION, X-LINKED, SYNDROMIC, CABEZAS TYPE	Tarpey et al. (2007)	NF-κB signaling	Hung et al. (2014); Song et al. (2021)
CUL7	273750	THREE M SYNDROME 1	Huber et al. (2005)	Ig class switch recombination	Luo et al. (2019)
HACE1	616756	SPASTIC PARAPLEGIA AND PSYCHOMOTOR RETARDATION WITH OR WITHOUT SEIZURES	Hollstein et al. (2015)	Antiviral immunity	Mao et al. (2016)
HERC1	617011	MACROCEPHALY, DYSMORPHIC FACIES, AND PSYCHOMOTOR RETARDATION	Nguyen et al. (2016)	MAP kinase and mTOR signaling	Sala-Gaston et al. (2020)
HERC2	615516	MENTAL RETARDATION, AUTOSOMAL RECESSIVE 38	Morice-Picard et al. (2016)	Genomic stability	Sala-Gaston et al. (2020)
HECW2	617268	NEURODEVELOPMENTAL DISORDER WITH HYPOTONIA, SEIZURES, AND ABSENT LANGUAGE	Berko et al. (2017)	Mitotic metaphase/anaphase transition, heterochromatin packaging	Lu et al. (2013); Krishnamoorthy et al. (2018)
HUWE1	309590	MENTAL RETARDATION, X-LINKED, SYNDROMIC, TURNER TYPE	Froyen et al. (2008)	Inflammasome, NF-κB signaling	Guo et al. (2020b); Ohtake et al. (2016)
IRF2BPL	618088	NEURODEVELOPMENTAL DISORDER WITH REGRESSION, ABNORMAL MOVEMENTS, LOSS OF SPEECH, AND SEIZURES	Tran Mau-Them et al. (2019)	Apoptosis, survival, and cell differentiation	Ramalho-Oliveira et al. (2019)
ITCH	613385	AUTOIMMUNE DISEASE, MULTISYSTEM, WITH FACIAL DYSMORPHISM	Lohr et al. (2010)	Inflammation, T-cell differentiation	Field et al. (2020)
LRSAM1	614436	CHARCOT-MARIE-TOOTH DISEASE, AXONAL, TYPE 2P	Guernsey et al. (2010)	Antibacterial autophagic response	Ng et al. (2011)
MAGEL2	615547	SCHAAF-YANG SYNDROME	Ates et al. (2019)	Immune infiltration	Arora et al. (2020)
MID1	300000	OPITZ GBBB SYNDROME	Quaderi et al. (1997)	T-cell differentiation, Antiviral immunity	Collison et al. (2013); Chen et al. (2021)
MID2	300928	MENTAL RETARDATION, X-LINKED 101	Geetha et al. (2014)	Cytokinesis	Zanchetta and Meroni (2019)
NEDD4L	617201	PERIVENTRICULAR NODULAR HETEROTOPIA 7	Broix et al. (2016)	Antiviral immunity	Gao et al. (2021)
NHLRC1	254780	EPILEPSY, PROGRESSIVE MYOCLONIC, 2B, INCLUDED	Chan et al. (2003)	Inflammatory cytokines	Lopez-Gonzalez et al. (2017)
PRKN	600116	PARKINSON DISEASE 2, AUTOSOMAL RECESSIVE JUVENILE	Kitada et al. (1998)	Antiviral immunity	Sliter et al. (2018)
RLIM	300978	TONNE-KALSCHEUER SYNDROME	Tonne et al. (2015)	Imprinted X chromosome inactivation	Gontan et al. (2018)
RNF125	616260	TENORIO SYNDROME	Tenorio et al. (2014)	Antiviral immunity, inflammasome IL-36 signaling	Oshiumi et al. (2010); Jia et al. (2017); Saha et al. (2018); Tang et al. (2020)
RNF168	611943	RIDDLE SYNDROME	Stewart et al. (2009)	Ig class switch recombination, Immune deficiency	Ramachandran et al. (2010); Chinn et al. (2017)
RNF170	608984	ATAXIA, SENSORY, 1, AUTOSOMAL DOMINANT	Valdmanis et al. (2011)	Antiviral immunity	Song et al. (2020)
RNF216	212840	GORDON HOLMES SYNDROME	Margolin et al. (2013)	TLR signaling, Antiviral immunity	Nakhaei et al. (2009); Kumazoe et al. (2017)

(Continued)

TABLE 1 | Continued

Gene	OMIM	Syndrome	References	Described regulator of:	References
STUB1	615768; 618093	SPINOCEREBELLAR ATAXIA, AUTOSOMAL RECESSIVE 16; SPINOCEREBELLAR ATAXIA 48	Shi et al. (2013); Genis et al. (2018)	TLR signaling, T-cell function, antiviral immunity, IL-4 signaling	Yang et al. (2011); Chen et al. (2013); Wei et al. (2014); Zhao et al. (2016); Zhou et al. (2018)
TRAF7	618164	CARDIAC, FACIAL, AND DIGITAL ANOMALIES WITH DEVELOPMENTAL DELAY	Tokita et al. (2018)	NF-κB signaling	Zotti et al. (2011)
TRIM2	615490	CHARCOT-MARIE-TOOTH DISEASE, AXONAL, TYPE 2R	Ylikallio et al. (2013)	New World arenavirus entry	Sarute et al. (2019)
TRIM36	206500	ANENCEPHALY	Singh et al. (2017)	Cell cycle progression	Miyajima et al. (2009)
TRIM37	253250	MULIBREY NANISM	Kallijarvi et al. (2002)	NF-κB signaling, Inflammation	Li et al. (2018); Zhao et al. (2021)
TRIM50	194050	WILLIAMS-BEUREN SYNDROME	Micale et al. (2008)	Clearance of aggresomes polyubiquitinated	Fusco et al. (2014)
TRIP12	617752	CLARK-BARAITSER SYNDROME	Zhang et al. (2017)	Epithelial-mesenchymal transition, DNA repair	Challa et al. (2021); Lee et al. (2021)
UBE3A	105830	ANGELMAN SYNDROME	Kishino et al. (1997)	Antiviral immunity	Furumai et al. (2019)
UBE3B	244450	KAUFMAN OCULOCEREBROFACIAL SYNDROME	Basel-Vanagaite et al. (2012)	Cell proliferation	Li et al. (2020)
UBR1	243800	JOHANSON-BLIZZARD SYNDROME	Zenker et al. (2005)	Protein quality control	Zenker et al. (2005)
UBR7	619189	LI-CAMPEAU SYNDROME	Li et al. (2021)	NLR activation, Stem cell function	Zhang et al. (2019); Srivastava et al. (2021)

The potential implication of the identified gene products in the regulation of innate and/or adaptive is indicated. When available, the OMIM (Online Mendelian Inheritance in Man®) disorder number is also reported.

any protein carrying N-terminal destabilizing motifs (also referred to as "N-degrons") such as arginine residues for degradation (Varshavsky, 2019). Substrates of the N-end rule pathway physiologically arise from limited proteolysis and encompass a wide variety of intracellular proteins fulfilling various functions in cell signaling, cellular homeostasis and apoptosis (Varshavsky, 2019). Herein, the multitude of pathways potentially affected by UBR1 and/or UBR7 loss-of-function mutations substantially challenges our understanding of NDD pathophysiology. In addition, one cannot exclude that the diseases may be triggered by the unspecific accumulation of N-end rule substrates that would affect cell function and/or integrity. In any case, CHIP, UBR1 and UBR7 exemplify the difficulty of deciphering the molecular pathogenesis of syndromes due to E3 ubiquitin ligase which have multiple substrates.

NDD-ASSOCIATED E3 UBIQUITIN LIGASES AND THEIR ROLES IN THE IMMUNE RESPONSE

Another E3 ubiquitin ligase potentially causing NDD with a wide range of substrates is ITCH, whose genetic disruption has been shown to cause a syndromic multisystem autoimmune disease referred to as autoimmune disease, multisystem, with facial dysmorphism (ADMFD) (Lohr et al., 2010). Interestingly, ADMFD is also a neurological disease with affected children exhibiting typical NDD features while developing autoimmune systemic responses at the same time. The immunological component of ADMFD is not surprising in view of the substantial number of ITCH cellular targets which play critical roles in T- and B-cell function. These notably include the T-cell receptor (TCR) chain-ζ as well as the RAR-related orphan receptor (ROR)-γt transcription factor, which control T-cell signaling and differentiation, respectively (Huang et al., 2010; Kathania et al., 2016).

The observation that NDD may be accompanied by immune manifestations is somehow intriguing and raises the question as to whether an unrestrained innate and/or adaptive immune response (i.e., autoinflammation and/or autoimmunity) might underlie the pathogenesis of NDD. Strikingly, besides ITCH, more than two-thirds of the E3 ubiquitin ligases reported to cause NDD have critical functions in the innate and adaptive immune systems. As listed in **Tables 1**, **2**, the E3 ubiquitin ligases CUL4B (Hung et al., 2014; Song et al., 2021), HUWE1 (Ohtake et al., 2016; Guo et al., 2020b), RNF216 (Kumazoe et al., 2017), STUB1 (Yang et al., 2011), TRAF7 (Zotti et al., 2011), TRIM37 (Li et al., 2018; Zhao et al., 2021), CRBN (Min et al., 2016; Yang et al., 2018), and the substrate recognition component FBXO7 (Kuiken et al., 2012) have been shown to regulate the expression of inflammatory cytokines mostly thanks to their capacity of modulating NF-κB signaling and/or the inflammasome. It is worth noting that, except HUWE1, all these ligases are described as inflammation negative regulators of these pathways (**Figure 2**), implying that any loss-of-function of any one of these genes would result in the sustained production of pro-inflammatory cytokines.

TABLE 2 | NDD-causing genes encoding CUL substrate receptors and associated syndromes.

Gene	E3 Ubiquitin ligase	OMIM	Syndrome	References	Described regulator of:	References
CCNF	SKP1-CUL1-F-box	619141	FRONTOTEMPORAL DEMENTIA AND/OR AMYOTROPHIC LATERAL SCLEROSIS 5	Williams et al. (2016)	HIV infectivity in CD4 + T-cells	Augustine et al. (2017)
CRBN	DDB1-CUL4-X-box	607417	MENTAL RETARDATION, AUTOSOMAL RECESSIVE 2	Higgins et al. (2004)	TLR signaling, T-cell function	Millrine et al. (2016); Min et al. (2016); Yang et al. (2018); Hesterberg et al. (2020)
DCAF8	DDB1-CUL4-X-box	610100	GIANT AXONAL NEUROPATHY 2	Klein et al. (2014)	Inflammatory cytokines	Peng et al. (2020)
ERCC8	DDB1-CUL4-X-box	216400 614621	COCKAYNE SYNDROME A UV-SENSITIVE SYNDROME 2	Nardo et al. (2009)	Inflammation	Ku and Cheng (2020)
FBXO7	SKP1-CUL1-F-box	260300	PARKINSON DISEASE 15, AUTOSOMAL RECESSIVE EARLY-ONSET	Shojaee et al. (2008)	NF-κB signaling	Kuiken et al. (2012)
FBXO11	SKP1-CUL1-F-box	618089	INTELLECTUAL DEVELOPMENTAL DISORDER WITH DYSMORPHIC FACIES AND BEHAVIORAL ABNORMALITIES	Gregor et al. (2018)	Inflammation, TGF-β signaling	Hardisty-Hughes et al. (2006); Tateossian et al. (2009)
FBXO28	SKP1-CUL1-F-box		DEVELOPMENTAL DELAY, DYSMORPHIC FEATURES, AND INTRACTABLE EPILEPSY	Balak et al. (2018)	Mitochondrial function	Zou et al. (2016)
FBXO31	SKP1-CUL1-F-box	615979	MENTAL RETARDATION, AUTOSOMAL RECESSIVE 45	Mir et al. (2014)	Stem cell differentiation	Baek et al. (2021)
FBXO38	SKP1-CUL1-F-box	615575	NEURONOPATHY, DISTAL HEREDITARY MOTOR, TYPE IID	Sumner et al. (2013)	T-cell function	Meng et al. (2018)
FBXW11	SKP1-CUL1-F-box	618914	NEURODEVELOPMENTAL, JAW, EYE, AND DIGITAL SYNDROME	Holt et al. (2019)	Ig class switch recombination	Luo et al. (2019)
KCTD13	BTB-CUL3-RBX1	611913; 614671	CHROMOSOME 16p11.2 DELETION SYNDROME; CHROMOSOME 16p11.2 DUPLICATION SYNDROME	Crepel et al. (2011)	Cell motility	Chen et al. (2009)
KLHL7	BTB-CUL3-RBX1	617055	PERCHING SYNDROME	Friedman et al. (2009)	Nucleolar integrity	Kim et al. (2017)
KLHL15	BTB-CUL3-RBX1	300982	MENTAL RETARDATION, X-LINKED 103	Mignon-Ravix et al. (2014)	DNA end resection	Ferretti et al. (2016)
RHOBTB2	BTB-CUL3-RBX1	618004	DEVELOPMENTAL AND EPILEPTIC ENCEPHALOPATHY 64	Straub et al. (2018)	Vesicle trafficking	Ji and Rivero (2016)

The potential implication of the gene products in the regulation of innate and/or adaptive is indicated. When available, the OMIM (Online Mendelian Inheritance in Man®) disorder number is also reported.

It is also understood that the E3 ubiquitin ligases HACE1 (Mao et al., 2016), MID1 (Chen et al., 2021), NEDD4L (Gao et al., 2021), PRKN (Sliter et al., 2018), RNF125 (Arimoto et al., 2007), RNF170 (Song et al., 2020), RNF216 (Nakhaei et al., 2009), STUB1 (Zhou et al., 2018), as well as UBE3A (Furumai et al., 2019) are involved in antiviral innate defense and the generation of type I interferon (IFN) responses (**Figure 2**). Again, besides NEDD4L, all these genes encode ligases involved in type I IFN negative feedback loops and, as such any dysfunction, would lead to uncontrolled type I IFN responses.

Some other NDD ubiquitin and/or CRL ligases seem to exert their activity predominantly during the adaptive immune response. These include CUL7 together with the substrate receptor FBXW11 (Luo et al., 2019) as well as RNF168 (Ramachandran et al., 2010) which regulate immunoglobulin switch recombination or CUL3 (Mathew et al., 2012), ITCH (Field et al., 2020), MID1 (Collison et al., 2013), STUB1 (Chen et al., 2013), CRBN (Hesterberg et al., 2020), and FBXO38 (Meng et al., 2018) that are involved in T-cell function and/or differentiation.

Despite the prominent implication of ligases in multiples levels of the innate immune system, only a very small number of NDD cases are associated with symptoms of autoinflammation. These include the Tenorio syndrome caused by RNF125 deficiency which, beside syndromic intellectual disability, leads to a severe inflammatory phenotype with recurrent episodes of

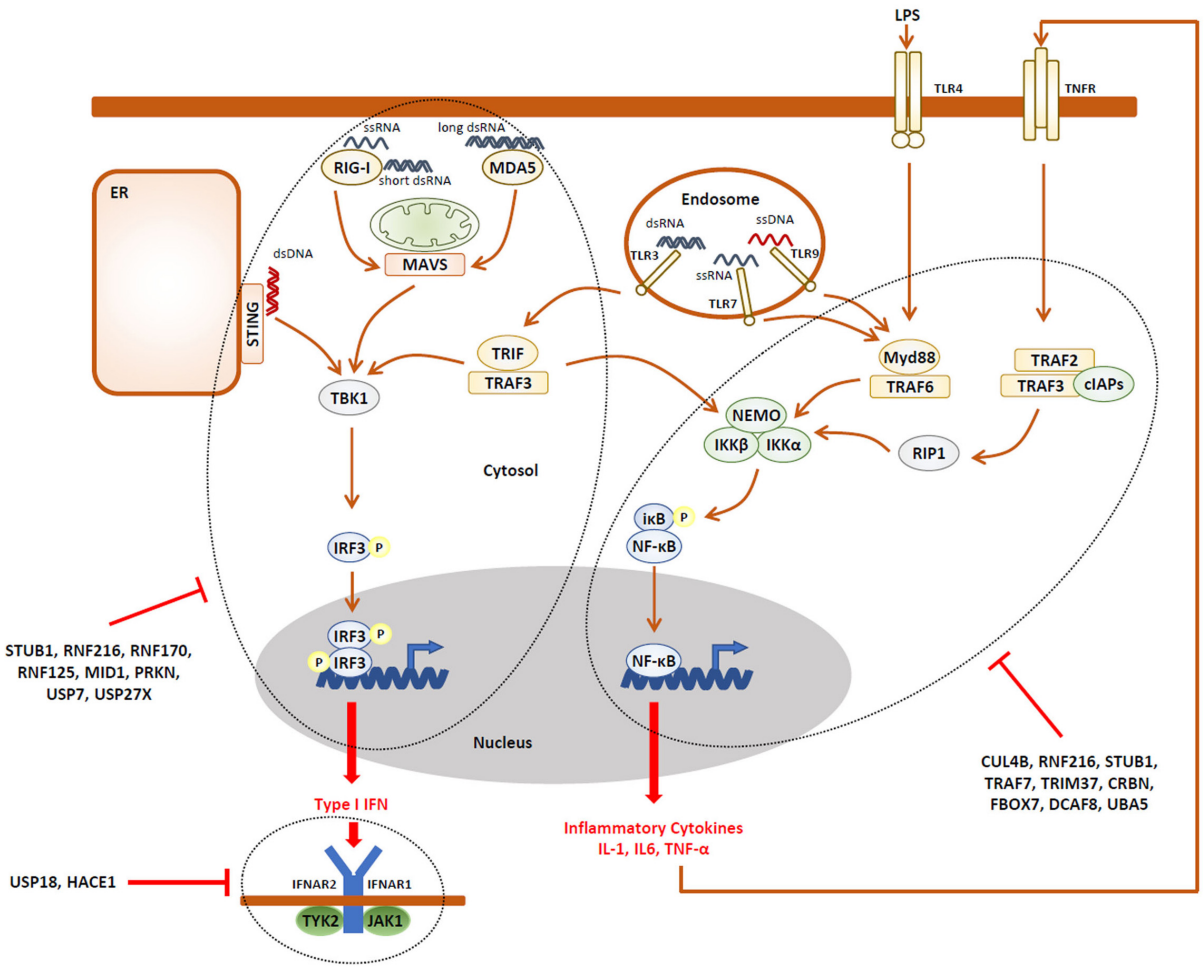

FIGURE 2 | A large fraction of the UPS gene products associated with NDD are negative regulators of the innate immune response. Activation of pathogen recognition receptors (PRR, i.e., TLR3, TLR4, TLR7, TLR9, RIG-1, MDA5, and STING) by pathogen-associated molecular patterns (PAMP, i.e., LPS, ssRNA, dsRNA, and CpG) results in the activation of intracellular signaling cascades which ultimately promote the nuclear translocation of the IRF3 and NF-κB transcriptions factors, as indicated. These, in turn, directly induce the expression of pro-inflammatory cytokines (i.e., TNF-α) and type I IFN which engage the innate arm of the immune system through autocrine and/or paracrine loops. Both the IRF3 and NF-κB activation pathways are down-regulated by many components of the UPS which have been associated with NDD, as indicated. In addition, the autocrine/paracrine action of type I IFN is subjected to negative regulation by the UPS genes USP18 and HACE1, as indicated.

conjunctivitis and stomatitis (Tenorio et al., 2014). The immune manifestations of the Tenorio syndrome clearly corroborate the fact that RNF125 acts as a negative regulator of innate immune signaling by targeting key pattern recognition receptors (i.e., RIG-1, MDA5) for degradation (Arimoto et al., 2007, 2018). Also found in this category of NDD are the Williams-Beuren and Cockayne syndrome which are characterized by intracranial calcifications (Neill and Dingwall, 1950; Knudtzon et al., 1987; Wilson et al., 2016), a typical trait of neuroinflammation (Saade et al., 2019). Ironically, and in contrast to the Tenorio syndrome, both Williams-Beuren and Cockayne syndromes are caused by genomic alterations in genes encoding E3 ubiquitin ligases (i.e., *TRIM50* and *ERCC8*) with no described function in the immune system. Non-etheless, a closer look at their cellular targets reveals that both of these ligases may intersect with host innate immune defenses. In effect, one major substrate of TRIM50 includes BECN1 (Fusco et al., 2018), an important component of the autophagy lysosomal degradation pathway that recruits autophagy proteins to the phagophore assembly site (Kang et al., 2011). Interestingly, it has been shown that BECN1 is capable of activating NF-κB (Leonard et al., 2019), suggesting that any perturbations of its turnover due to TRIM50 deficiency might result in sustained inflammatory responses. In a similar manner, because of its implication in transcription-coupled nucleotide excision repair (TC-NER) in response to ultraviolet (UV) irradiation (Nardo et al., 2009), ERCC8 may render the cells susceptible for autoinflammation. In fact, it is conceivable that ERCC8 loss-of-function might result in abnormal cytosolic accumulation of damaged transcripts, which in turn may be sensed as non-self RNA by immune cells.

The lack of peripheral immune manifestations in NDD caused by the disruption of other E3 ubiquitin ligases may seem

surprising at first sight, but it does not necessarily preclude the absence of ongoing autoinflammation and/or autoimmunity in these patients. Indeed, immune-inflammatory parameters have been frequently detected in NDD subjects seemingly devoid of clinical inflammatory symptoms. For instance, an elevated pro-inflammatory cytokine blood profile has been reported in patients with ASD (Eftekharian et al., 2018; Matta et al., 2019), epilepsy (Riazi et al., 2010), schizophrenia (Potvin et al., 2008; Miller et al., 2011; Tourjman et al., 2013), BP (Benedetti et al., 2020), and ADHD (Zhou et al., 2017). This also holds true for both Aicardi-Goutières and Down syndromes, two NDD which fail to exhibit clinical features of systemic inflammation but fall into the category of interferonopathies because of their sustained production of type I IFN (Crow and Manel, 2015; Livingston and Crow, 2016; Sullivan et al., 2016; Sullivan et al., 2017; Waugh et al., 2019).

Deubiquitinating Enzymes (DUB) in NDD

As illustrated in **Table 3**, a total of ten DUB have been reported as diseases-causing genes for various forms of NDD. Strikingly, eight of them have described roles in the immune system. These include STAMBP (Bednash et al., 2017, 2021), UCHL1 (Karim et al., 2013), USP7 (Daubeuf et al., 2009; Colleran et al., 2013; Palazon-Riquelme et al., 2018), and USP9X (Xiang et al., 2019) which have all been shown to modulate the expression of pro-inflammatory cytokines thanks to their capacity of interfering with the NF-κB signaling and/or inflammasome pathways. Other DUB exert a more specific action on type I IFN responses, rendering them essential at the very first line of innate antiviral defense. One prominent member of this family is undoubtedly USP18 which negatively regulates type I IFN signaling by competing with Janus kinase 1 (JAK1) for binding to IFN α/β receptor 2 (IFNAR2) (Malakhova et al., 2006). The observation that USP18 loss-of-function mutations give rise to brain malformations in patients with pseudo-Torch syndrome (Meuwissen et al., 2016) strongly suggests a cause-and-effect relationship between type I IFN and neurodevelopmental disabilities. Further support for this notion comes from the identification of USP7 and USP27X as disease-causing genes for the Hao-Fountain syndrome and X-linked mental retardation, respectively, (Hao et al., 2015; Hu et al., 2016). Both of these genes encode DUB that stimulate a type I IFN negative feedback mechanism by removing K63-linked poly ubiquitin chains on critical components of the antiviral signaling pathway such as RIG-I (Tao et al., 2020) and TBK1 (Cai et al., 2018).

The association between type I IFN in NDD pathogenesis is, however, challenged by the fact that two DUB identified as NDD-causing have been also described as potent inducers of type I IFN. These include ATXN3 and OTUD5, respectively, causing the Machado-Joseph disease and a X-linked multiple congenital anomalies-neurodevelopmental syndrome (Kawaguchi et al., 1994; Beck et al., 2021; Tripolszki et al., 2021). Indeed, by removing proteolytic poly ubiquitin chains from the cytosolic DNA sensor STING, OTUD5 facilitates antiviral innate signaling and the subsequent transcription of type I IFN genes (Guo et al., 2020a). Likewise, ATXN3 has been shown to exacerbate type I antiviral response via the deubiquitination and stabilization of histone deacetylase 3 (HDAC3) (Feng et al., 2018). As such, any loss-of-function of any of these DUB would mitigate type I IFN responses and contradicts the view that NDD is associated with increased inflammation. However, it is highly likely that ATX3 deficiency exert its pathogenic effect independent of its deubiquitination function, since Machado-Joseph disease is a triplet (CAG encoding glutamine, Q) repeat expansion disorder whereby ATXN3 mutant proteins accumulate as toxic insoluble protein aggregates (Chai et al., 1999). Quite on the contrary, it has been shown that cell lines expressing expanded ATXN3 were characterized by increased transcription of pro-inflammatory cytokines (Evert et al., 2001; Evert et al., 2006). As for OTUD5, its recently described implication in DNA damage-induced transcriptional repression (de Vivo et al., 2019) opens the possibility that its genomic disruption would generate a danger signal leading to inflammation via excessive cytosolic mRNA accumulation.

Very little is known about the biological function and/or cellular targets of OTUD7A and OTUD6B, whose genetic lesions cause the chromosome 15q13.3 deletion syndrome and an intellectual developmental disorder with dysmorphic facies, seizures, and distal limb anomalies (Santiago-Sim et al., 2017; Uddin et al., 2018), respectively. Interestingly, common to both disorders is a decreased proteasome function ultimately resulting in the aggregation of ubiquitin-modified protein (Santiago-Sim et al., 2017; Garret et al., 2020). These studies suggest that OTUD7A and OTUD6B are directly or indirectly involved in the regulation of proteasome-mediated proteolysis and that their deficiencies would result in cellular situations very similar neurodegeneration ones. A distant and indirect member of the DUB family associated with NDD phenotypes is USP22 which participates in the pathogenesis of spinocerebellar ataxia 7 caused by poly Q repeats in the ATX7 gene (David et al., 1997). Both UPS22 and ATX7 are parts of the Spt-Ada-Gcn5 Acetyl transferase (SAGA) complex which promotes gene transcription via histone acetylation and deubiquitination activities (Melo-Cardenas et al., 2016). It is argued that the generation of poly Q Ataxin-7 protein aggregates substantially affects the access of USP22 to its cellular substrates notably histone H2B (Henry et al., 2003), a dysfunction likely contributing to disease onset. Most interestingly, it has been shown that USP22 gene silencing is accompanied by activation of the JAK-STAT1 signaling pathway (Han et al., 2020), thereby raising the possibility that type I IFN might be a component of spinocerebellar ataxia 7.

Proteasomes in NDD

As shown in **Table 4**, the most recent identified group of UPS genes associated with NDD include those encoding proteasome subunits. In this short list are found the *PSMD12*, *PSMC3* and *PSMB1* genes that cause the Stankiewicz-Isidor syndrome (STISS), a neurosensory syndrome combining deafness and cataract as well as a disorder characterized by microcephaly, intellectual disability, developmental delay and short stature, respectively, (Kury et al., 2017; Ansar et al., 2020; Kroll-Hermi et al., 2020). Like most of the NDD due to genomic alterations of UPS genes, these syndromes are

TABLE 3 | NDD-causing genes encoding DUB and associated syndromes.

Gene	OMIM	Syndrome	References	Described regulator of:	References
ATXN3	109150	MACHADO-JOSEPH DISEASE	Kawaguchi et al. (1994)	Antiviral immunity	Feng et al. (2018)
OTUD5	301056	MULTIPLE CONGENITAL ANOMALIES-NEURODEVELOPMENTAL SYNDROME, X-LINKED	Beck et al. (2021); Tripolszki et al. (2021)	Antiviral immunity	Guo et al. (2020a)
OTUD6B	617452	INTELLECTUAL DEVELOPMENTAL DISORDER WITH DYSMORPHIC FACIES, SEIZURES, AND DISTAL LIMB ANOMALIES	Santiago-Sim et al. (2017)	B-lymphocyte proliferation	Xu et al. (2011)
OTUD7A	612001	CHROMOSOME 15q13.3 DELETION SYNDROME	Hoppman-Chaney et al. (2013)	DNA repair	Wu et al. (2019)
STAMBP	614261	MICROCEPHALY-CAPILLARY MALFORMATION SYNDROME	Hori et al. (2018)	Inflammasome	Bednash et al. (2017, 2021)
UCHL1	615491, 613643	SPASTIC PARAPLEGIA 79, PARKINSON DISEASE 5, AUTOSOMAL DOMINANT	Leroy et al. (1998)	TLR signaling	Karim et al. (2013)
USP7	616863	HAO-FOUNTAIN SYNDROME	Hao et al. (2015)	TLR signaling, antiviral immunity, NF-κB signalling, T-cell differentiation, Inflammasome	Daubeuf et al. (2009); Colleran et al. (2013); van Loosdregt et al. (2013); Cai et al. (2018); Palazon-Riquelme et al. (2018)
USP9X	300919; 300968	MENTAL RETARDATION, X-LINKED 99; MENTAL RETARDATION, X-LINKED 99, SYNDROMIC, FEMALE-RESTRICTED	Homan et al. (2014)	T-cell signaling; TLR signaling	Naik et al. (2014); Naik and Dixit (2016); Xiang et al. (2019)
USP18	617397	PSEUDO-TORCH SYNDROME 2	Meuwissen et al. (2016)	Antiviral immunity	Ritchie et al. (2004)
USP27X	300984	MENTAL RETARDATION, X-LINKED 105	Hu et al. (2016)	Antiviral immunity	Guo et al. (2019); Tao et al. (2020)

The potential implication of the gene products in the regulation of innate and/or adaptive is indicated. When available, the OMIM (Online Mendelian Inheritance in Man®) disorder number is also reported.

TABLE 4 | NDD-causing genes encoding proteasome subunits and associated syndromes.

Gene	OMIM	Syndrome	References	Described regulator of	References
PSMD12	617516	STANKIEWICZ-ISIDOR SYNDROME	Kury et al. (2017)	Inflammation	Ebstein et al. (2019); Cetin et al. (2021); Goetzke et al. (2021)
PSMC3		NEUROSENSORY SYNDROME COMBINING DEAFNESS AND CATARACT	Kroll-Hermi et al. (2020)		
PSMB1		MICROCEPHALY, INTELLECTUAL DISABILITY, DEVELOPMENTAL DELAY AND SHORT STATURE	Ansar et al. (2020)		

The potential implication of the gene products in the regulation of innate and/or adaptive is indicated. When available, the OMIM (Online Mendelian Inheritance in Man®) disorder number is also reported.

seemingly devoid of systemic signs of immune dysregulation. This observation is even more surprising considering the fact that proteasome loss-of-function mutations have been described to cause autoinflammatory diseases referred to as proteasome-associated autoinflammatory syndromes (PRAAS) or chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature (CANDLE) (Agarwal et al., 2010; Arima et al., 2011; Kitamura et al., 2011; Liu et al., 2012; Brehm et al., 2015; Poli et al., 2018; de Jesus et al., 2019; Sarrabay et al., 2019; Kataoka et al., 2021). In contrast to the NDD alterations that may affect 20S or 19S proteasome complexes, the CANDLE/PRAAS mutations are exclusively located in the 20S core particle and/or proteasome assembly chaperones (Ebstein et al., 2019). Common to all CANDLE/PRAAS subject is a type I IFN gene signature characterized by increased amounts of transcripts encoding canonical IFN-stimulated

TABLE 5 | NDD-causing genes encoding components of the Umf1-conjugation pathway and associated syndromes.

Gene	OMIM	Syndrome	References	Described regulator of	References
UBA5	617132; 617133	DEVELOPMENTAL AND EPILEPTIC ENCEPHALOPATHY 44; SPINOCEREBELLAR ATAXIA, AUTOSOMAL RECESSIVE 24	Duan et al. (2016); Muona et al. (2016)	NF-κB signaling	Li et al. (2017, 2019); Xi et al. (2013)
UFC1	618076	NEURODEVELOPMENTAL DISORDER WITH SPASTICITY AND POOR GROWTH	Nahorski et al. (2018)		

The potential implication of the gene products in the regulation of innate and/or adaptive is indicated. When available, the OMIM (Online Mendelian Inheritance in Man®) disorder number is also reported.

genes such as *ISG15*, *SIGLEC-1*, *IFI44L*, *IFIT1*, *IFI27*, and *RSAD2* (Brehm and Krüger, 2015). Interestingly, resetting the immune system of patients with mutation in the proteasome assembly maturation protein (POMP) via hematopoietic stem cell transplantation (HSCT) could successfully reverse the clinical and molecular features of CANDLE/PRAAS (Martinez et al., 2021), indicating that the signature is mostly generated by immune cells. Although some patients may exhibit signs of cognitive impairment, CANDLE/PRAAS are usually not dominated by typical neuropsychological and biological features of a neurodevelopmental disorder, thereby making them distinct from classical NDD. Conversely, and unlike CANDLE/PRAAS, NDD due to lesions in *PSMD12*, *PSMC3* and *PSMB1* genes fail to develop systemic autoinflammation, which prevent them from falling into autoinflammatory disease categories. The reasons why proteasomes loss-of-function mutations lead to two clinically distinct phenotypes are unclear and warrant further investigations. Clearly, the divergence between the two diseases is not dictated by the location of the affected subunit within the 26S proteasome, as initially assumed (Ebstein et al., 2019). The notion that CANDLE/PRAAS develop peripheral autoimmunity is not unexpected given the pleiotropic role of proteasomes in multiple inflammatory signal cascades (Cetin et al., 2021; Goetzke et al., 2021). On the contrary, the lack of systemic manifestations in NDD due to proteasome loss-of-function mutations is intriguing, but again does not necessarily imply the absence of autoinflammation in some tissues and/or the generation of atypical inflammatory signatures that may have been overlooked.

UFMylation in NDD

As shown in **Table 5**, the last and smallest group of UPS-related genes responsible for NDD comprises the two E1 ubiquitin-activating enzymes *UBA5* (Duan et al., 2016; Muona et al., 2016) and *UFC1* (Nahorski et al., 2018). Both of these proteins belong to the recently described Ubiquitin-fold modifier 1 (Ufm1)-conjugation system whose biological relevance remains to be fully understood. Because the only E3 Ufm1 ligase identified so far (i.e., UFL1) is recruited at the cytosolic side of the endoplasmic reticulum (ER) membrane (Wu et al., 2010), it is thought that Ufm1 modification is involved in ER protein quality control and/or homeostasis (Adamson et al., 2016). This notion is in line with recent studies showing that proteins involved in these processes such as RPL26 and RPN1 are cellular targets of the (Ufm1)-conjugation pathway (Walczak et al., 2019; Liang et al., 2020). Most importantly, it seems that Ufm1 modification at the ER represses the unfolded protein response (UPR) (Liang et al., 2020), a pathway known to cause sterile inflammation (Ebstein et al., 2019). It is therefore conceivable that Ufm1 loss-of-function might result in sustained overactivation of the UPR and expression of inflammatory markers. This assumption is in agreement with recent reports showing that Ufm1 attenuates inflammation induced by LPS (Li et al., 2017, 2019). Although patients with UBA5 loss-of-function mutations fail to show noticeable symptoms of inflammation, one can again not exclude that these syndromes are devoid of inflammatory process.

CONCLUSION AND FUTURE DIRECTIONS

In this review, we have identified 62 reported monogenic NDD directly caused by lesions in genes encoding components of the UPS (**Tables 1–5**). To our surprise, 37 of these genes encode products that have been shown to regulate the immune system at various levels (**Tables 1–5**). Specifically, 20 of them are negative regulators of the two major (i.e., NF-κB and IRF) pathways in inflammation and antiviral response as well as type I IFN signaling (**Figure 2**). We believe that this number is likely underestimated, as many cellular targets of the identified ubiquitin ligases and/or DUB encompass proteins involved in DNA/RNA processing which may alert the immune system upon dysfunction through the generation of dangers signals. Altogether, this analysis strengthens the straightforward assumption that uncontrolled inflammation contributes to the pathogenesis of psychiatric disorders including NDD, although this remains to be formally demonstrated. One general contradiction stemming from our work is the fact that a great majority of NDD patients does not exhibit typical symptoms of chronic inflammation. However, suspecting subtle and stealthy levels of inflammation remains a challenge for pediatricians and it is likely that children apparently devoid of clinical signs of inflammation are not tested for immune disorders. In this regard, the absence of standardized diagnostic assays for a number of pro-inflammatory cytokines, particularly type I IFN, makes also the detection of specific and atypical inflammatory

signatures difficult. One further possible explanation for this discrepancy may be that the UPS components affected in NDD exhibit a tissue-specific distribution, thereby promoting a more localized inflammation rather than a systemic one. In view of the neuronal phenotype of these disorders, it is highly likely that most of these genes are expressed in the CNS including microglia cells and astrocytes which might represent a potential source of inflammation in response to UPS dysfunction. As such, it is conceivable that inflammation might be restricted to the cerebrospinal fluid (CSF) in these patients. Future work aiming to address the role of pro-inflammatory mediators on neuron differentiation and/or function will help improve our understanding of disease pathogenesis and identify therapeutic targets for NDD.

AUTHOR CONTRIBUTIONS

FE conceived, wrote, and edited the manuscript. SK, JJP, and EK participated in data analysis and provided intellectual input into the manuscript. FE and SK have designed the figures and tables.

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Interruption of Endolysosomal Trafficking After Focal Brain Ischemia

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A typical neuron consists of a soma, a single axon with numerous nerve terminals, and multiple dendritic trunks with numerous branches. Each of the 100 billion neurons in the brain has on average 7,000 synaptic connections to other neurons. The neuronal endolysosomal compartments for the degradation of axonal and dendritic waste are located in the soma region. That means that all autophagosomal and endosomal cargos from 7,000 synaptic connections must be transported to the soma region for degradation. For that reason, neuronal endolysosomal degradation is an extraordinarily demanding and dynamic event, and thus is highly susceptible to many pathological conditions. Dysfunction in the endolysosomal trafficking pathways occurs in virtually all neurodegenerative diseases. Most lysosomal storage disorders (LSDs) with defects in the endolysosomal system preferentially affect the central nervous system (CNS). Recently, significant progress has been made in understanding the role that the endolysosomal trafficking pathways play after brain ischemia. Brain ischemia damages the membrane fusion machinery co-operated by N-ethylmaleimide sensitive factor (NSF), soluble NSF attachment protein (SNAP), and soluble NSF attachment protein receptors (SNAREs), thus interrupting the membrane-to-membrane fusion between the late endosome and terminal lysosome. This interruption obstructs all incoming traffic. Consequently, both the size and number of endolysosomal structures, autophagosomes, early endosomes, and intra-neuronal protein aggregates are increased extensively in post-ischemic neurons. This cascade of events eventually damages the endolysosomal structures to release hydrolases leading to ischemic brain injury. Gene knockout and selective inhibition of key endolysosomal cathepsins protects the brain from ischemic injury. This review aims to provide an update of the current knowledge, future research directions, and the clinical implications regarding the critical role of the neuronal endolysosomal trafficking pathways in ischemic brain injury.

Keywords: brain ischemia-reperfusion injury, membrane trafficking, N-ethylmaleimide sensitive fusion protein, cathepsin B, late endosome, early endosome, autophagosome, autophagic flux

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INTRODUCTION

The endolysosomal system is a major catabolic pathway for recycling cellular waste products. Newer evidence has placed this system at the center stage of many research fields. This system has previously been viewed as a relatively static acidic compartment containing hydrolytic enzymes for cellular waste degradation, but now has emerged as a highly dynamic organelle capable of sensing and controlling multiple cellular processes (Repnik et al., 2013, 2015; Inpanathan and Botelho, 2019). The endolysosomal system has sophisticated organizations constantly sensing diverse intra- and extracellular stimuli to adjust their numbers, size, and position, as described in detail in several recent reviews (Condon and Sabatini, 2019; Lawrence and Zoncu, 2019; Ballabio and Bonifacino, 2020). In addition to degradation of waste protein aggregates, damaged organelles, and extracellular materials, this system plays a key role in inflammatory and immune responses, antigen presentation, plasma membrane repair, exosome release, cellular adhesion and migration, extracellular matrix (ECM) remodeling, programmed cell death, tumor invasion and metastasis, and neurodegeneration (Wang et al., 2018; Winckler et al., 2018; Inpanathan and Botelho, 2019; Lie and Nixon, 2019; Alu et al., 2020; de Araujo et al., 2020; Giovedi et al., 2020; Navarro-Romero et al., 2020; Qureshi et al., 2020; Song et al., 2020).

A typical neuron consists of a soma, a single axon with numerous nerve terminals, and multiple dendritic trunks with numerous branches. On average, there are 7,000 distal synaptic connections from each of the hundreds of billions of neurons present in the human brain to other neurons. The neuronal endolysosomal degradation compartments are located in the soma region. That means that all autophagosomal and endosomal cargos from 7,000 synaptic connections must be transported to the soma region for degradation. Therefore, the degradation of cargo in polarized neurons by endolysosomal compartments is a rigorous event, vulnerable to many pathological conditions.

In this review, we focus on the neuronal endolysosomal pathway after stroke. We provide a basic overview of updated knowledge on membrane trafficking from the endocytic and autophagic pathways to the endolysosomal compartments. Next, we illustrate the features of neuronal endolysosomal trafficking. Finally, we explore the current knowledge of dysfunctional endolysosomal compartments in animal models of brain ischemia.

PART 1: ENDOCYTIC AND AUTOPHAGIC TRAFFICKING TO THE ENDOLYSOSOMAL COMPARTMENTS

Endocytic Pathway

The primary function of the endocytic pathway is recycling and bulk degradation of internalized materials and redundant cellular components (Repnik et al., 2013). The endocytic pathway encompasses three membrane compartments: the early endosome, recycling endosome, and late endosome. This pathway begins with invagination of a segment of the cell surface membrane, typically coated with proteins such as clathrin or caveolin, to form endocytic vesicles. The endocytic vesicles either fuse with existing early endosomes to deliver their cargo or undergo multiple rounds of homotypic fusion to generate a new early endosome (Rizzoli et al., 2006). The early endosome serves as the primary sorting compartment to distribute the engulfed cargos to the trans-Golgi network, the recycling endosome, and the late endosome. Engulfed cargos include lipids, membrane proteins, signaling molecules, and extracellular substances.

In the recycling route of the endocytic pathway, the early endosome converts a part of its structure into a recycling endosome to reassemble engulfed membrane proteins for reuse back in plasma membranes (Hyttinen et al., 2013). In the degradation route, the early endosome undergoes a maturation process to become a late endosome or fuses with an existing late endosome to deliver the cargo (Hyttinen et al., 2013). The transition between early and late endosome requires switching proteins, e.g., exchanging Rab5 with Rab7, is accompanied by structural changes, such as forming of the intraluminal membranous vesicles (ILV) (Poteryaev et al., 2010). The late endosome itself also undergoes a series of transformations and comes in various forms, such as the multivesicular body (MVB) (Bissig and Gruenberg, 2013). During the transformation process, the late endosome receives newly synthesized hydrolytic enzymes and structural components from the trans-Golgi network and degradative cargos from the endocytic and autophagic (see below) pathways (Repnik et al., 2013; Bright et al., 2016). Therefore, the late endosome is often akin to the stomach of the cell. At the final mature stage, the late endosome fuses with a terminal lysosome to become an endosome–lysosome hybrid, i.e., endolysosome, where the endocytosed materials are broken up into basic components, such as amino acids, for cell reuse (Bright et al., 2016). After degradation of cargo, the endolysosome converts to a terminal lysosome in a process referred to as lysosome re-formation (Bright et al., 2016).

Autophagy Pathway

Macroautophagy, herein referred to as autophagy, is a cellular bulk degradation mechanism to recycle intracellular waste protein aggregates and damaged organelles and is essential to maintain cell homeostasis (Liu et al., 2010). Autophagy begins with the nucleation of a membrane sack termed phagophore. This nucleation process requires multiple autophagy-related (ATG) protein and lipid complexes, such as the ATG7-ATG12-ATG16 complex, as well as the microtubule-associated protein 1A/1B-light chain 3 (LC3I)-phosphatidylethanolamine (PE) conjugate referred to as LC3II. The phagophore engulfment of waste ubiquitinated protein aggregates and damaged organelles is mediated by adaptor proteins known as autophagic receptors, such as p62/sequestosome-1 (SQSTM1), optineurin, neighbor of BRCA1 gene 1 (NBR1), and ubiquilin-2. These autophagic receptors share a common ubiquitin-binding domain (UBD) and an LC3-interacting region (LIR). They use the UBD to recognize ubiquitinated protein aggregates or ubiquinated proteins on damaged organelle membranes, and the LIR to interact with LC3II on the phagophore membrane (Deng et al., 2017). The most widely studied autophagy receptor is p62/SQSTM1. The

phagophore expands during engulfment and eventually seals both ends to give rise to a double-membrane autophagosome. The autophagosome then fuses with the late endosome to form an intermediate organelle, termed amphisome. Next, the amphisome undergoes membrane fusion with a terminal lysosome to become an autolysosome where cargo are degraded. In an alternative route, the autophagosome can directly fuse with either an endolysosome or a lysosome to become an autolysosome to degrade cargo (Zhao and Zhang, 2019).

The autophagic degradation activity can be measured by the autophagic cargo degradation rate, also known as autophagy flux (Klionsky et al., 2021). During the autophagic degradation process, autophagic cargo and autophagic proteins such as LC3II and autophagy receptors (e.g., p62/SQSTM1) are degraded together. Given this, the protein levels and distribution patterns of LC3II and p62/SQSTM1 are often used to assess the autophagic flux. Thus, an increased level of p62/SQSTM1 immunolabeled intracellular aggregates or LC3-immunopositive puncta on tissue sections often indicates disrupted degradation activity or reduced autophagic flux (Runwal et al., 2019).

Endolysosomal System

As mentioned above, the endolysosomal system is the extension of the trans-Golgi network and the biosynthetic, endocytic, and autophagic pathways. In this review, the endolysosomal system refers to the gamut of organelles, encompassing the late endosome, endolysosome, and terminal lysosome (**Figure 1**) (Bright et al., 2016; Bissig et al., 2017). Endolysosomal compartments and endolysosomal structures are interchangeable.

There remains some misapprehension of the endolysosomal system. In some studies, the late endosome and endolysosome have been vaguely batched together as the lysosome (Lie and Nixon, 2019). The protein compositions among all three endolysosomal compartments are almost identical, hence this common misconception. Furthermore, because the endolysosomal hydrolytic enzymes and structural proteins are delivered from the trans-Golgi network to the late endosome and then further to endolysosome and lysosome, the late endosome has been considered as the precursor of the endolysosome and lysosome. For that reason, the commonly used lysosomal markers such as cathepsins and lysosomal membrane-associated proteins (LAMPs) are major components of the late endosome, endolysosome, and lysosome. There are no specific protein markers that can discern the late endosome from the endolysosome (Lie and Nixon, 2019). M6PR and Rab7 are the only two proteins currently known to be present in the late endosome and endolysosome, but not in the terminal lysosome (Saftig and Klumperman, 2009). Under transmission electron microscopy (TEM), the late endosome contains ILVs and membrane whorls or fragments, the lysosome appears homogeneous with few membrane fragments, and the endolysosome has morphological characteristics from both the late endosome and terminal lysosome (Tjelle et al., 1996; Huotari and Helenius, 2011). Despite these morphological characteristics, the ultrastructure of the late endosome, endolysosome, and lysosome under EM often remains atypical and thus difficult to differentiate (Griffiths, 1996; Repnik et al., 2013). Another misconception of the lysosome is that they are the major cellular degradation organelle. However, recent studies show that the endolysosome is the principal intracellular site of acid hydrolase activity for degradation of early endosomal and autophagic cargos, whereas the terminal lysosome acts as a store of acid hydrolases and is cathepsin-inactive (Bright et al., 2016).

All endolysosomal compartments have acidic lumens generated by their transmembrane vacuolar adenosine triphosphatase (V-ATPase) (Luzio et al., 2014). The V-ATPase is present throughout the endolysosomal compartments, albeit at different densities, to create a pH range from 6.0 in the late endosome to 4.5 in the lysosome (Yamashiro and Maxfield, 1987). Although the late endosome receives incoming acid hydrolytic enzymes from the Golgi and the degradation cargos from the early endosome and autophagosome, they cannot degrade these waste cargos efficiently because of its higher intraluminal pH (6.0). They must be acidified transiently ("kiss-and-run") or permanently by fusing with the more acidic terminal lysosome (pH 4.5) to become a hybrid endolysosome (Bright et al., 2016).

After degradation of the cargo, an endolysosome transforms or reforms into a new terminal lysosome. This new terminal lysosome is ready to fuse with a late endosome for the next round of an endolysosome—terminal lysosome cycle (Bright et al., 2016; Bissig et al., 2017; de Araujo et al., 2020). Cells require a dynamic equilibrium of late endosomes, endolysosomes, and terminal lysosomes to maintain homeostasis. An imbalance among endolysosomal compartments leads to a release of their intraluminal hydrolases into the cytoplasm and extracellular space, resulting in cell death (Inpanathan and Botelho, 2019).

The lumens of endolysosomal compartments harbor at least 60 different acid hydrolase enzymes with an optimal pH between 4 and 5, including proteases, nucleases, phosphatases, and lipases. The endolysosomal compartments also contain more than 100 highly glycosylated transmembrane proteins (Xu and Ren, 2015; de Araujo et al., 2020). LAMP1 and LAMP2 are the most abundant transmembrane proteins and make up about 50% of the protein content in the endolysosomal compartment (Luzio et al., 2014). The highly glycosylated transmembrane proteins protect themselves and the lipid membrane from degradation by intraluminal acidic hydrolases. As described in detail in several recent reviews (Condon and Sabatini, 2019; Lawrence and Zoncu, 2019; Ballabio and Bonifacino, 2020), the microphthalmia-associated transcription factor (MiTF)/transcription factor (TF) family, such as transcription factor EB (TFEB), regulates the expression of most endolysosomal proteins. Phosphorylation of TFEB by mammalian target of rapamycin complex 1 (mTORC1) retains TFEB on the endolysosomal outer membranes. To respond to an increase in endolysosomal degradation demand, TFEB is dephosphorylated and translocated to the nucleus to increase the transcriptions of hydrolases (e.g., cathepsins), transmembrane proteins such as LAMPs, and the V-ATPase proton pump complexes.

Cathepsins

Cathepsins are the most abundant cellular proteases found in the lumen of all three endolysosomal compartments (Yadati et al.,

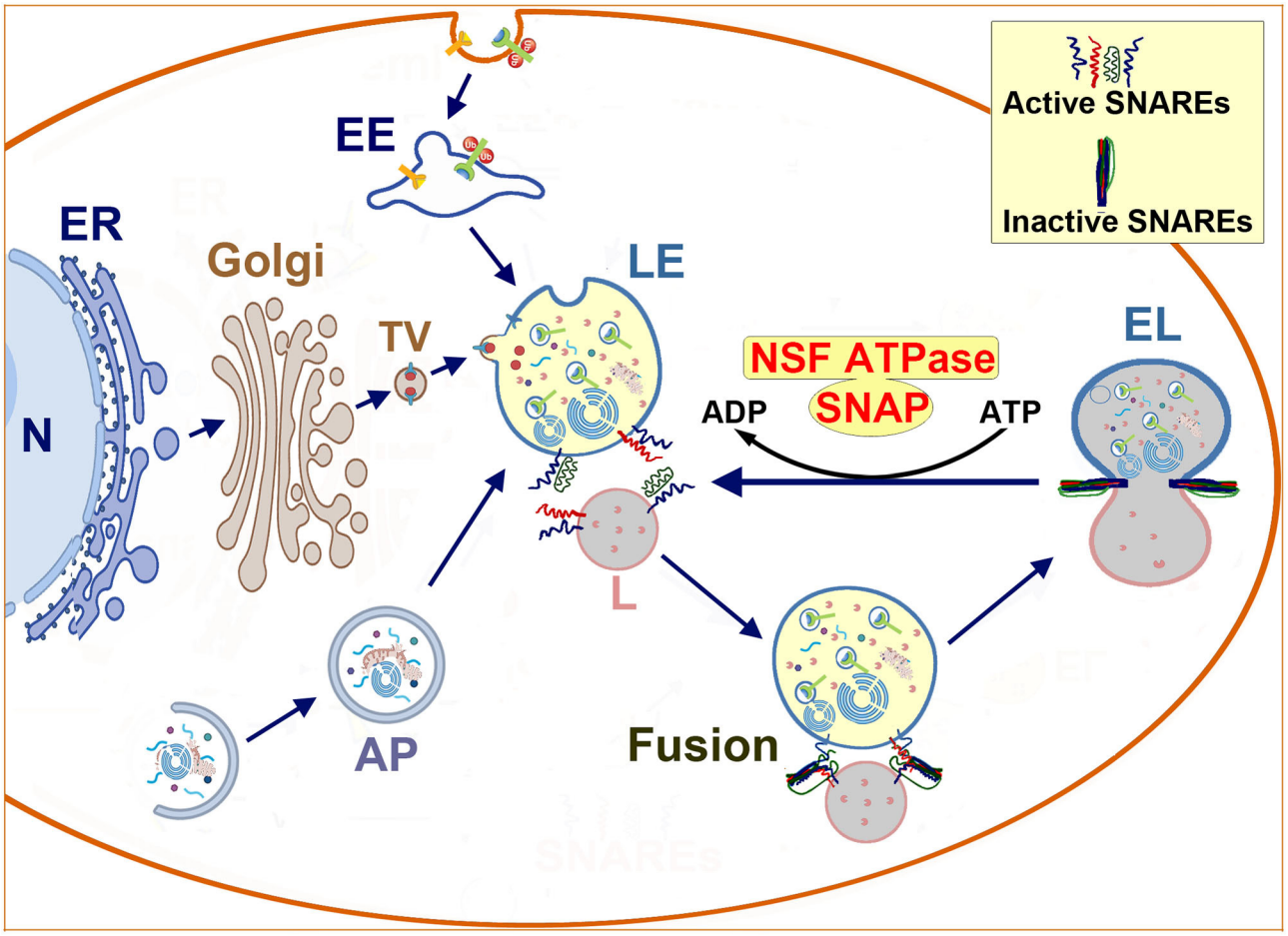

FIGURE 1 | Schematic diagram of endolysosomal trafficking: endolysosomal hydrolytic enzymes, membrane components, and structural proteins are synthesized on the ER-associated polyribosomes, and modified in the ER and the Golgi lumen. Next, they are transported via vesicles to the late endosome. The late endosome also receives incoming cargos from the endocytic and autophagic pathways. The enzyme- and cargo-loaded late endosome fuses with the terminal lysosome to form an endolysosome to digest cargos. Afterwards, the endolysosome reforms into a terminal lysosome for the next round of fusion. The membrane fusion is mediated by the NSF-SNAP-SNAREs machinery and assisted by other proteins such as HOPS complexes and Rab7. Interactions between SNAREs from two opposite lipid membranes bring two membranes together to execute the fusion. After fusion, SNAREs form inactive complexes that must be dissociated and reactivated by the cooperative action of NSF ATPase and SNAP. N, nucleus; ER, endoplasmic reticulum; Golgi, Golgi apparatus; TV, transport vesicle; EE, early endosome; AP, autophagosome; LE, late endosome; L, lysosome; NSF, N-ethylmaleimide Sensitive Factor (ATPase); SNAP, soluble NSF attachment protein; SNARE, SNAP receptor.

2020). They are classified into three families: serine proteases (A and G), aspartic proteases (D and E), and cysteine cathepsins (B, C, F, H, K, L, O, S, V, X, and W) (Yadati et al., 2020). With a few exceptions, cathepsins show the highest activity in the low pH environment of the endolysosomal lumen. Cathepsins B, L, and D are the most abundant in the endolysosomal compartments (Turk et al., 2000). Cathepsin B (CTSB) is the most abundant cathepsin in the brain (Petanceska et al., 1994). Most cysteine cathepsins exhibit a predominantly endopeptidase activity. Cathepsin B exhibits both exopeptidase and endopeptidase activities favorably at acidic and neutral/alkaline pH values, respectively (Turk et al., 2012). Cathepsin B retains protease activity at the neutral pH found in the extracellular milieu, particularly in the presence of large ECM proteins (Buck et al., 1992; Cavallo-Medved et al., 2011; Turk et al., 2012).

All cathepsins are synthesized as pre-proenzymes on ER-associated polyribosomes. Pre-procathepsins are composed of three segments: a signal peptide, a propeptide, and a mature enzyme. During synthesis, pre-procathepsins are imported into the ER lumen. Inside the ER lumen, pre-procathepsins are cleaved by a signal peptidase to become procathepsins. Procathepsins are then glycosylated with high levels of mannose within the ER before being transported to the Golgi apparatus. In the Golgi, the mannose residues are modified to become mannose-6-phosphate moieties (M6P). The M6P-tagged procathepsins are recognized and bound by M6P-receptors (M6PR), packed together into clathrin-coated vesicles, and transported to the late endosomes (Bright et al., 2016) (**Figure 1**). Once in the acidic environment of the late endosome, M6PRs release the M6P-procathepsins and then are transported back

to the trans-Golgi networks to be reused (Abazeed et al., 2005). Inside the acidic environment (pH 6.0) of the late endosome, the propeptides of procathepsins undergo autocleavage or are cleaved by other cathespins to become mature cathepsins. Cathepsin maturation continues even after the late endosome fuses with a terminal lysosome to become an endolysosome. In the endolysosome, the mature forms of cathepsins are further processed into double heavy chain forms and a light chain. The double heavy chain forms of cathepsins are proteolytically active (Repnik et al., 2013).

NSF, SNAP, and SNARE-Mediated Membrane Fusion

All eukaryotic cells employ membrane-bound compartments to shuttle macromolecules among organelles or to move them in and out of cells through a process known as membrane trafficking (Yuan et al., 2018a). **Figure 1** shows a schematic diagram summarizing the neuronal endolysosomal trafficking pathways and the membrane fusion between a late endosome and a terminal lysosome. The neuronal endolysosomal trafficking pathways include: (i) the delivery of macromolecules through the Golgi to the late endosome; (ii) the endocytic pathway; and (iii) the autophagic pathway. At the trafficking destination, the membrane-bound compartments must fuse to their target membrane for cargo assembly or delivery.

The membrane-to-membrane fusion or simply membrane fusion is the process by which two initially distinct lipid bilayers merge to become a single integrated structure. The membrane fusion is mediated by the cooperative action of several protein complexes and phosphoinositide lipids (Brocker et al., 2010). As shown in **Figure 1**, the core protein complex is comprised of: (i) N-ethyl-maleimide sensitive factor (NSF), (ii) soluble NSF attachment protein (SNAP), and (iii) soluble NSF attachment protein receptors (SNAREs) (Bombardier and Munson, 2015).

Soluble NSF attachment protein receptors are fusion proteins and can be divided into two categories: vesicle or v-SNAREs, and target or t-SNAREs. Interactions between v-SNAREs and t-SNAREs from two opposite lipid membranes form a trans-SNARE complex, bringing two membranes nearby for their phospholipid membranes to merge into a single organelle (**Figure 1**). After fusion, SNAREs form an inactive stable cis-complex and must be dissociated by NSF adenosine triphosphatase (ATPase) to become the individual active trans-conformations for the next round of membrane fusion (Baker and Hughson, 2016; Yoon and Munson, 2018). This reactivation requires SNAREs to connect the NSF ATPase mediated by an adaptor protein SNAP (Hong and Lev, 2014) (**Figure 1**).

N-ethylmaleimide sensitive factor is a homohexamer, with each subunit composed of an amino-terminal domain followed by two AAA+ (ATPases associated with various cellular activities) domains termed D1 and D2. The D2 domain has slow adenosine triphosphate (ATP)-hydrolytic activity to maintain the NSF hexameric structure. The D1 domain is thought to be the main engine that uses ATP hydrolysis for SNARE complex disassembly and reactivation (Yoon and Munson, 2018). There is only a single form of NSF in mammalian cells (Whiteheart et al., 1994). Therefore, deficiency in NSF brings membrane fusion activity to a halt (Wilson et al., 1992; Whiteheart et al., 1994; Mohtashami et al., 2001). In comparison, SNAREs are both abundant and redundant with more than 60 types in yeasts and mammalian cells (Whiteheart et al., 1994). Of the 38 SNAREs present in humans, 30 have been identified in the Golgi and endolysosomal systems (Wilson et al., 1992). The Golgi and endolysosomal compartments contain the highest concentration of NSF and SNAREs, whereas other subcellular organelles such as the ER recruits little NSF (Robinson et al., 1997; Dalal et al., 2004). This distribution suggests that the Golgi and endolysosomal compartments are among the most dynamic organelles with the highest membrane fusion activity (Dingjan et al., 2018).

N-ethylmaleimide sensitive factor-SNAP-SNARE machinery-mediated membrane fusion also requires assistance from several other categories of proteins (Sudhof, 2007). These proteins include coiled-coil tethering complexes, multisubunit tethering complexes, small Rab GTPases, and phosphoinositide lipids (Brocker et al., 2010). In most cases, tethering proteins on opposite membranes connect before v-SNARE and t-SNARE engagement (Pfeffer, 1999; Baker and Hughson, 2016). Examples of these proteins include early endosome antigen 1 (EEA1) which is a coiled-coil membrane tethering protein containing an FYVE domain. The EEA1 FYVE domain binds to phosphatidylinositol-3-phosphate [PtdIns(3)P] on early endosomal membranes (Gaullier et al., 1998). Rab5 is a small GTPase that interacts with EEA1 and is required for the early stages of endosomal membrane-related fusion (Haas et al., 2005). The homotypic fusion and vacuole protein sorting (HOPS) is a multisubunit tethering complex in which complex subunit interactions are required before SNARE-mediated membrane fusion between late endosomes and terminal lysosomes can occur. Rab7 is another small GTPase that interacts with Rab interacting lysosomal protein (RILP) and the HOPS complex during the late endosome and terminal lysosome fusion (Lin et al., 2014).

PART 2. NEURONAL ENDOLYSOSOMAL SYSTEM

While endolysosomal trafficking has been studied extensively in non-neuronal cells, recent advances have been made in understanding the role of endolysosomal trafficking in highly polarized neurons. Neurons have adapted the same endolysosomal trafficking pathways to accommodate their specific morphological complexity and activity requirements (Winckler et al., 2018). Most neurons require a constant firing of action potentials and a fast turnover of membrane proteins (Harris et al., 2012). The neuronal endolysosomal compartments require extremely high degradative capacities for processing the waste from numerous distal nerve terminals and dendritic branches. These features underscore the need for highly efficient endolysosomal trafficking activities and intricate waste transport systems (Boland et al., 2008; Cai et al., 2010; Lee et al., 2011; Cheng et al., 2015; Gowrishankar et al., 2015; Maday and Holzbaur, 2016; Tammineni and Cai, 2017; Yap et al., 2018; Yuan et al., 2018a).

The extraordinarily high demand of endolysosomal trafficking activities in neurons relative to other cellular systems is supported by emerging gene knockout studies. Knockout of key ATG proteins, ATG5 and ATG7, primarily induces neuronal death (Hara et al., 2006; Komatsu et al., 2006). Rab7 knockout in mice causes an explicit buildup of neuronal late endosomes or autophagosomes, leading to neurodegeneration (Hyttinen et al., 2013).

Sophisticated neuronal waste transport systems are required to cope with the long-distance trafficking from distal synaptic connections to the soma region where the endolysosomal degradation compartments are located (Maday et al., 2012; Cheng et al., 2015; Maday and Holzbaur, 2016; Giovedi et al., 2020). Treatment with bafilomycin A1, which targets the V-ATPase, blocks the acidification of the endolysosomal compartments, resulting in the accumulation of autophagosomes exclusively within the soma region. This result supports the concept that the neuronal soma region is the primary site of cargo degradation (Maday and Holzbaur, 2016). As demonstrated below, brain ischemia also leads to a buildup of autophagosomes and endosomes within the soma region, but not in the neuropil area where distal neuronal processes and synapses are distributed (see **Figures 3–6**).

A dysfunctional endolysosomal system has been observed across a broad spectrum of neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (Winckler et al., 2018; Lie and Nixon, 2019; Giovedi et al., 2020; Navarro-Romero et al., 2020; Qureshi et al., 2020). Lysosomal storage disorders (LSDs) favorably affect neurons as well (Navarro-Romero et al., 2020; Qureshi et al., 2020; Song et al., 2020). Recent studies also show that stroke brain injury results from damage to endolysosomal trafficking (Yuan et al., 2018a). These studies suggest that dysfunction of endolysosomal trafficking may play a key role in most, if not all, neuronal death processes, although the mechanism underlying the deficiencies varies among neurological disorders.

PART 3. INTERRUPTION OF ENDOLYSOSOMAL TRAFFICKING LEADS TO STROKE BRAIN INJURY

Alteplase (tPA) and endovascular treatment may improve clinical outcomes. However, only 5–10% of stroke patients are eligible for these reperfusion therapies. Furthermore, these reperfusion therapies rely on removing vascular blockage and can inadvertently lead to an ischemia-reperfusion injury. At present, there exist no therapies directed toward brain ischemia-reperfusion injuries.

Smith et al. (1984) found that 10 min of global brain ischemia in a rat model led to selective and delayed neuronal death in some populations of hippocampal, striatal, and neocortical neurons at 2–3 days after reperfusion. Compared to the brief episode of global brain ischemia (prolonged) focal brain ischemia (stroke) with or without reperfusion leads to a well-described progression from early infarction in the striatum to delayed infarction in the dorsolateral cortex overlying the striatum (Barber et al., 2004; Carmichael, 2005). About 80% of neurons die in the non-perfused core with complete ischemia within 4 h, whereas about 40% of neurons gradually die in the hypoperfused penumbral region over a period of 4–24 h after focal brain ischemia (Fifield and Vanderluit, 2020). The breakdown of the blood brain barrier, microglia activation, monocyte/neutrophil infiltration, and astrogliosis were just observed until 24 h after focal brain ischemia (Fifield and Vanderluit, 2020). In general, animals cannot survive a global brain ischemic episode lasting longer than 25–30 min. A brief episode of global brain ischemia is mostly used to model the brain ischemia after a transient cardiac arrest in humans (Yuan et al., 2018a). Alternatively, animals can survive prolonged focal brain ischemia with or without reperfusion. Focal brain ischemia in animals is exclusively used to model an ischemic stroke in humans (Hu et al., 2001).

Deficiency in the endolysosomal system is observed virtually in all neurodegenerative disorders and in a significant number of LSDs (Wang et al., 2018; Winckler et al., 2018; Lie and Nixon, 2019; de Araujo et al., 2020; Giovedi et al., 2020; Navarro-Romero et al., 2020; Qureshi et al., 2020). Recent studies show that interruption of endolysosomal trafficking also leads to post-stroke brain ischemia-reperfusion injury. The interruption of endolysosomal trafficking in post-stroke neurons leads to: (i) the complete fragmentation of the Golgi apparatus; (ii) a massive buildup of enlarged endolysosomal compartments filled with undigested material; (iii) a massive increase in autophagosomes due to the blockade of autophagic flux; (iv) a massive accumulation of enlarged early endosomal structures due to the blockade of the late endosomal fusion; and (v) a significant buildup of protein aggregates (Hu et al., 2000b, 2001; Yuan et al., 2018a,b).

Figure 2 shows that, under TEM, complete fragmentation of the Golgi apparatus and a massive accumulation of protein aggregates and endolysosomal structures are observed in these post-ischemic neurons. These protein aggregates and endolysosomal structures become the foremost intra-neuronal structures after both global (Hu et al., 2000b; Liu and Hu, 2004) and focal brain ischemia (Hu et al., 2001; Zhang et al., 2006) (**Figure 2**).

Inactivation of NSF Leads to Interruption of Neuronal Endolysosomal Trafficking After Brain Ischemia

As described in Part 1 of this review, the NSF-SNAP-SNARE machinery is the prerequisite for membrane fusion (see **Figure 1**). Interactions between v-SNAREs and t-SNAREs from two opposite lipid membranes execute membrane fusion (Yoon and Munson, 2018). After fusion, SNAREs form inactive stable cis-complexes, which must be dissociated and then reactivated by NSF for the next round of membrane fusion (Baker and Hughson, 2016; Yoon and Munson, 2018). There is only a single form of NSF in mammalian cells (Whiteheart et al., 1994). Therefore, deficiency in NSF brings membrane fusion activities to a halt (Wilson et al., 1992; Whiteheart et al., 1994; Mohtashami et al., 2001; Yuan et al., 2018a,b). This halt on membrane fusion

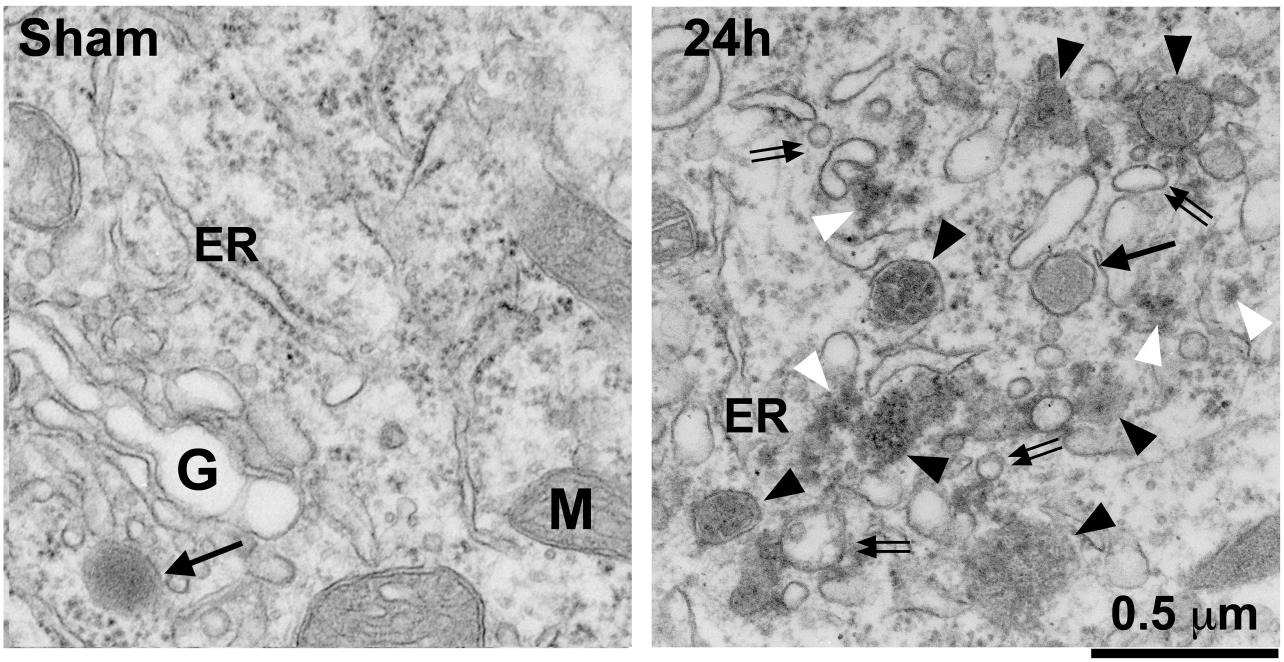

FIGURE 2 | Electron microscopy of Golgi fragmentation and buildup of endolysosomal structures and protein aggregates after stroke. The ultrathin neocortical sections were obtained from a sham-operated control rat and a rat subjected to 2 h of MCAO followed by 24 h of reperfusion. ***Sham:*** A sham neuron showing normal rough endoplasmic reticulum (ER), mitochondria (M), Golgi apparatus (G), and lysosome (arrow). ***24 h*:** A 24 h reperfused neuron showing complete fragmentation of the Golgi apparatus into vesicles or vacuoles (double arrows) and buildup of various sized late endosomes (black arrowheads) and protein aggregates (white arrowheads). Arrow points to a terminal lysosome. Scale bar = 0.5 μm.

activity handicaps the endolysosomal trafficking system from performing its job leading to neuronal dysfunction and injury.

There are a few *in vivo* studies referencing the NSF-SNAP-SNARE machinery after brain ischemia. Most of these studies are reported by our group. Liu et al. (Liu and Hu, 2004) found that functional NSF was depleted because it was deposited into inactive Triton X100-insoluble protein aggregates as early as after 30 min of reperfusion in the hippocampal CA1 neurons. This persisted until neuronal death occurred at 2–3 days of reperfusion following the initial ischemic episode. In comparison, NSF remained active in the dentate gyrus neurons that survived the same ischemic episode. The inactive deposition of NSF was accompanied by complete fragmentation of the Golgi stacks and accumulation of large quantities of intracellular vesicular structures (mainly due to Golgi fragmentation) in hippocampal CA1 neurons. This study showed that permanent depletion of active NSF likely led to delayed neuronal death in CA1 neurons even after just a brief episode of global brain ischemia.

Yuan et al. (2018b) found that 20 min of global brain ischemia led to the selective and complete inactivation of NSF ATPase in post-ischemic neurons. They discovered that NSF inactivation leads to a massive accumulation of Golgi fragments, intracellular vesicles, and cargo-laden late endosomes in affected post-ischemic neurons. They also found that the presence of 33 kDa CTSB was significantly increased in the cytosolic fraction likely released from late endosomes, and these neurons with increased cytosolic CTSB underwent delayed neuronal death. However, CTSB release was mostly limited inside neurons, suggesting that the release was likely on a microscale level. They suggested that the microscale release of cathepsins might not cause catastrophic destruction of neuronal structures or tissue infarction indiscriminately; instead, they might specifically activate the truncated Bax-like BH3 protein (tBid) and BCL2-associated X protein (Bax) pathway. As a result, neuronal mitochondrial outer membrane permeabilization (MOMP) occurs, leading to delayed neuronal death after a brief episode of global brain ischemia (Yuan et al., 2018a).

Recent work from our laboratory showed that (prolonged) focal brain ischemia caused a large-scale release of CTSB that damaged all neuronal structures indiscriminately. As a result, the post-ischemic neurons rupture, releasing their contents which include CTSB, ubiquitinated protein aggregates, and p62-protein aggregates into the extracellular space.

Yuan et al. (2021) showed that NSF was irreversibly depleted or inactively deposited in virtually all penumbral neurons after 2 h of middle cerebral artery occlusion (MCAO) in the rat model. **Figure 3** shows that NSF was irreversibly depleted from virtually all penumbral neurons after 2 h of MCAO in the rat model. Brain sections of 2 h of MCAO followed by 24 h of reperfusion were double-immunolabeled with mouse anti-CTSB (red) and rabbit anti-NSF (green) antibodies (**Figure 3**). Both CTSB and NSF immunostainings were predominantly distributed in neurons (**Figure 3**, arrows). In the non-ischemic

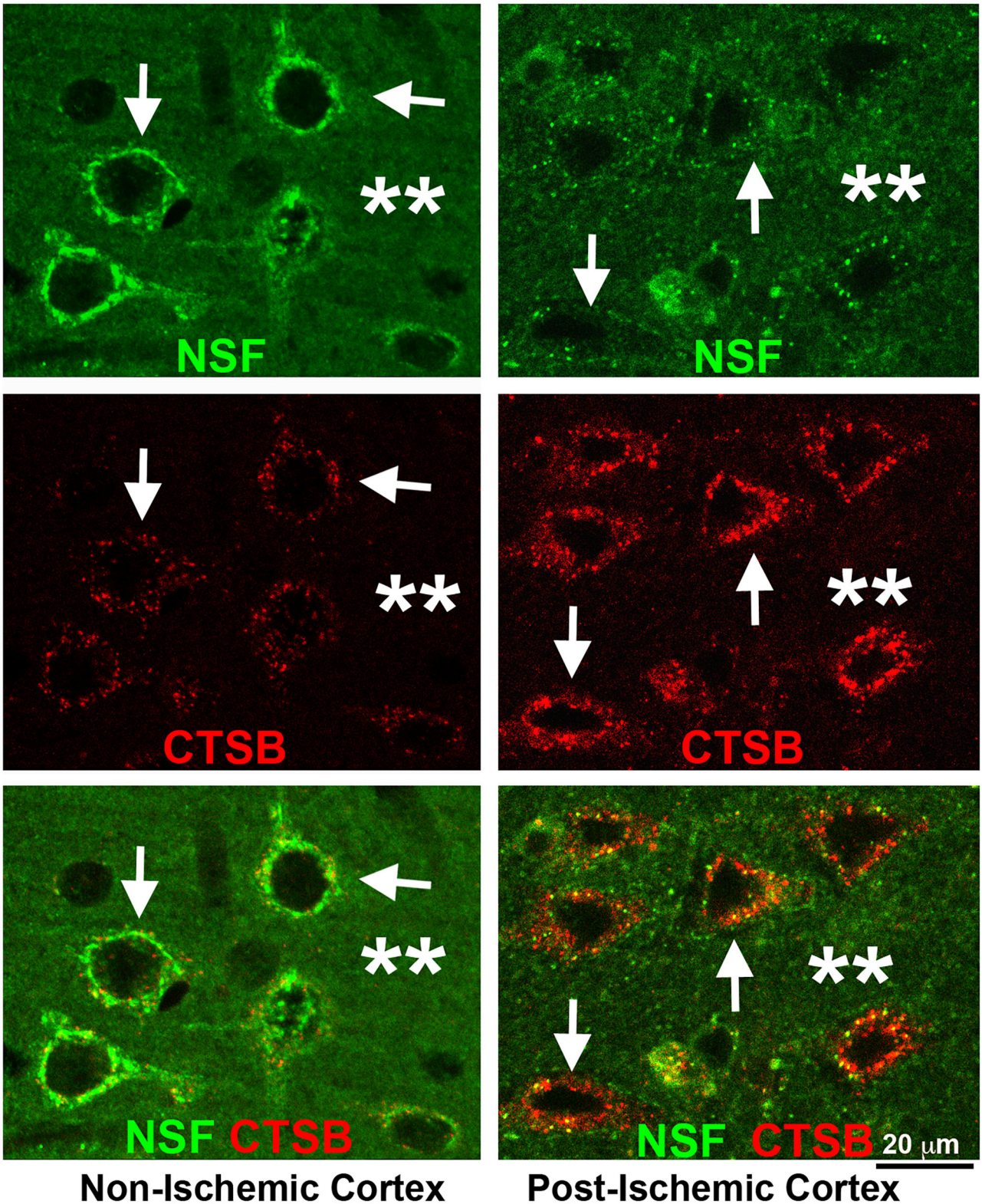

FIGURE 3 | Confocal imaging of neuronal NSF depletion and CTSB buildup after stroke. Brain section obtained from a rat subjected to 2 h of MCAO followed by 24 h of reperfusion, double-labeled with NSF (green) and CTSB (red) antibodies, and examined by confocal microscopy. Left panels display the non-ischemic contralateral neocortex; Right panels show the post-ischemic ipsilateral neocortex. **Upper row:** NSF immunolabeling; **Middle row:** CTSB immunolabeling; **Lower row:** NSF (green) and CTSB (red) double-immunolabeling. Arrows point to neuronal soma; and stars denote the immunostaining outside neuronal soma.

contralateral cortex, anti-NSF immunoreactivity was distributed in the perinuclear region and apical dendritic trunk (**Figure 3**, Non-Ischemic Cortex, upper left, NSF, green, arrows), as well as in the neuropil region (**Figure 3**, Non-Ischemic Cortex, upper left, NSF, green, stars). Anti-CTSB antibody labeled the endolysosomal structures as small dots in the same perinuclear region and apical dendritic trunk (**Figure 3**, middle left, CTSB, red, arrows), but CTSB immunolabeling was virtually absent in the neuropil or extracellular space between neuronal soma of the non-ischemic contralateral cortex (**Figure 3**, middle left, CTSB, red, stars). Furthermore, there was little colocalization between NSF (green) and CTSB (red) immunoreactivities in the non-ischemic contralateral neocortical neurons (**Figure 3**, lower left, green vs. red, arrows). In contrast, in the post-ischemic ipsilateral cortex, NSF immunostaining was mostly depleted from inside the neuronal soma and dendritic trunks, only leaving a few NSF-immunopositive small dots in the perinuclear area (**Figure 3**, Post-ischemic cortex, upper right, NSF, green, arrows). In the same post-MCAO penumbral neurons, the size, intensity, and number of CTSB-immunostained endolysosomal structures were drastically increased (**Figure 3**, Post-ischemic cortex, middle right, CTSB, red, arrows). Cathepsin B-immunostaining was also distributed outside neuronal soma (**Figure 3**, Post-ischemic cortex, middle right, CTSB, red, stars), indicating that some damaged neurons had ruptured and released their contents including CTSB into the extracellular space. Furthermore, NSF (green) and CTSB (red) appeared partially colocalized in the perinuclear region of the penumbral neurons (**Figure 3**, lower right, yellow, small arrows). These results suggest that NSF ATPase was irreversibly depleted virtually from all penumbral neurons, resulting in a massive buildup of CTSB-containing endolysosomal structures. Penumbral neurons then rupture and release their contents into the extracellular space, causing catastrophic destruction of neuronal structures indiscriminately.

Mechanism Underlying Post-Ischemic NSF Inactivation

As described above, the depletion or disappearance of intra-neuronal NSF immunoreactivity was mainly due to NSF deposition into a detergent (Triton X100)/salt-insoluble aggregates after both global and focal brain ischemia (Liu and Hu, 2004; Yuan et al., 2018b, 2021; Liu et al., 2021). These NSF aggregates are such dense structures that they were not accessible by the NSF antibody during the immunostaining of brain sections (Hu et al., 2000b; Liu and Hu, 2004; Zhang et al., 2006; Yuan et al., 2018b, 2021).

N-ethylmaleimide sensitive factor (ATPase) requires binding to ATP or non-hydrolysable ATP analogs to maintain its active soluble symmetrical homohexamer conformation (Moeller et al., 2012). All previous studies of NSF activity have consistently shown that the cytosolic free NSF is in its active form, whereas NSF deposition into Triton X100-insoluble aggregates loses its ATPase activity (Mohtashami et al., 2001; Sanyal and Krishnan, 2001). Cellular NSF forms a symmetrical homohexamer that undergoes drastic conformational change from an ATP- to a non-ATP-binding state (Yu et al., 1998; Moeller et al., 2012). N-ethylmaleimide sensitive factor requires ATP for stability (Moeller et al., 2012; Morgan and Burgoyne, 2015). Without ATP as a cofactor, NSF undergoes inactive aggregation in nucleotide-free solutions (Block et al., 1988; Whiteheart et al., 1992; Moeller et al., 2012; Morgan and Burgoyne, 2015). The underlying mechanism for NSF inactive deposition is likely due to an NSF ATPase conformational change from a compact cylindrical structure to an "aggregation-prone" open structure under ATP depleted conditions (Hanson et al., 1997; Yu et al., 1998; Moeller et al., 2012). Adenosine triphosphate is depleted within a couple of minutes following brain ischemia (Siesjo et al., 1999). Therefore, ATP depletion after brain ischemia leads to the inactive deposition of NSF hexamer ATPase into Triton X100-insoluble aggregates as described above (Hu et al., 2000b; Liu and Hu, 2004; Zhang et al., 2006; Yuan et al., 2018b, 2021). N-ethylmaleimide sensitive factor also formed inactive aggregates due to a conformational change in tissue samples from mice with genetically induced leucine-rich repeat kinase 2 (LRRK2)-deficiency in a Parkinson's disease mouse model (Pischedda et al., 2021). N-ethylmaleimide sensitive factor is persistently deposited in the Triton X100-insoluble aggregate form until neuronal death occurs during the post-ischemic phase, suggesting that post-ischemic NSF aggregation is an irreversible process (Yuan et al., 2018b, 2021).

Complete Fragmentation of Golgi Complex After Focal Brain Ischemia

The Golgi apparatus, also known as the Golgi complex or simply the Golgi, processes and packages proteins into membrane-bound transport vesicles destined for the secretory and endocytic pathways. The Golgi is organized into the first cisterna called cis-Golgi network, medial cisternae, and the final cisterna known as the trans-Golgi network. The cis-Golgi network receives incoming proteins from transport vesicles derived from the ER. The proteins are further modified in the cis-Golgi network, middle cisternae, and trans-Golgi network. These proteins are then packed into transport vesicles at the trans-Golgi network and relayed to their final destinations.

The fragmentation of the Golgi stacks and the buildup of membranous whorls were first observed under EM more than three decades ago from brain tissue after a brief episode of global brain ischemia (Petito and Pulsinelli, 1984; Rafols et al., 1995; Hu et al., 2000a). We now know that these cytoplasmic membranous whorls likely represent the significant buildup of cargo-laden endolysosomal structures (Hall et al., 1997; Hu et al., 2000a, 2001; Yuan et al., 2018a). Despite this knowledge, only a few direct studies focus on the Golgi's morphological and functional characteristics after focal brain ischemia in animal stroke models. A recent study from our laboratory demonstrated that the Golgi apparatus was severely disintegrated and eventually dissolved in post-ischemic neurons destined for death after 2 h of MCAO in a rat model (Yuan et al., 2021).

Figure 4 shows that the Golgi networks disintegrated into small dots and dissolved into diffuse immunostainings while the cytoplasmic endolysosomal structures markedly accumulate

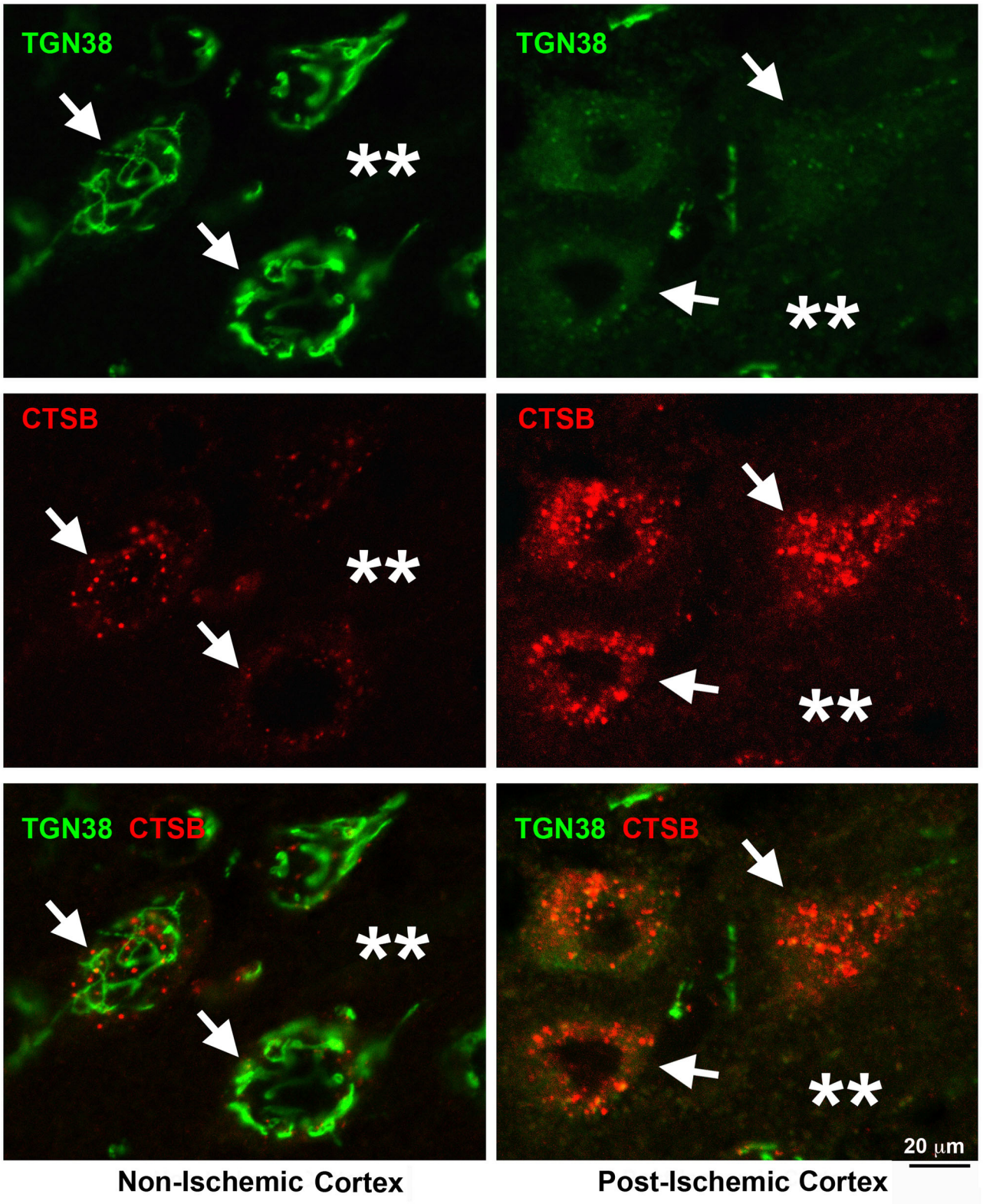

FIGURE 4 | Confocal imaging of neuronal Golgi fragmentation after stroke. Brain section obtained from a rat subjected to 2 h of MCAO followed by 24 h of reperfusion, double-labeled with TGN38 (green) and CTSB (red) antibodies, and examined by confocal microscopy. Left panels display the contralateral non-ischemic cortex; Right panels show the ipsilateral post-ischemic cortex. **Upper row**: TGN38 immunolabeling; **Middle row**: CTSB immunolabeling; **Lower row:** TGN38 (green) and CTSB (red) double-immunolabeling. Arrows point to neuronal soma; and stars denote the immunostaining outside neuronal soma.

in penumbral neurons after MCAO. Brain sections from a rat subjected to 2 h of MCAO followed by 24 h of reperfusion were double-immunolabeled with TGN protein 38 (TGN38, a Golgi marker) and CTSB antibodies (**Figure 4**). In non-ischemic contralateral neocortical neurons, TGN38 immunoreactivity was distributed as twisted tubular networks (**Figure 4**, Non-ischemic cortex, upper left, TGN38, green, arrows) while CTSB immunoreactivity showed as tiny dots in the perinuclear region and apical dendrites (**Figure 4**, Non-ischemic cortex, middle left, CTSB, red, arrows). There was little overlap between TGN38 and CTSB (**Figure 4**, Non-ischemic cortex, lower left, green vs. red, arrows). In contrast, TGN38 disintegrates at 24 h of reperfusion following 2 h of MCAO (**Figure 4**, Post-ischemic cortex, upper right, TGN38, green, arrows), while CTSB increased in size, intensity, and number (**Figure 4**, Post-ischemic cortex, middle right, CTSB, red, arrows). Furthermore, TGN38 appeared to be overlapping with the CTSB structures in post-ischemic neurons (**Figure 4**, Post-ischemic cortex, lower right, arrows). Post-ischemic neurons became polygonal in shape and were surrounded with higher extracellular levels of TGN38 and CTSB immunoreactivities (**Figure 4**, Post-ischemic cortex, lower right, stars), suggesting that some penumbral neurons ruptured, releasing their contents into the extracellular space.

Evidence suggests that the Golgi cisterns irreversibly disintegrate and eventually dissolve prior to penumbral neurons' rupture after 2 h of focal brain ischemia. As a result, the transport of hydrolytic enzymes and structural proteins from the trans-Golgi network to the late endosome is impeded, retaining CTSB in the fragmented Golgi and contributing to the endolysosomal deficiency. Furthermore, the Golgi structural damage releases toxic CTSB into the cytoplasm and extracellular space, increasing stroke brain injury.

Interruption of Autophagic Flux After Brain Ischemia

Autophagy is a major degradation pathway responsible for removing abnormal protein aggregates and damaged organelles (Liu et al., 2010). Increases in autophagic components, such as LC3-II, beclin-1, TFEB, and aggregated p62/SQSTM1 were consistently observed in neurons of various brain ischemia and hypoxia animal models (Nitatori et al., 1995; Adhami et al., 2007; Carloni et al., 2008; Rami et al., 2008; Wen et al., 2008; Liu et al., 2010, 2018).

As mentioned above, the most common methods for measuring the autophagic cargo degradation rate or autophagic flux employ a combination of techniques including Western blotting and confocal or fluorescence microscopic analysis. These methods measure the levels and immunolabeling patterns of autophagosome-associated proteins, such as LC3-II and autophagy receptors (e.g., p62/SQSTM1). The increased levels of LC3-II and p62/SQSTM1 often indicate that autophagic flux is disrupted or autophagic degradation activity is reduced (Runwal et al., 2019). Autophagic flux can also be measured by EM (Liu et al., 2010; du Toit et al., 2018). Electron microscopy has been considered to be the gold standard in many autophagy research applications because it has the advantage of allowing a direct assessment of autophagosomes in the specimen (Liu et al., 2010; du Toit et al., 2018). Electron microscopy manifestation of the accumulation of mature autophagosomes located in close proximity to enlarged endolysosomal compartments often indicates that the autophagic flux is interrupted, mostly owing to the deficiency in the fusion and degradation steps of the autophagic pathway (Abada et al., 2017). Although EM has an advantage in viewing endolysosomal structures, relative to Western blotting and confocal microscopy, EM methods require multiple steps of sample preparation and specific EM equipment (Liu et al., 2010).

Presently, there are two opposing hypotheses in literature stating that post-ischemic autophagy either increases or decreases brain ischemia reperfusion injury. However, there is no unified theory to reconcile these two opposing hypotheses. In the autophagic pathway, autophagosomes must fuse with endolysosomal compartments for degradation of the autophagic cargos. This fusion is mediated by the NSF-SNAP-SNARE machinery (Abada et al., 2017). Therefore, depletion of NSF in post-ischemic neurons predictably leads to the blockade of autophagic flux at the fusion step between autophagosomes and the endolysosomal compartments.

This NSF-depletion hypothesis is supported by recent work from our laboratory. **Figure 5** shows the massive buildup of p62/SQSTM1 immunostained structures in post-ischemic neurons and the nearby extracellular space after focal brain ischemia. Brain sections from a rat subjected to 2 h of MCAO followed by 24 h of reperfusion were double-immunolabeled with p62/SQSTM1 and CTSB antibodies. p62/SQSTM1 immunoreactivity was evenly distributed and occasionally seen as small dots in non-ischemic contralateral neocortical neurons (**Figure 5**, Non-ischemic cortex, upper left, p62, green, arrows), while CTSB immunolabeled as small dots and was primarily distributed in the perinuclear and apical dendritic regions (**Figure 5**, Non-ischemic cortex, middle left, CTSB, red, arrows). There was little immunostained CTSB in the neuropil and extracellular space (**Figure 5**, Non-ischemic cortex, middle left, CTSB, red, stars) and virtually no overlap between p62/SQSTM1 and CTSB immunoreactivities (**Figure 5**, Non-ischemic cortex, lower left, green vs. red, arrows). In contrast, in the post-ischemic ipsilateral penumbral neocortical neurons, both p62/SQSTM1 (**Figure 5**, Post-ischemic cortex, upper right, p62, green, arrows) and CTSB (**Figure 5**, Post-ischemic cortex, middle right, CTSB, red, arrows) immunoreactivities were markedly upregulated in size, number, and intensity. Furthermore, both p62/SQSTM1 and CTSB immunoreactivities appeared also in the neuropil and extracellular space (**Figure 5**, Post-ischemic cortex, upper and middle right, double-arrows, and stars). There remained little colocalization between p62/SQSTM1 and CTSB both inside and outside of post-ischemic penumbral neuronal soma (**Figure 5**, lower right, p62 and CTSB, green vs. red, arrows, double-arrows, and stars). These results strongly suggest that the accumulation of p62/SQSTM1-immunostained autophagosomes was located in close proximity to the CTSB-positive endolysosomal compartments but that they were not fused together after focal brain ischemia. Evidence demonstrates that the autophagic flux is interrupted because of deficient fusion between autophagosomes and endolysosomal compartments owing to NSF depletion in post-ischemic neurons.

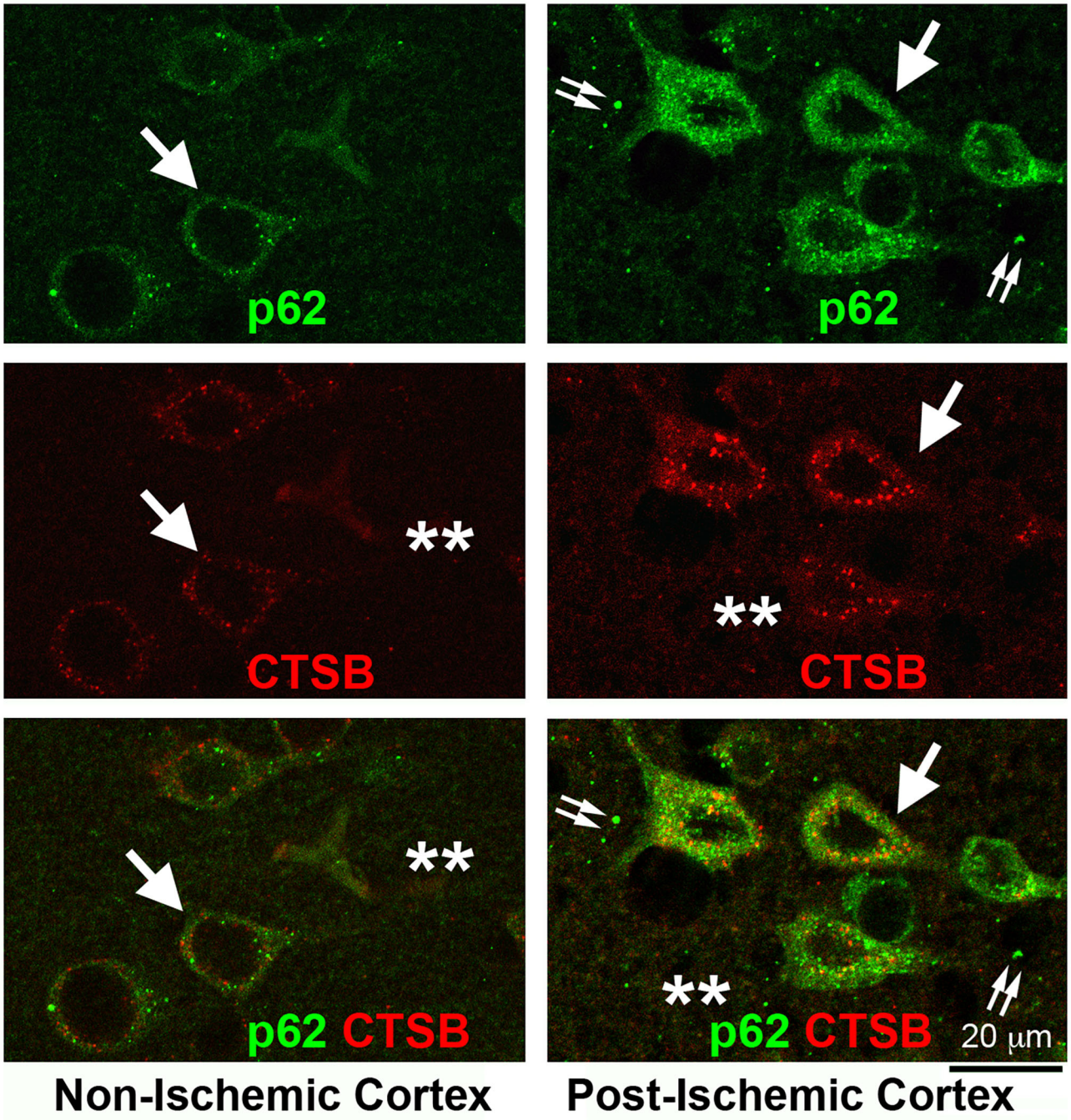

FIGURE 5 | Confocal imaging of neuronal buildup of p62/SQSTM1 and CTSB after stroke. Brain section obtained from a rat subjected to 2 h of MCAO followed by 24 h of reperfusion, double-labeled with p62/SQSTM1 (green) and CTSB (red) antibodies, and examined by confocal microscopy. Left panels display the contralateral non-ischemic cortex; Right panels show the ipsilateral post-ischemic cortex. **Upper row:** p62/SQSTM1 immunolabeling; **Middle row:** CTSB immunolabeling; **Lower row**: p62/SQSTM1 (green) and CTSB (red) double-immunolabeling. Arrows point to p62/SQSTM1 and CTSB immunolabeling in neuronal soma; double-arrows indicate the p62/SQSTM1 immunolabeling outside of neuronal soma; and stars denote the immunostaining outside of neuronal soma.

Buildup of EEA1 Endosomal Structures After Brain Ischemia

There are only a few studies of the endocytic pathway after brain ischemia. (Vaslin et al., 2009, 2011) showed that all tracers were taken up by endocytosis selectively into post-ischemic neurons destined for death in a rat focal brain ischemia model. The endocytosed tracers were colocalized with enhanced immunolabeling for EEA1 and clathrin in the same population of

penumbral neurons after focal brain ischemia. The results suggest a drastic endosomal structure buildup after focal brain ischemia (Vaslin et al., 2009). Huang et al. (2013) showed an accumulation of internalized aquaporin 4 (AQP4) in the increased EEA1-immunolabeled structures at 1 h and then accumulation in the LAMP1-immunolabeled structures at 3 h after focal brain ischemia in the rat model. This finding suggested that AQP4 was internalized and delivered from the EEA1-immunopositive early endosome to the LAMP1-immunopositive endolysosomal compartments after brain ischemia. These studies demonstrated a buildup of early (EEA1-immunopositive) and late (LAMP1-immunopositive) endosomal structures that occur in post-ischemic neurons destined for death after focal brain ischemia. However, similar to the increased autophagosomal structures, the increase in cargo-carrying endosomal structures may not only suggest boosted endocytic activity, but also more likely an indication of the reduced degradation of endocytosed structures in post-ischemic neurons.

Depletion of NSF blocks the fusion between the late endosomes and terminal lysosomes. As a result, the endosomal structures accumulate after focal brain ischemia. **Figure 6** shows that immunoreactivities of both EEA1 and CTSB were drastically increased in penumbral neurons after focal brain ischemia. Brain sections from a rat subjected to 2 h of MCAO followed by 24 h of reperfusion were double-immunolabeled with EEA1 and CTSB antibodies. In the non-ischemic contralateral neocortex, EEA1 immunoreactivity was distributed as small dots throughout the soma, dendritic trunks, as well as, to a significant lesser degree, the neuropil region (**Figure 6**, upper left, EEA1, green, arrow, and stars), while CTSB immunoreactivity was limited to only the perinuclear and apical dendritic regions (**Figure 6**, Non-Ischemic Cortex, middle left, CTSB, red, arrow). There were partial colocalizations between EEA1 and CTSB immunoreactivities in non-ischemic contralateral neocortical neurons (**Figure 6**, Non-Ischemic Cortex, lower left, green vs. red, arrow). In contrast, in post-ischemic ipsilateral penumbral neocortical neurons, both EEA1 (**Figure 6**, Post-Ischemic Cortex, upper right, EEA1, green, arrows) and CTSB (**Figure 6**, middle right, CTSB, red, arrows) immunoreactivities were markedly increased in size, number, and intensity in the perinuclear region. The EEA1 and CTSB immunoreactivities were mostly colocalized in the perinuclear region of post-ischemic penumbral neurons (**Figure 6**, Post-Ischemic Cortex, lower right, EEA1, and CTSB, green vs. red, arrows). The EEA1 and CTSB immunoreactivities were also found in the extracellular space (**Figure 6**, right panels, stars). These results suggest that increased EEA1-immunopositive early endosomes matured to become the CTSB-immunopositive late endosomes but were not able to fuse with terminal lysosomes owing to NSF depletion in post-ischemic neurons.

NSF Inactivation Is Responsible for the Golgi, Endosomal, and Autophagic Defects

The following evidence supports the hypothesis that NSF inactivation is responsible for the Golgi, endosomal, and autophagic defects observed after brain ischemia. N-ethylmaleimide sensitive factor was originally identified by Rothman's laboratory based on an assay of Golgi fragmentation in a cell free system (Block et al., 1988). Treatment of the cell free system with alkylating agent N-ethylmaleimide (NEM) leads to Golgi fragmentation. The Golgi fragmentation can then be rescued by adding back an appropriately prepared cytosol fraction. The protein in the cytosol fraction responsible for the rescue was termed NEM-sensitive factor (NSF). For their discoveries of NSF-SNAP-SNARE machinery regulating vesicle traffic, the Nobel Prize in Physiology or Medicine 2013 was awarded jointly to James E. Rothman, Randy W. Schekman, and Thomas C. Südhof.

As shown in **Figures 1**, **7**, the LE-to-L fusion is NSF dependent (Mullock et al., 1998). Mutation of the first NSF ATP hydrolysis site (replacement of the aminoacid 329 glutamate with glutamine or E329Q mutation) leads to complete cellular NSF ATPase inactivation via a dominant-negative mechanism (Whiteheart et al., 1994). This dominant-negative inhibition is because the incorporation of a single E329Q NSF into an NSF ATPase hexamer completely eliminates NSF ATPase activity (Whiteheart et al., 1994). Expressing E329Q NSF in cell cultures leads to similar fragmentation of Golgi stacks into dispersed vesicular structures as those observed in post-ischemic neurons (Dalal et al., 2004; Yuan et al., 2018b). Expression of the E329Q NSF dominant-negative mutant in cell cultures also leads to similar NSF deposition as that seen in post-ischemic tissue samples into the two major systems: (i) the Golgi apparatus, and (ii) the endolysosomal system (that are co-immunolabeled with LAMP2) (Dalal et al., 2004). Meanwhile, other subcellular structures such as the ER are minimally affected by expression of E329Q NSF in cell cultures (Dalal et al., 2004). Therefore, the Golgi and endolysosomal systems are primarily damaged by expression of the E329Q NSF dominant-negative mutant (Dalal et al., 2004). These studies suggest that the Golgi and endolysosomal structures are the most dynamic membrane trafficking organelles containing the highest concentrations of NSF, SNAP, and SNARE proteins (Dalal et al., 2004). In comparison, other subcellular organelles such as ER membranes recruit little NSF despite the known ability of SNAREs to bind α-SNAP and NSF in these subcellular organelles (Dalal et al., 2004). These E329Q NSF dominant-negative mutant studies clearly indicate that the Golgi and endolysosomal systems depend on NSF-mediated membrane fusion to maintain their normal structures and functions, whereas other subcellular organelles have only a minimal need for the NSF-SNAP-SNARE machinery to support their normal structures (Dalal et al., 2004). This conclusion is further enforced by NSF knockdown studies. Knockdown of NSF in normal immortalized human kidney (HK2) cells primarily results in: (i) fragmentation of the Golgi apparatus; (ii) impairment of trafficking of lysosomal hydrolases to late endosomes; and (iii) a large scale buildup of EEA1-immunopositive early endosomes and Rab7-immunopositive late endosomes under confocal microscopy, as well as various endolysosomal structures containing whole organelles, membrane whorls, and electron-dense aggregates under EM (Lanning et al., 2018). Knockdown of NSF or α-SNAP also leads to a significant buildup of sealed autophagosomes located in close proximity to lysosomes but not fused with them (Abada et al., 2017; Lanning et al.,

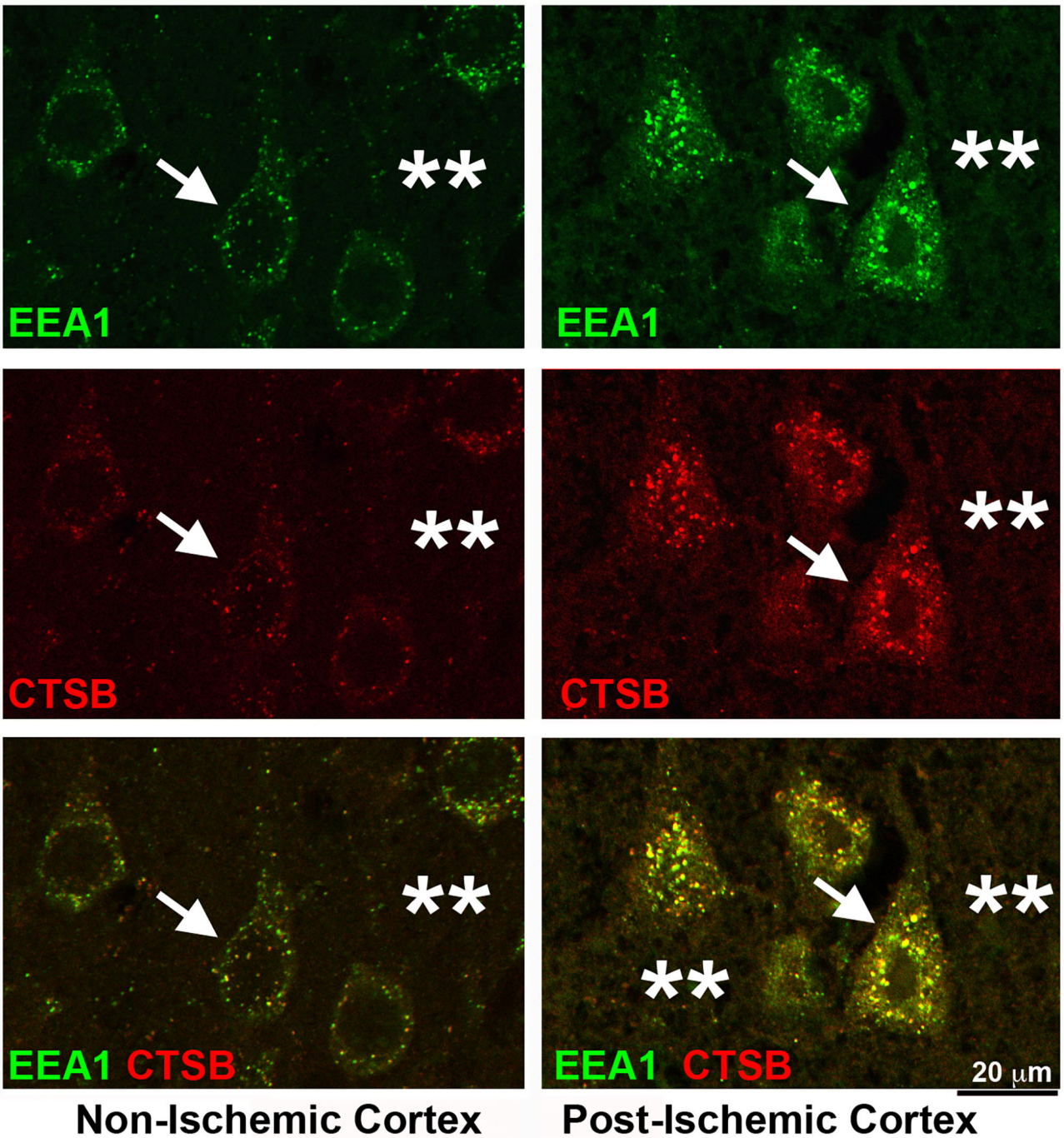

FIGURE 6 | Confocal imaging of neuronal buildup of EEA1 and CTSB after stroke. Brain section obtained from a rat subjected to 2 h of MCAO followed by 24 h of reperfusion, double-labeled with EEA1 (green) and CTSB (red) antibodies and examined by confocal microscopy. Left panels display the contralateral non-ischemic cortex; Right panels show the ipsilateral post-ischemic cortex. **Upper row**: EEA1 immunolabeling; **Middle row**: CTSB immunolabeling; **Lower row**: EEA1 (green) and CTSB (red) double-immunolabeling. Arrows point to neuronal soma; and stars denote the immunostaining outside neuronal soma.

2018). All of the NSF inactivation-induced changes (e.g., Golgi fragmentation and buildup of endolysosomal structures) observed in cell cultures can also be found in post-ischemic neurons (**Figures 2–5**).

Biochemical studies further showed that brain ischemia primarily led to deposition of Golgi and endolysosomal proteins into Triton-insoluble protein aggregates, including Golgi marker proteins (GM130, giantin, and GS28), endosomal

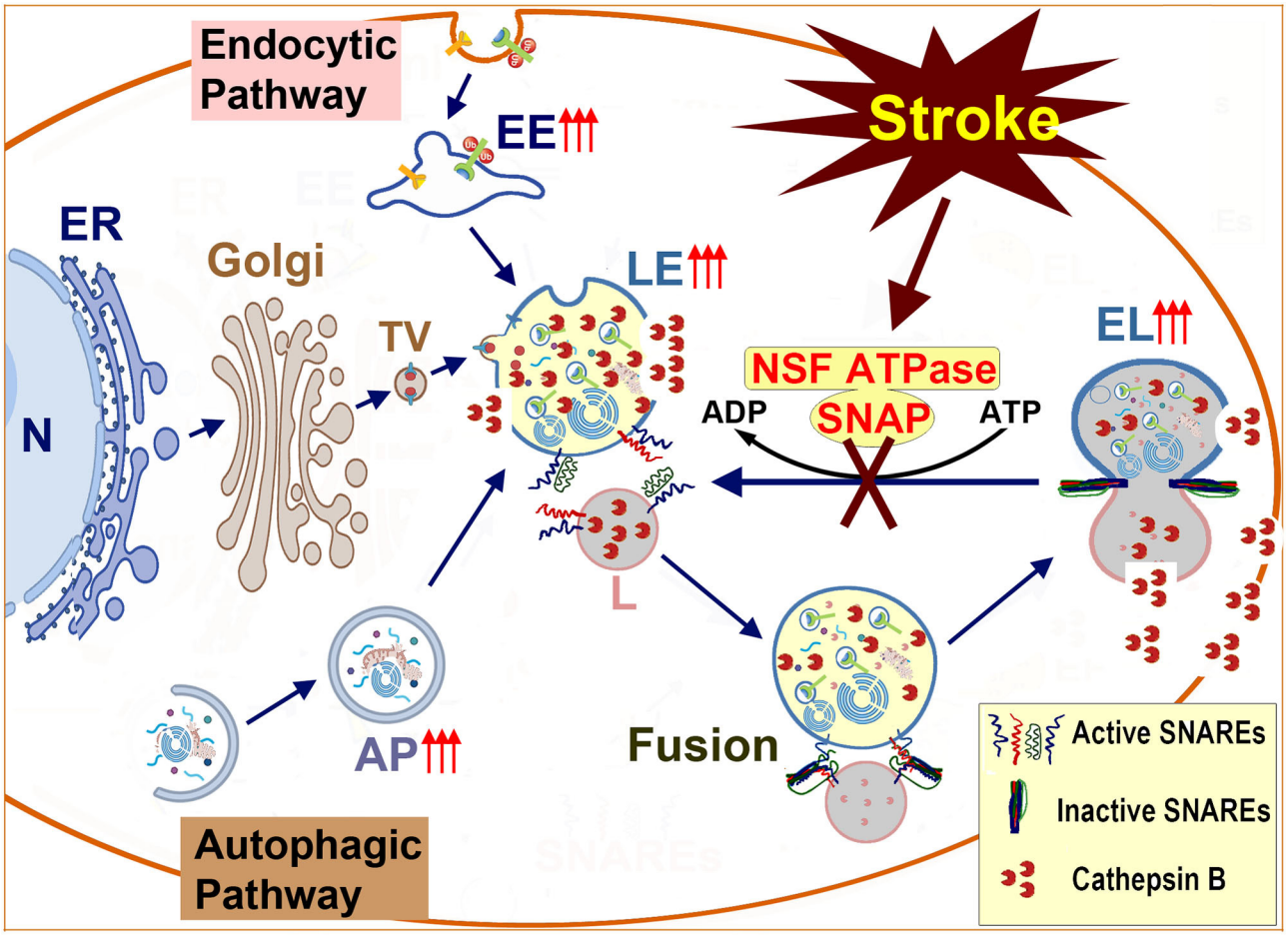

FIGURE 7 | Schematic diagram of endolysosomal trafficking after brain ischemia: endolysosomal hydrolytic enzymes, membrane components, and structural proteins are synthesized on the ER-associated polyribosomes, modified in the ER and the Golgi lumen, and transported to the late endosome. The late endosome also receives incoming cargos from the endocytic and autophagic pathways. The enzyme- and cargo-loaded late endosome fuses with the terminal lysosome to form an endolysosome to digest cargos. After, the endolysosome reform into a terminal lysosome for the next round of fusion. The membrane fusion is mediated by the NSF-SNAP-SNAREs machinery and assisted by other proteins such as HOPS complexes and Rab7. Brain ischemia inactivates NSF ATPase, prevents the regeneration of active SNAREs, and interrupts incoming traffic from both the autophagic and endocytic pathways. This interruption leads to a buildup of damaged LE and EL to release cathepsins such as CTSB and brain ischemia reperfusion injury. N, Nucleus; ER, Endoplasmic Reticulum; Golgi, Golgi apparatus; TV, Transport Vesicle; EE, Early Endosome; AP, Autophagosome; LE, Late Endosome; L, Lysosome; NSF, N-ethylmaleimide Sensitive Factor (ATPase); SNAP, Soluble NSF Attachment Protein; SNARE, SNAP Receptor.

marker proteins (syntaxin13 and EEA1), autophagic marker proteins (LC3II and p62), and endolysosomal marker proteins (LAMP1/3 and CTSB) (Liu et al., 2010; Yuan et al., 2018a). In comparison, membrane trafficking proteins unrelated to Golgi and endolysosome systems such as synaptic syntaxin 1 and synaptotagmin1, plasma membrane syntaxin 2, exocytic Rab3, and the ER p97 were unchanged after brain ischemia (Yuan et al., 2018a). Multiple lines of evidence support that NSF inactivation after brain ischemia leads to Golgi apparatus fragmentation and a large-scale buildup of early endosomes, autophagosomes, late endosomes, and endolysosomes (Hu et al., 2000b; Liu and Hu, 2004; Zhang et al., 2006; Yuan et al., 2018b, 2021) (**Figure 7**).

Release of Golgi and Endolysosomal Components Into the Extracellular Space After Brain Ischemia

The following observations show support that CTSB, as well as TGN38, p62, and ubiquitin may be released into the extracellular space: (i) CTSB, TGN38, p62, and ubiquitin immunoreactivities that were normally located inside sham-operated control neurons, were found abnormally distributed outsides of neuronal soma in the brain region where selective neuronal death occurs after brain ischemia (**Figures 2–5**) (Yuan et al., 2021); (ii) cellular contents and endolysosomal structures were found outside of ruptured neurons under EM (Yuan et al., 2021); and (iii) Western blot analysis showed that CTSB protein

was significantly increased in the 165,000 g supernatant fraction prepared from post-ischemic brain tissue, but was virtually absent in the same supernatant fraction prepared from sham-operated control brain tissue. This suggests that CTSB might leak out from the endolysosomal compartment into the cytosol or extracellular space (Yuan et al., 2018b, 2021).

Microscale vs. Large Scale Release of Cathepsins After Brain Ischemia

N-ethylmaleimide sensitive factor depletion leads to an ever-increasing buildup of endolysosomal compartments. A prolonged buildup of endolysosomal compartments leads to endolysosomal membrane damage or rupture, leading to the release of cathepsins (Yuan et al., 2018a). For most endolysosomal dysfunction-related diseases, the mechanisms underlying the endolysosomal damage remains unknown (Wang et al., 2018; de Araujo et al., 2020). Recent results from our laboratory demonstrated that the endolysosomal damage could be caused by the interruption of the fusion between late endosomes and terminal lysosomes and the interruption of the deliveries of lipids, hydrolytic enzymes, and structural proteins from the Golgi to the endolysosomal compartments. These interruptions were caused by NSF inactivation (Yuan et al., 2018a). For these reasons, the endolysosomal compartments were filled with undigested materials and may eventually rupture to release cathepsins after brain ischemia.

There are several studies describing the role of released cathepsins after brain ischemia. Nearly all studies focused on CTSB, while only a couple of studies discussed cathepsins D, E, H, and L. Cathepsin B is the most abundant cathepsin in the brain (Pungercar et al., 2009; Turk et al., 2012). Nitatori et al. (1995) showed that immunoreactivities of cathepsins B, H, and L increased in hippocampal CA1 pyramidal neurons at day three of reperfusion after a brief episode of global brain ischemia in a gerbil model. Nakanishi et al. (1993) showed that levels of cathepsins D and E were unchanged in both the hippocampal and neostriatal neurons before day three of reperfusion after a brief episode of forebrain ischemia. Kohda et al. (1996) showed that the enzymatic activity of CTSB increased while the enzymatic activity of cathepsin L decreased in the CA1 neurons at 24 h after a brief episode of global brain ischemia in a monkey model. Hill et al. (1997) showed that the distribution of CTSB changed from lysosomal to cytoplasmic after a brief episode of global brain ischemia. The studies mentioned above consistently demonstrated that both the levels and activities of CTSB were increased, while those of cathepsins D and E remained unchanged in the hippocampal CA1 neurons destined for death after a brief episode of global brain ischemia in animal models.

Seyfried et al. (1997) showed that increased CTSB immunoreactivity was detected exclusively in the penumbral neurons 2 h post-reperfusion following a 2 h MCAO in rats. Furthermore, continuous intraventricular infusion of stefin A, a weak CTSB inhibitor, before 2 h of MCAO significantly reduced infarct volume, relative to that of a vehicle-treated control group. The authors suggested that increased neuronal CTSB during 2 h of post-reperfusion contributed to neuronal cell death (Seyfried et al., 1997). Anagli et al. (2008) showed that CTSB activity increased predominantly in the ischemic hemisphere after MCAO in rats. Furthermore, post-ischemic treatment with cysteine protease inhibitor 1 (CP-1) reduced infarct volume, neurological deficits, and CTSB activity in the brain. Chaitanya and Babu (2008) showed that the CTSB levels significantly increased in post-ischemic neurons at 1 and 12 h post-reperfusion after MCAO in rats. Ni et al. (2015) showed that focal brain hypoxia caused extensive brain injury in neonatal wild-type mice, but not in CTSB knockout mice. Hossain et al. (2020) showed a time-dependent decrease in CTSD protein levels and activity in the mouse brain after MCAO. The aforementioned studies demonstrate that both the levels and activities of CTSB were upregulated, whereas the level of cathepsin D was decreased in the ischemic region after focal brain ischemia in animal models.

Evidence supports that a microscale amount of CTSB is released from mildly damaged endolysosomal compartments after a brief episode of global brain ischemia. This amount of CTSB release may be limited inside neurons to cause a non-rupture type of cell death after a brief episode of global brain ischemia (Wang et al., 2018; Yuan et al., 2018a). When compared to a brief episode of global brain ischemia (10–15 min), prolonged focal brain ischemia (e.g., 1–2 h) results in a more significant release of CTSB. Our recent work showed that CTSB was released on a large scale from substantially more damaged endolysosomal compartments into the cytoplasm and eventually into the extracellular space (see **Figures 3–6**). This large-scale CTSB release causes direct tissue destructive infarction (Alu et al., 2020).

In addition to the endolysosomal structures, CTSB is also released from damaged Golgi fragments. Previous cell culture studies show that the 46 kDa pro-CTSB in the Golgi can autocleave its own propeptide to become a 33 kDa active CTSB, which in turn can cleave the propeptide of another pro-CTSB to initiate a chain reaction. This chain reaction eventually leads to a substantially accelerated production and release of active CTSB from damaged Golgi fragments (Pungercar et al., 2009; Turk et al., 2012). Yuan et al. (2018b) also showed that pro-CTSB was significantly reduced while mature CTSB was substantially upregulated in neocortical tissue samples after a brief episode of global brain ischemia. Released CTSB can further damage the Golgi and endolysosomal structures, resulting in a cycle of amplified CTSB and neuronal injury.

Previous studies show that CA-074me and E64d might offer neuroprotection by inhibiting CTSB after brain ischemia (e.g., Yamashima et al., 1998; Xu et al., 2016). However, these treatments increase the endolysosomal CTSB half-life and concentration by about 2–3 fold (Katunuma, 2010). E64d is not a CTSB-specific inhibitor. Numerous previous studies indicate that CA-074 and CA-074me are not CTSB-specific inhibitors either, despite previous claims (Montaser et al., 2002; Mihalik et al., 2004; Wieczerzak et al., 2007; Reich et al., 2009). More importantly, E64d and CA-074me inhibitory effects are dependent on pH. They exhibit strong inhibitory activity at pH 4.5 present in the endolysosomal luminal acidic environment, but are ineffective when CTSB is released into the neutral pH of the cytoplasm or extracellular space (Cathers et al., 2002). The endolysosomal luminal CTSB is required to degrade toxic

materials, while released CTSB in the cytosolic and extracellular space is harmful. Therefore, inhibition of endolysosomal luminal cathepsin activities likely leads to deficiency in the endolysosomal degradation activities, causing increased dysfunction of the endocytic and autophagic pathways and more cell death after brain ischemia. An ideal inhibitor would limit its inhibitory capacity to the harmful released CTSB in the cytoplasm and extracellular space, while having minimal impact on the endolysosomal luminal degradation activities.

CONCLUDING REMARKS

It is well-established that a brief episode of cardiac arrest or global brain ischemia leads to delayed neuronal death selectively in most hippocampal CA1 and some neocortical pyramidal neurons (Smith et al., 1984; Hu et al., 2000b; Yuan et al., 2018a). In comparison, stroke or focal brain ischemia with or without reperfusion results in destructive infarction in the brain during the 1 to 24-h post-stroke period (Hu et al., 2001). The mechanism underlying brain ischemia reperfusion injury after both cardiac arrest and stroke remains only partially understood. The latest studies show evidence that brain ischemia inactivates neuronal NSF ATPase. This inactivation results in a cascade of endolysosomal structural damage and the release of digestive enzymes such as CTSB, leading to more structural damage.

There are significant similarities and also differences in the cell death mechanisms in the context of NSF inactivation and endolysosomal structural damage between global and focal brain ischemia animal models. In the global brain ischemia models, milder endolysosomal structural damage or microscale cytosolic CTSB release induces the cell death pathways such as the MOMP cell death pathways (Wang et al., 2018; Yuan et al., 2018b). In comparison, CTSB is released on a large scale from the endolysosomal compartments into the cytoplasm and eventually into the extracellular space after stroke (see **Figures 3**–**6**). This large-scale CTSB release results in tissue destruction or infarction (Alu et al., 2020). Due to these differences, treatment strategies against a brief episode of global brain ischemia should target the microscale release of CTSB in the cytosol. In contrast, management of the large-scale CTSB release from both intracellular and extracellular sources is likely to be more effective against stroke brain injury.

Although significant progress has been made in understanding endolysosomal trafficking in post-ischemic neurons, many questions remain. For example, the interruption of endolysosomal trafficking was observed mainly in post-stroke penumbral neurons in which pathology progresses somewhat slowly for observing NSF depletion and accumulation of endolysosomal structures. Therefore, it is unknown if endolysosomal rupture also occurs in rapidly dying neurons of the ischemic core. Additionally, the interruption of endolysosomal trafficking after stroke has primarily been observed in neuronal soma. It remains unclear whether the endolysosomal trafficking defects also occur in the axons and dendrites during the post-ischemic phase.

This review focuses on early intra-neuronal endolysosomal pathological events leading to neuronal death during the first 1 to 24 h after stroke onset. In comparison to early intra-neuronal pathological events, non-neuronal cells change, such as the blood-brain barrier breakdown, microglia activation, monocyte/neutrophil infiltration, and astrogliosis, which mainly occur from 24 h onward after focal brain ischemia (Fifield and Vanderluit, 2020). Changes in endolysosomal trafficking are likely to affect non-neuronal pathological events as well. For example, endolysosomal activities should be significantly upregulated in non-neuronal cells such as reactive microglia and astrocytes or infiltrated macrophages after stroke. The potential differences between a brief episode of global brain ischemia and prolonged focal brain ischemia, as well as between neurons and non-neuronal cells, increases the complexities in developing treatments that target endolysosomal trafficking after brain ischemia.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

All experimental procedures involving animal use were approved by the Animal Use and Care Committees at the University of Miami Millier School of Medicine and University of Maryland School of Medicine.

AUTHOR CONTRIBUTIONS

KH wrote parts 1 and 2 of the manuscript. BH planned the conception of the review and wrote part 3 of the manuscript. BG, LS, AA, HT, and CL contributed to the proofreads and revisions of the manuscript. All authors contributed to the article and approved the submitted version.

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Proteomic Analysis Reveals Sex-Specific Protein Degradation Targets in the Amygdala During Fear Memory Formation

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Ubiquitin-proteasome mediated protein degradation has been widely implicated in fear memory formation in the amygdala. However, to date, the protein targets of the proteasome remain largely unknown, limiting our understanding of the functional significance for protein degradation in fear memory formation. Additionally, whether similar proteins are targeted by the proteasome between sexes has yet to be explored. Here, we combined a degradation-specific K48 Tandem Ubiquitin Binding Entity (TUBE) with liquid chromatography mass spectrometry (LC/MS) to identify the target substrates of the protein degradation process in the amygdala of male and female rats following contextual fear conditioning. We found that males (43) and females (77) differed in the total number of proteins that had significant changes in K48 polyubiquitin targeting in the amygdala following fear conditioning. Many of the identified proteins (106) had significantly reduced levels in the K48-purified samples 1 h after fear conditioning, suggesting active degradation of the substrate due to learning. Interestingly, only 3 proteins overlapped between sexes, suggesting that targets of the protein degradation process may be sex-specific. In females, many proteins with altered abundance in the K48-purified samples were involved in vesicle transport or are associated with microtubules. Conversely, in males, proteins involved in the cytoskeleton, ATP synthesis and cell signaling were found to have significantly altered abundance. Only 1 protein had an opposite directional change in abundance between sexes, LENG1, which was significantly enhanced in males while lower in females. This suggests a more rapid degradation of this protein in females during fear memory formation. Interestingly, GFAP, a critical component of astrocyte structure, was a target of K48 polyubiquitination in both males and females, indicating that protein degradation is likely occurring in astrocytes following fear conditioning. Western blot assays revealed reduced levels of these target substrates following fear conditioning in both sexes, confirming that the K48 polyubiquitin was targeting these proteins for degradation. Collectively, this study provides strong

evidence that sex differences exist in the protein targets of the degradation process in the amygdala following fear conditioning and critical information regarding how ubiquitin-proteasome mediated protein degradation may contribute to fear memory formation in the brain.

Keywords: ubiquitin, proteasome, amygdala, sex difference, memory, consolidation

INTRODUCTION

Post-traumatic stress disorder (PTSD) is a chronic anxiety disorder that affects nearly 5% of the world population. Despite this, treatment options are limited and none currently exist that can consistently reverse the symptoms of this disorder in all patients (Kessler et al., 2017, 2018). Importantly, females are more likely than males to develop PTSD despite not reporting experiencing more traumatic events (Breslau et al., 1997; Olff, 2017), though the mechanisms controlling this sex-dependent predisposition remain equivocal. A better understanding of the mechanisms controlling these sex differences in PTSD is important for developing therapeutic strategies that are effective in both male and female patients.

The ubiquitin-proteasome system (UPS) is a highly conserved pathway that controls the majority of protein turnover in cells (Bedford et al., 2010; Hegde, 2010; Reis et al., 2013; Hegde et al., 2014; Livneh et al., 2016; Collins and Goldberg, 2017) and dysregulation of this system has been widely implicated in a variety of neurological, neurodegenerative and psychiatric disorders (Dennissen et al., 2012; Zheng et al., 2016). This pathway is tightly regulated as proteins must first be "tagged" with the small regulatory protein ubiquitin *via* various E1, E2, and E3 ligases (Hershko and Ciechanover, 1998; Glickman and Ciechanover, 2002). Proteins may be monoubiquitinated or obtain multiple ubiquitin modifications, called polyubiquitination, the latter of which usually leads to degradation, though not all polyubiquitinated proteins are degraded. Ubiquitin can form different chains due to the presence of multiple lysine (K) residues on it which act as linkage sites (Dikic et al., 2009; Akutsu et al., 2016; Grice and Nathan, 2016). Lysine 48 (K48) polyubiquitin chains are highly abundant in cells and act as the canonical degradation signal (Newton et al., 2008).

Numerous studies conducted during the last two decades provide strong evidence to support a critical role of the UPS in the formation of fear memories that may underlie PTSD. It is well established that both degradation-specific (K48) polyubiquitin chains and proteasome activity are enhanced following fear learning in various brain regions, including the amygdala, hippocampus and prefrontal cortex (Lopez-Salon et al., 2001; Jarome et al., 2011; Rosenberg et al., 2016; Cullen et al., 2017; Orsi et al., 2019; Dulka et al., 2020). Additionally, the infusion of proteasome inhibitors into these brain regions leads to impaired memory for several different aversive and non-aversive behavioral paradigms (Rodriguez-Ortiz et al., 2011; Reis et al., 2013; Figueiredo et al., 2015; Furini et al., 2015; Rosenberg et al., 2016; Cullen et al., 2017). However, the majority of studies have been conducted solely in male rodents, though recent work from our group shows that a sex effect exists in the role of the UPS in fear memory formation (Devulapalli et al., 2021). This study demonstrated that while males, but not females, had increased UPS-mediated protein degradation during fear memory formation, genetic manipulations of ubiquitin and the proteasome impaired memory in both sexes, suggesting that despite these differences, protein degradation *via* the UPS is in fact necessary for fear memory formation in males and females. These findings indicate that males and females may differ in the functional role of ubiquitin-proteasome activity during fear memory formation, though this has never been tested. Furthermore, males and females differ in the age-related engagement of the protein degradation process following fear memory retrieval (Dulka et al., 2021), further emphasizing the importance of sex in studying UPS function during fear memory formation and disease-associated memory loss.

Although evidence supports an important role of protein degradation in fear memory formation, the proteins being targeted by the proteasome remain largely unknown. To date, only 4 proteins have been identified as putative targets of the proteasome during any stage of memory storage, including the synaptic scaffolds SHANK and GKAP, the transcriptional repressor IκB, and the RNA-induced silencing complex (RISC) factor MOV10 (Lopez-Salon et al., 2001; Lee et al., 2008; Jarome et al., 2011). All of these proteins were identified *via* candidate-driven approaches that rely on protein precipitation methods followed by immunoblotting, which can be non-specific and lack the sensitivity of unbiased proteomic approaches such as liquid chromatography mass spectrometry (LC/MS). Additionally, the primary method used in these studies, GST-S5a pull downs (Kamitani et al., 1997), cannot distinguish between the different types of polyubiquitin modifications, which have diverse functions in cells. The recent development of Tandem Ubiquitin Binding Entities (TUBEs) allow direct capture of specific forms of polyubiquitinated proteins in cells (Hjerpe et al., 2009; Mattern et al., 2019), which when combined with LC/MS can provide an unbiased, whole proteome analysis of linkage-specific protein polyubiquitination in cell lysates (Lopitz-Otsoa et al., 2012). However, to the best of our

Abbreviations: ATP1A2, ATPase Na+/K+ Transporting Subunit Alpha 2; CCDC177, Coiled-Coil Domain Containing 177; CTSD, Cathepsin D; Cyfip2, Cytoplasmic FMR1 Interacting Protein 2; ERK5, Extracellular-Signal-Regulated Kinase 5; GFAP, Glial Fibrillary Acidic Protein; GKAP, guanylate kinase-associated protein; IκB, Inhibitory kappa B; KIF1C, Kinesin Family Member 1C; LENG1, Leukocyte Receptor Cluster Member 1; MAP, mitogen-activated protein; MOV10, Moloney leukemia virus 10; SHANK, SH3 and multiple ankyrin repeat domains 3; SHH, Sonic hedgehog; SOX2, SRY-box transcription factor 2; SPTBN1, Spectrin Beta, Non-Erythrocytic 1; STX8, syntaxin 8; THOP1, Thimet Oligopeptidase 1; TPPP, Tubulin polymerization-promoting protein; VTI1B, vesicle transport through interaction with t-SNAREs 1B; WNT, Wingless/Integrated; YWHA, 14-3-3 phospho-serine/phosphor-threonine binding proteins.

knowlegde, no unbiased examination of degradation-specific K48 polyubiquitinated proteins has ever been completed in the brain or during fear memory formation.

In order to better understand the functional role of the UPS in fear memory formation, the proteins being targeted for degradation (K48) following learning must be elucidated. Additionally, due to previous evidence suggesting differences in UPS levels between sexes, it is imperative that protein targets be identified in both sexes to evaluate whether the UPS acts in similar or different pathways to support fear memory formation. To address this gap in knowledge, we identified protein targets of K48 polyubiquitination in the basolateral amygdala of male and female rats during fear memory formation using K48-specific TUBEs in combination with LC/MS. We identified numerous protein targets of K48 polyubiquitination following learning, with very little overlap between sexes, implicating a variety of different cellular processes as being regulated by sex- and degradation-specific K48 polyubiquitination during fear memory formation.

METHODS AND MATERIALS

Subjects

All experiments used 8–9 week old male or female Sprague Dawley rats obtained from Envigo (Frederick, MA). Animals were housed two per cage with free access to water and rat chow. The colony was maintained under a 12:12-h light/dark cycle. All experiments took place during the light portion of the cycle. All procedures were approved by the Virginia Polytechnic Institute and State University Institutional Animal Care and Use Committee (protocol #18-019) and conducted with the ethical guidelines of the National Institutes of Health.

Apparatus

The 2 identical Habitest chambers used for contextual fear conditioning have been previously described in detail (Orsi et al., 2019). Habitest chamber consisted of a steel test cage with front and back Plexiglas walls and a grid shock floor above a plastic drop pan. The right wall of the chamber consisted of a house light in the top back corner, which remained on during the behavioral procedures, and an infrared light in the top middle, which was not illuminated during this project. The left wall of the chamber consisted of a high-bright light, which was not illuminated during this project. All remaining slots of both walls were filled with blank metal panels. A USB camera was mounted on a steel panel outside the back Plexiglas wall of the chamber, angled at ∼45°. The entire chamber was housed in an isolation cubicle with an acoustic liner and a house fan, which remained active during all behavioral procedures. Shock was delivered through the grid floor *via* a Precision Animal Shocker under the control of FreezeFrame 4 software, which also analyzed animal behavior in real-time. A freezing threshold of 2.0 was used as the scoring parameter for all animals. All video was recorded and stored for later analysis. The chamber walls were wiped with 70% isopropanol before use.

Behavioral Procedures

Rats underwent contextual fear conditioning training and testing as described previously (Devulapalli et al., 2019, 2021; Orsi et al., 2019) in a Habitest chamber. Both experimental and naïve animals were handled for 4 days prior to behavioral training; the first two days occurred in the animal housing room and the second two days occurred in an adjacent room where behavioral training was to occur. Following this, the experimental group animals were placed into the fear conditioning apparatus and after a 1 min baseline, received 4 unsignaled footshock (1.0 mA, 1 sec) presentations. After a 1 min post-shock period, the animals were returned to their homecages. Naïve animals did not undergo the fear conditioning procedure and were not exposed to the conditioning chamber. We recently reported that male and female Sprague Dawley rats perform similarly on this task and do not differ in shock reactivity (Devulapalli et al., 2021), eliminating concerns of any biochemical effects seen between sexes being due to differences in behavioral performance or sensory processing.

Tissue Collection

One or 4 h after the training session, experimental rats were overdosed on isoflurane in a necrosis chamber and the brain rapidly removed and immediately frozen on dry ice. During the same time on the training day, naïve animals were overdosed on isoflurane and the brain rapidly removed in the same manner as the experimental group. Tissue containing the basolateral amygdala (BLA) was then dissected out by blocking the brain in a rat brain matrix (Harvard Apparatus, Holliston, MA) incubated with dry ice. All dissected tissue was frozen at −80°C until needed.

Tandem Ubiquitin Binding Entity (TUBE) Assays

BLA tissue was homogenized in buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5% IGEPAL, 0.02% SDS, 70 mM NEM, 1 μl/ml protease inhibitor cocktail, and 1 μl/ml phosphatase inhibitor cocktail) designed to preserve endogenous polyubiquitinated chains. Then a high affinity K48 polyubiquitin-selective tandem ubiquitin binding entity (K48-TUBE, 100 μl, #UM407M, Life Sensors, Malvern, PA) conjugated to beads was washed in buffer (100 mM Tris-HCL, 150 mM NaCl, 5 mM EDTA, 0.08% NP-40) and a 500 μl mixture of protein (300 μg), protease inhibitor (1 μg/ml), and Wash Buffer was added, followed by incubation for 2 h on rotator at 4°C. Samples were then washed twice and incubated at 96°C for 5 min at 800 rpm in 1X sample buffer (Bio-rad, Hercules, CA). After cooling at room temperature, the supernatant was collected and stored at −80°C for mass spectrometry analysis.

Liquid Chromatography Mass Spectrometry (LC/MS)

Optima™ LC/MS grade solvents, Pierce™ trypsin protease (MS grade), were from Fisher Scientific (Waltham, MA). S-Trap™ micro columns were from ProtiFi (Farmingdale, NY). Triethylammonium bicarbonate, pH 8.5 (TEAB), *o*-phosphoric acid, and formic acid were from MilliporeSigma (St. Louis, MO).

Protein samples were acidified by the addition of 11.1 μl 12% (v/v) *o*-phosphoric acid then protein precipitated by the addition of 725 μl LC/MS grade methanol and incubation at −80°C overnight. Precipitated protein was collected at the bottom of the sample tubes by centrifugation at room temperature for 15 mins at 13,000 x g. All but ∼150 μl of the liquid from each sample was removed and discarded. Precipitated protein in each sample tube was then homogenized in the remaining liquid by scraping the sides of the tube with a pipette tip and repeated pipetting. The protein homogenate from each sample was then loaded onto an S-Trap™ micro column by centrifugation at room temperature for 1 min at 1000 x g. Each S-Trap™ micro column was washed four times with 150 μl LC/MS grade methanol at room temperature for 1 min at 1000 x g. Pierce™ trypsin protease (0.8 μg in 25 μl 50 mM TEAB) was loaded on top of each S-Trap™ micro column and incubated for 4 h at 37°C. A second aliquot of trypsin (0.8 μg in 25 μl 50 mM TEAB) was loaded on top of each S-Trap™ micro column and incubated overnight at 37°C.

Peptides were recovered by sequential washing of the spin column with 25 μl 50 mM TEAB, 25 μl solvent A (2:98 LC/MS grade acetonitrile: LC/MS grade water supplemented with 0.1% (v/v) formic acid), and 25 μl solvent B (80:20 LC-MS grade acetonitrile: LC-MS grade water supplemented with 0.1% (v/v) formic acid). Acetonitrile was removed using a centrifugal vacuum concentrator then peptide concentration was determined by measuring the absorbance at 215 nm using a DS-11 FX+ spectrophotometer/fluorometer (DeNovix, Wilmington, DE). Samples were diluted to 0.5 mg/ml using solvent A and 2 μl (1 μg, females) or 4 μl (2 μg, males) were analyzed using LC-MS/MS and each sample was analyzed twice yielding technical duplicates. The higher concentration used for males was due to a greater streptavidin signal than seen in females. As noted below, final values are normalized to streptavidin to account for this difference.

Samples were first loaded onto a precolumn (Acclaim PepMap 100 (Thermo Scientific, Waltham, MA), 100 μm x 2 cm) after which flow was diverted to an analytical column (50 cm μPAC (PharmaFluidics, Woburn, MA). The UPLC/autosampler utilized was an Easy-nLC 1,200 (Thermo Scientific, Waltham, MA). Flow rate was maintained at 150 nl/min and peptides were eluted utilizing a 2 to 45% gradient of solvent B in solvent A over 88 min. The mass spectrometer utilized was an Orbitrap Fusion Lumos Tribid™ from Thermo Scientific (Waltham, MA). Spray voltage on the μPAC compatible Easy-Spray emitter (PharmaFluidics, Woburn, MA) was 1,300 volts, the ion transfer tube was maintained at 275°C, the RF lens was set to 30% and the default charge state was set to 3.

MS data for the m/z range of 400–1,500 was collected using the orbitrap at 120,000 resolution in positive profile mode with an AGC target of 4.0e5 and a maximum injection time of 50 ms. Peaks were filtered for MS/MS analysis based on having isotopic peak distribution expected of a peptide with an intensity above 2.0e4 and a charge state of 2–5. Peaks were excluded dynamically for 15 s after 1 scan with the MS/MS set to be collected at 45% of a chromatographic peak width with an expected peak width (FWHM) of 15 s. MS/MS data starting at m/z of 150 was collected using the orbitrap at 15,000 resolution in positive centroid mode with an AGC target of 1.0e5 and a maximum injection time of 200 ms. Activation type was HCD stepped from 27 to 33.

Data were analyzed utilizing Proteome Discoverer 2.5 (Thermo Scientific, Waltham, MA) combining a Sequest HT and Mascot 2.7 (Matrix Science, Boston, MA) search into one result summary for each sample. Both searches utilized the UniProt reference *R. norvegicus* proteome database (downloaded July 28, 2020) and a common protein contaminant database provided with the Proteome Discoverer (PD) software package. Each search assumed trypsin-specific peptides with the possibility of 2 missed cleavages, a precursor mass tolerance of 10 ppm and a fragment mass tolerance of 0.1 Da. Sequest HT searches also included the PD software precursor detector node to identify MSMS spectra containing peaks from more than one precursor. Sequest HT searches included a fixed modification of carbamidomethyl at Cys and the variable modifications of oxidation at Met and loss of Met at the N-terminus of a protein (required for using the INFERYS rescoring node). Peptide matches identified by Sequest HT were subjected to INFERYS rescoring to further optimize the number of peptides identified with high confidence.

Mascot searches included the following dynamic modifications: oxidation of Met, acetylation of the protein N-terminus, cyclization of a peptide N-terminal Gln to pyro-Glu, N-ethylmaleimide at Cys, DeStreak (β-mercaptoethanol) at Cys, GlyGly at Lys, and deamidation of Asn/Gln residues.

Protein identifications were reported at a 1% false discovery rate (high confidence) or at a 5% false discovery rate (medium confidence) based on searches of decoy databases utilizing the same parameters as above. The software matched peptide peaks across all runs and protein quantities are the sum of all peptide intensities associated with the protein. Values were normalized to Streptavidin. Technical duplicates were averaged then biological replicates were averaged before determination of the trained to naïve ratio. Due to the loading differences between male and female samples (noted above), a simple *t*-test was used to determine *p*-values comparing the 5 trained males to the 5 naïve males and the 5 trained females to the 5 naïve females with $p < 0.05$ as threshold for a protein as being significantly different between group. All data and related files were submitted to the PRIDE archive with accession number PXD027544.

Mass Spectrometry Pathway Analysis

All quantified ubiquitinated proteins were analyzed for identifying pathways and networks using Ingenuity Pathway Analysis (IPA) software (QIAGEN, Redwood City, CA) core analysis based on the user dataset as the reference set. The top canonical pathways and molecular networks associated with the uploaded dataset were listed along with the *p*-values calculated using a right tailed Fisher's exact test.

Antibodies

Antibodies included synaptotagmin-1 (1:1000; #14558S Cell Signaling Technology, Danvers, MA), Cathepsin-D (1:1000, #69854, Cell Signaling Technology), Actin (1:1000; #4967, Cell

Signaling Technology) and GFAP (1:5000; #ab190288, Abcam, Waltham, MA).

Western Blot Analysis

Western blots were performed with 7% Acrylamide gels using 10 μg of protein as described previously (Orsi et al., 2019). Membranes were transferred with a Turbo Transfer System (Biorad) and incubated in a 50:50 blocking buffer (50% Licor TBS blocking buffer and 50% TBS + 0.1% Tween-20) for 1 h at room temperature, followed by overnight incubation in primary antibody in 50:50 blocking buffer at 4°C. Membranes were then washed 3 times for 10 min with TBS + 0.1% Tween-20 (TBSt) and incubated in secondary antibody (1:20,000; goat anti-rabbit or goat anti-mouse IgG1 800CW) in 50:50 blocking buffer for 45 min. After two 10 min washes in TBSt, the membranes were washed in TBS, imaged using the Odyssey Fc (LI-COR, Lincoln, NE) and visualized proteins were analyzed using Image Studio Ver 5.2. Samples were normalized to Actin.

Statistical Analyses

All data are presented as mean with standard error, with scatter plots to identify individual samples (except in line graphs). Training data were analyzed with 2-way ANOVA and Fisher LSD *post-hoc* tests and western blot analysis was done with one-tailed *t*-test, which was based on the a priori prediction of the conformational experiment for the LC/MS data. Statistical outliers were defined as those samples that were two or more standard deviations from the mean and were determined by the outlier function in Prism.

RESULTS

Identification of Sex-Specific K48 Polyubiquitin Protein Targets in the Amygdala After Fear Conditioning

To investigate the potential role of K48 polyubiquitination in fear memory formation, we trained male and female rats to contextual fear conditioning and collected the BLA 1 h later, which has consistently been reported as the peak time point for increased K48 polyubiquitination levels in the amygdala following fear conditioning (Jarome et al., 2011, 2013; Orsi et al., 2019; Devulapalli et al., 2021). Associative control animals were not used as we have consistently shown that exposing rats to the context and shock alone or in a non-associative manner (immediate shock training) do not engage protein degradation in the amygdala, as measured by changes in functional proteasome activity and K48 and proteasome-specific protein polyubiquitination (Jarome et al., 2011, 2013; Orsi et al., 2019). Proteins containing K48 polyubiquitination were then enriched using a K48-specific TUBE (Hjerpe et al., 2009) followed by protein identification using LC/MS ($N = 5$ per group per sex). This allowed an unbiased proteomic analysis of K48-specific polyubiquitin protein targets in the amygdala following fear conditioning. Behavioral performance of male and female rats during training is shown in **Figure 1A**. We found a main effect for Time [$F_{(4,32)} = 46.53$, $P < 0.0001$], but not Sex [$F_{(1,8)} = 0.7709$, $P = 0.4055$] and there was not a Time X Sex interaction [$F_{(4,32)} = 0.6453$, $P = 0.6342$], indicating that male and female rats performed similar during the training session. Notably, while several labs have reported conflicting results in whether a sex difference exists on this task using the same species and strain as us (Graham et al., 2009; Russo and Parsons, 2021), we have previously reported that male and female rats show similar fear to the context during the testing session when using identical training parameters (Devulapalli et al., 2021). In total, we found 1,261 and 476 K48 polyubiquitinated proteins in females and males, respectively, of which 378 overlapped between sexes (**Figure 1B**). The larger amount of K48 polyubiquitin targets in the amygdala of females is consistent with our recent work showing that females have naturally higher levels of protein degradation in the amygdala in comparison to males (Devulapalli et al., 2021). Of these proteins, 77 in females and 43 in males had significantly altered expression levels of K48 polyubiquitination following fear conditioning based on the altered levels of protein identified in the samples (**Supplementary Table 1**), representing only a small fraction of the total K48 polyubiquitinated proteins in each dataset (Females = 6.1%; Males = 9.0%). Though these proteins varied across the subcellular compartments, a majority (> 50%) were localized to the cytoplasm in both sexes (**Table 1**).

Next, we separated these significantly altered K48 polyubiquitination protein targets by whether they had increased or decreased abundance in our TUBE purified samples following fear conditioning. The protein degradation process rapidly increases in the amygdala following fear conditioning, showing significant changes within 30 min and peaking at 1 h (Jarome et al., 2011). Since our proteomic analysis examined only the 1 h time point, we defined proteins with increased abundance in our enriched samples as targets that are about to be degraded while those with decreased abundance were defined as having actively undergone degradation; confirmation for this assumption will be discussed below (**Figure 6**). Importantly, we find that proteomic analyses on non-degradation polyubiquitin chains (M1) reveals few proteins with decreased abundance (Musaus et al., 2021), supporting that reduced levels in our K48 purified samples is likely indicative of the degradation process. Of the proteins with significantly altered levels, we identified 11 with increased abundance in K48-purified samples after fear learning, with 4 proteins found in females and 7 in males (**Figure 1C**), but no mutual proteins between the sexes. Conversely, both females and males had a higher number of proteins with significantly lower abundance in K48-purified samples following fear conditioning: 73 proteins of our dataset were identified in females and 36 proteins in males, with 3 of these proteins overlapping between sexes (**Figure 1D**). These observations suggest that there is a unique K48-mediated protein degradation pattern concerning protein targets between the sexes and that the learning-induced changes in the protein degradation process likely occur very rapidly following fear conditioning. All four proteins with increased abundance in K48-purified samples in females are shown in **Figure 1E** ($P < 0.05$), of which one protein, VTI1B, had a 593-fold increase compared to naïve controls. On the contrary, no proteins were identified to have a fold change higher than 4 in males (**Figure 1F**). Together, these results reveal that, contrary to our recent report which used broad immunoblotting

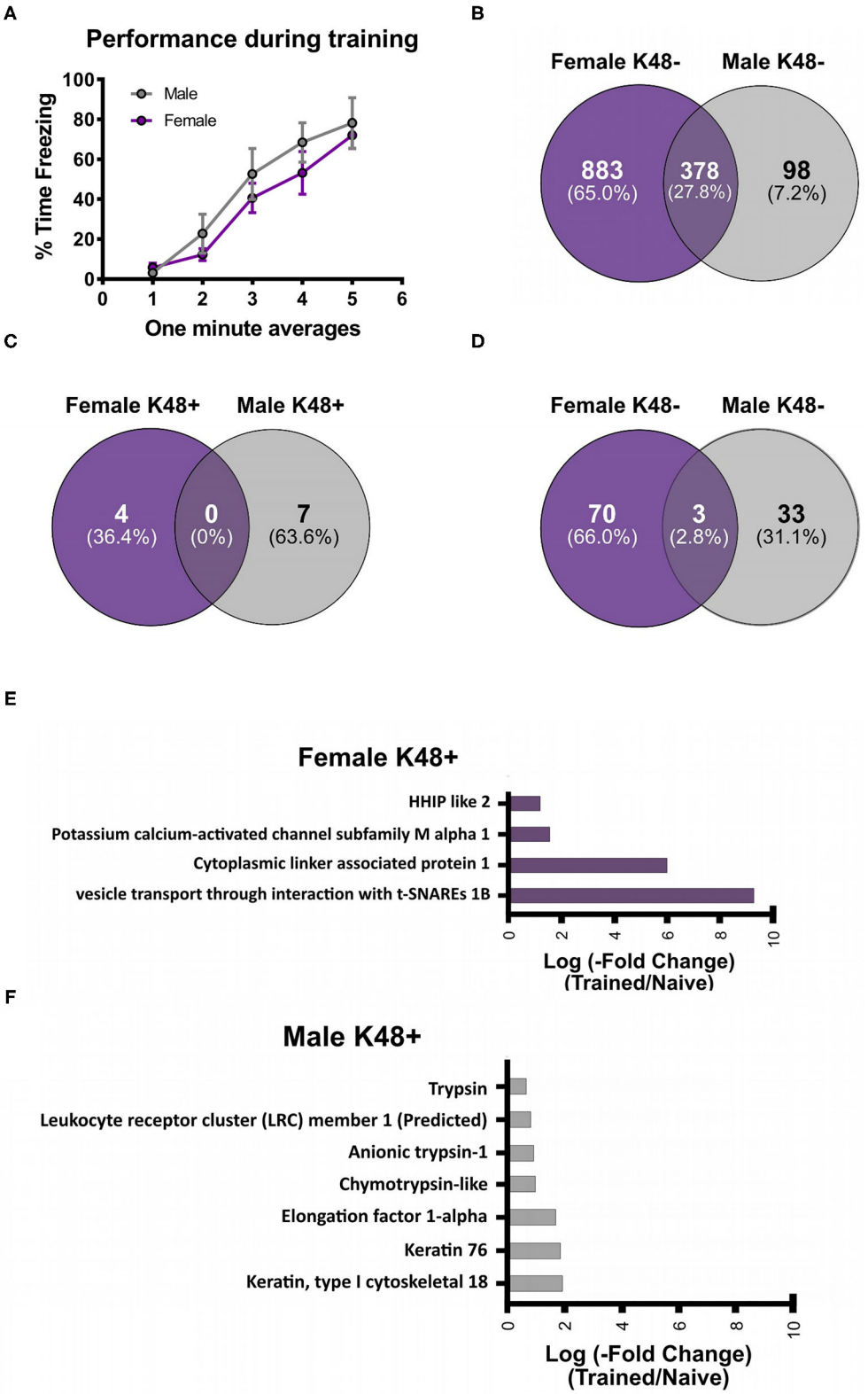


FIGURE 1 | Sex-specific changes in K48 polyubiquitin protein targets in the amygdala of male and female rats during fear memory formation. Male and female Sprague-Dawley rats were trained to a foreground contextual fear conditioning task ($n = 5$ per sex) and the basolateral amygdala (BLA) was collected 1 h later.

(Continued)

FIGURE 1 | Samples were purified with a K48-specific tandem ubiquitin binding entity (K48-TUBE), target proteins identified with liquid chromatography mass spectrometry (MC/MS) and compared with naïve rats ($n = 5$ per sex). **(A)** Behavioral performance of animals during the training session. **(B)** Venn diagram showing the total K48 polyubiquitinated proteins identified in female (purple) and male (gray) BLA tissue. **(C,D)** Venn diagram showing the number of proteins that had increased **(C)** or decreased **(D)** abundance in BLA K48-purified samples 1 h after fear conditioning. **(E,F)** List of the proteins that had increased levels in K48-purified samples 1 h after training in females **(E)** and males **(F)** with their corresponding log value (-fold change).

TABLE 1 | Subcellular localization of K48 polyubiquitin targets.

Sex	Cytoplasm	Nucleus	Extracellular Space	Plasma Membrane	Other	Total
Male	24	3	2	7	7	43
Female	41	15	4	11	6	77

approaches (Devulapalli et al., 2021), females do show significant upreguation of the protein degradation process in the amygdala during fear memory formation. Importantly, despite these mutual increases in protein degradation in the amygdala of males and females, the protein targets are largely unique between sexes.

Sex Differences in the Functional Networks of K48 Polyubiquitin Protein Targets During Fear Memory Formation

After identifying the specific protein targets of K48 polyubiquitination in males and females, we first used IPA software to identify downstream targets of VTI1B, which is involved in vesicle transport pathways, an important process in synaptogenesis signaling pathways, to better interpret the dramatic increase in abundance observed in K48-purified samples from female BLA tissue. Since the increase in protein abundance following K48 purification indicates subsequent degradation of this substrate, we predicted the outcome of VTI1B loss on its downstream targets. Based on our analysis, 5 proteins interact with VTI1B or play a role in downstream pathway targets, of which syntaxin 8 (STX8) is positively regulated by VTI1B (**Figure 2**). Importantly, STX8 is mainly involved in protein trafficking *via* vesicle fusion and exocytosis by binding to proteins, such as VTI1B. These findings suggest a role for degradation in presynaptic plasticity in the female amygdala.

Next, we performed core analysis on our male and female K48 datasets to determine whether any of the proteins that were identified as having significantly altered abundance in K48-purified samples interacted with each other in a network or a particular signaling pathway. We first examined the female dataset and IPA detected 5 different networks and one signaling pathway. The top network (**A**) and the canonical pathway (**B**) are shown in **Figure 3**. The total number of proteins in this network are 35, with the majority of them (23 proteins, indicated by green) showing decreased abundance, 2 showing an increase (red), and 10 (part of network but not identified by LC/MS) having no change in abundance following K48 purification. Additionally, many of these proteins are associated with microtubules and transportation within the cell, such as MAPs, TPPP, and KIF1C. Unlike the network of protein-protein interaction, only two proteins are contributing to the identified signaling pathway, transcriptional regulatory network in embryonic stem cells: H3.3 histone A (H3-3A/H3-3B) and SRY-box transcription factor 2 (SOX2). Both of these proteins have lower abundance in the K48-purified sample after fear conditioning.

Next, we repeated the networks and pathways analyses for the male dataset. Similar to the female analysis, IPA detected 5 networks that contained interactions of the proteins identified as having significantly altered levels in K48-purified samples after fear learning. Moreover, the proteins with decreased abundance following K48 purification were more abundant in the top network (shown in green; **Figure 4A**). There are 35 proteins in this network in total, of which 17 had decreased, 3 had increased, and 15 (part of network but not identified by LC/MS) had no changes in abundance in K48-purified samples 1 h following training. Some of these identified proteins are associated with the cytoskeleton, ATP synthesis, and cell signaling (i.e., neurotransmitter transduction), such as the YWHA family, SPTBN1, and ATP1A2. However, proteins in the other 4 networks are also involved in some of the canonical pathways (**Figure 4B**); neuroprotective role of THOP1 in Alzheimer's disease, ERK5 signaling, and inhibition of ARE-mediated mRNA degradation pathway are among these pathways detected by IPA. Collectively, these results suggest that the protein targets of K48 polyubiquitination may have sex-specific functional roles during fear memory formation.

Sex Independent K48 Polyubiquitin Targets During Fear Memory Formation

We further examined the overlap in K48 polyubiquitin targets in the BLA of male and female animals. There were 3 proteins identified in both the male and female datasets as having significantly reduced levels in K48-purified samples following training (GFAP, Cyfip2, and CCDC177, **Figure 5A**). Of these, both GFAP and Cyfip2 are involved in cytoskeleton and cellular structural changes, but the function of CCDC177 is still unknown. Conversely, 1 protein, LENG1, was identified as having increased abundance in male K48-purified samples and decreased abundance in female K48-purified samples 1 h post-training, suggesting a more rapid degradation in the females, although the function of this protein is not well understood. Next, we looked at the downstream targets of these 4 proteins. IPA failed to detect any targets for CCDC177 and LENG1, which is consistent with the lack of available information regarding their

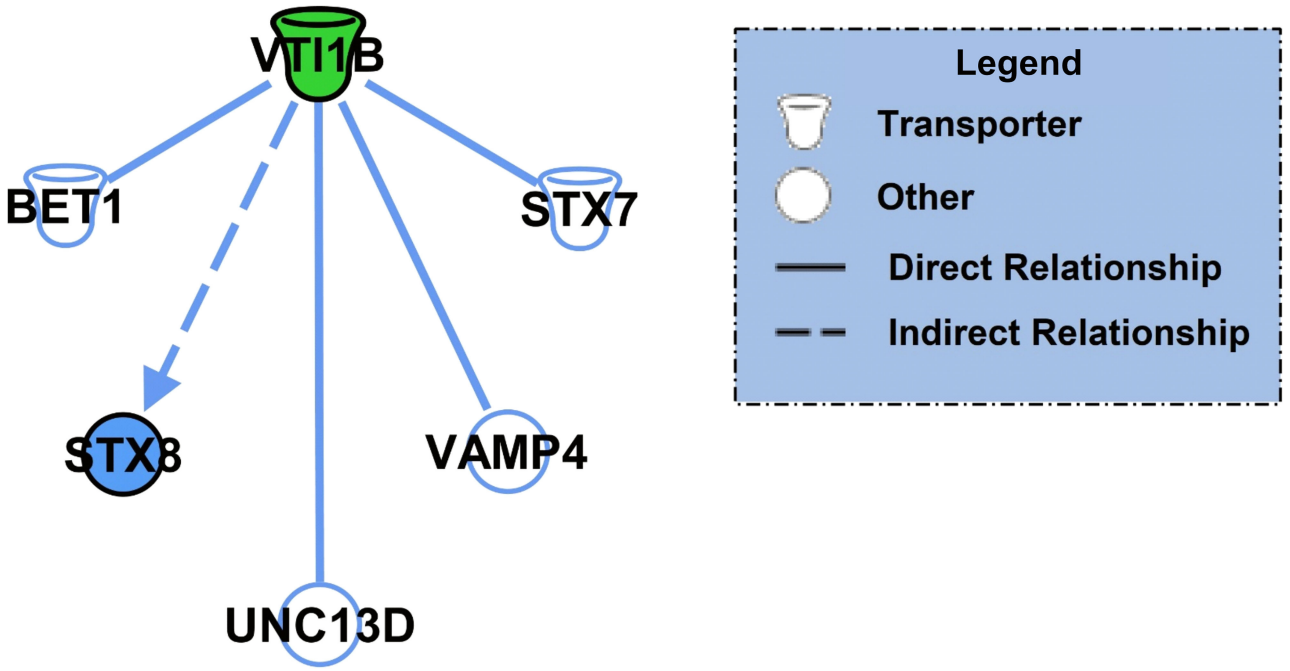
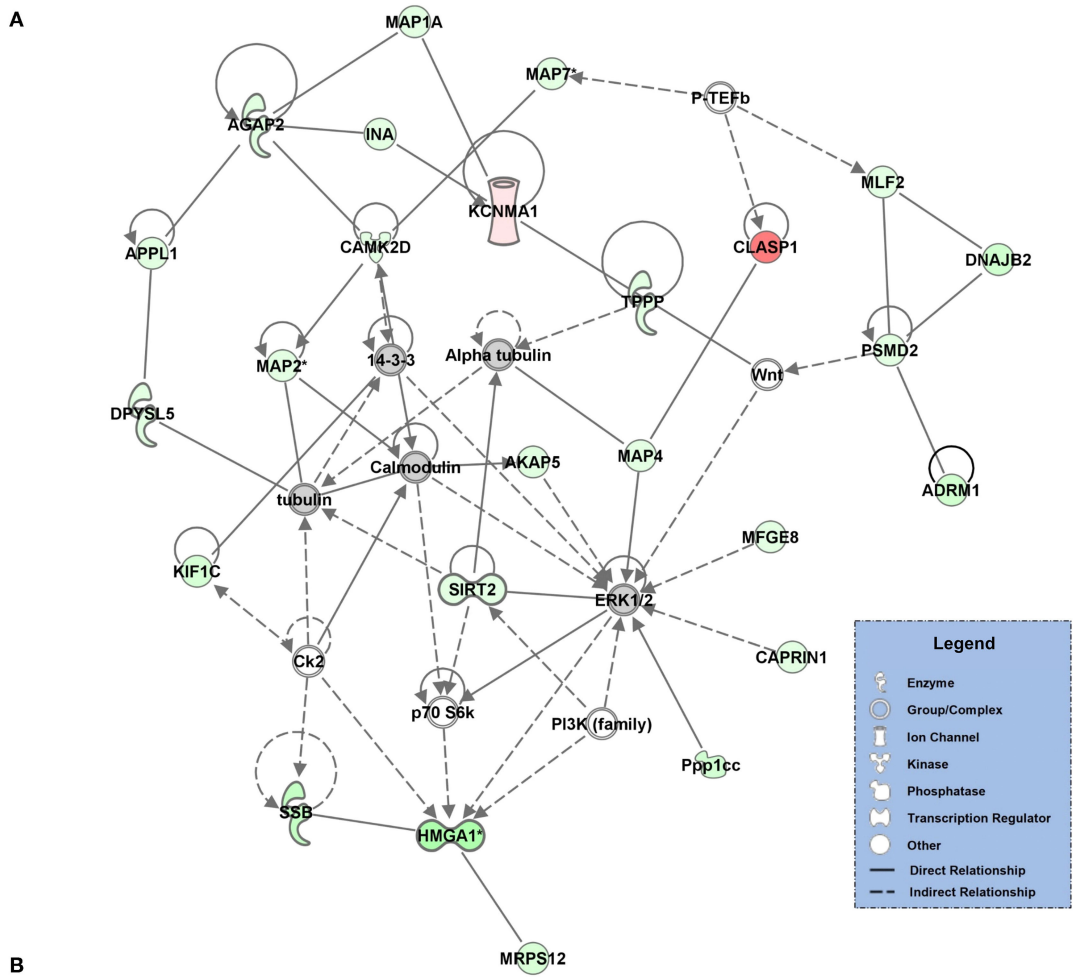


FIGURE 2 | The downstream effectors of the putative K48 polyubiquitin target protein in the amygdala of females. Using IPA, the downstream proteins of VTI1B, which had the largest increase in abundance following K48 purification 1 h after the training in females, were identified. Green denotes a decrease in the protein expression levels, which leads to inhibition of the effector protein (shown in blue). Direct relationships are shown with solid lines and the indirect relationship is indicated by a dotted line.

function, but networks were generated for Cyfip2 (**Figure 5B**) and GFAP (**Figure 5C**). Reductions in the Cyfip2 protein do not change the activity or levels of its downstream target in rats. However, GFAP is a well known intermediate filament protein primarily expressed in astrocytes and interacts with many other proteins, mainly to maintain the structural integrity of astrocytes. Changes in this protein result in dramatic changes in a range of other proteins, such as transcription regulators, ion channels, and transmembrane receptors. Collectively, these findings suggest that distinct sets of proteins are targeted by K48-polyubiquitination after the fear learning to facilitate synaptic plasticity and morphological changes needed in females and males, except for a few proteins that their specific role is not well studied in memory and neuronal plasticity.

K48 Polyubiquitin Protein Targets Have Reduced Expression Following Fear Conditioning

To confirm the LC/MS results and that the K48 polyubiquitin mark was targeting substrates for degradation, we assessed the expression levels of candidate proteins with lower abundance in K48-TUBE enriched samples using western blotting. We first examined GFAP since it was the only major target that was altered in the same direction in both sexes (approximately -two-fold change in both; $N = 5$ per group per sex) and is a crucial component of astrocyte morphology. To increase power in this analysis, we included additional amygdala of naïve rats or those collected 1 h following contextual fear conditioning. In the amygdala of the males, there was a significant reduction in GFAP levels ($t_{14} = 1.907$, $p = 0.0387$, N = 8 per group; **Figure 6A**). Conversely, females did not show any significant changes in GFAP levels ($t_{14} = 0.804$, $p = 0.2174$, $N = 8$ per group; **Figure 6B**). To confirm this, next, we quantified Cathepsin D (CTSD) levels, one of the proteins with a high fold-change reduction in female K48-TUBE enriched samples. Consistent with the GFAP result, there were no changes in the trained group CTSD levels compared to the naïve animals ($t_{14} = 0.7264$, $p = 0.2398$; **Figure 6C**). While unexpected, it is possible that increases in the degradation of a target substrate could take time to be reflected as a change in total protein level (Lee et al., 2008), especially considering that proteasome activity remains elevated for at least 4 h post-training (Jarome et al., 2013). Additionally, protein degradation is a multi-step process, which begins with deubiquitination of the protein before it enters the 19S cap of the proteasome, followed by degradation in the 20S core. Thus, it is possible that the proteins identified as having a lower level in the K48-TUBE enriched samples may have lost the K48 polyubquitination mark, but not yet been degraded, supporting the idea of active degradation. To confirm that the proteins identified in female K48-TUBE purified samples were undergoing degradation, we trained another group of females for contextual fear conditioning and collected their amygdala 4 h post-training ($N = 7$–8 per group). We found a significant reduction in both GFAP ($t_{13} = 3.272$, $p = 0.0030$; **Figure 6D**) and CTSD ($t_{13} = 3.338$, $p = 0.0027$; **Figure 6E**) levels, but not



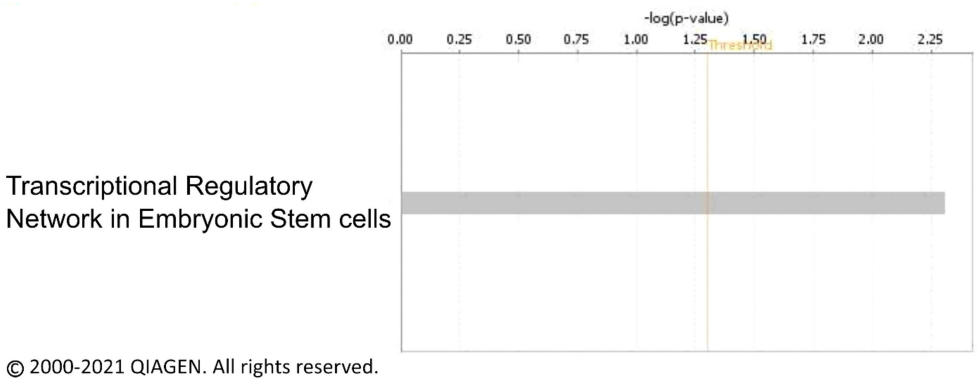
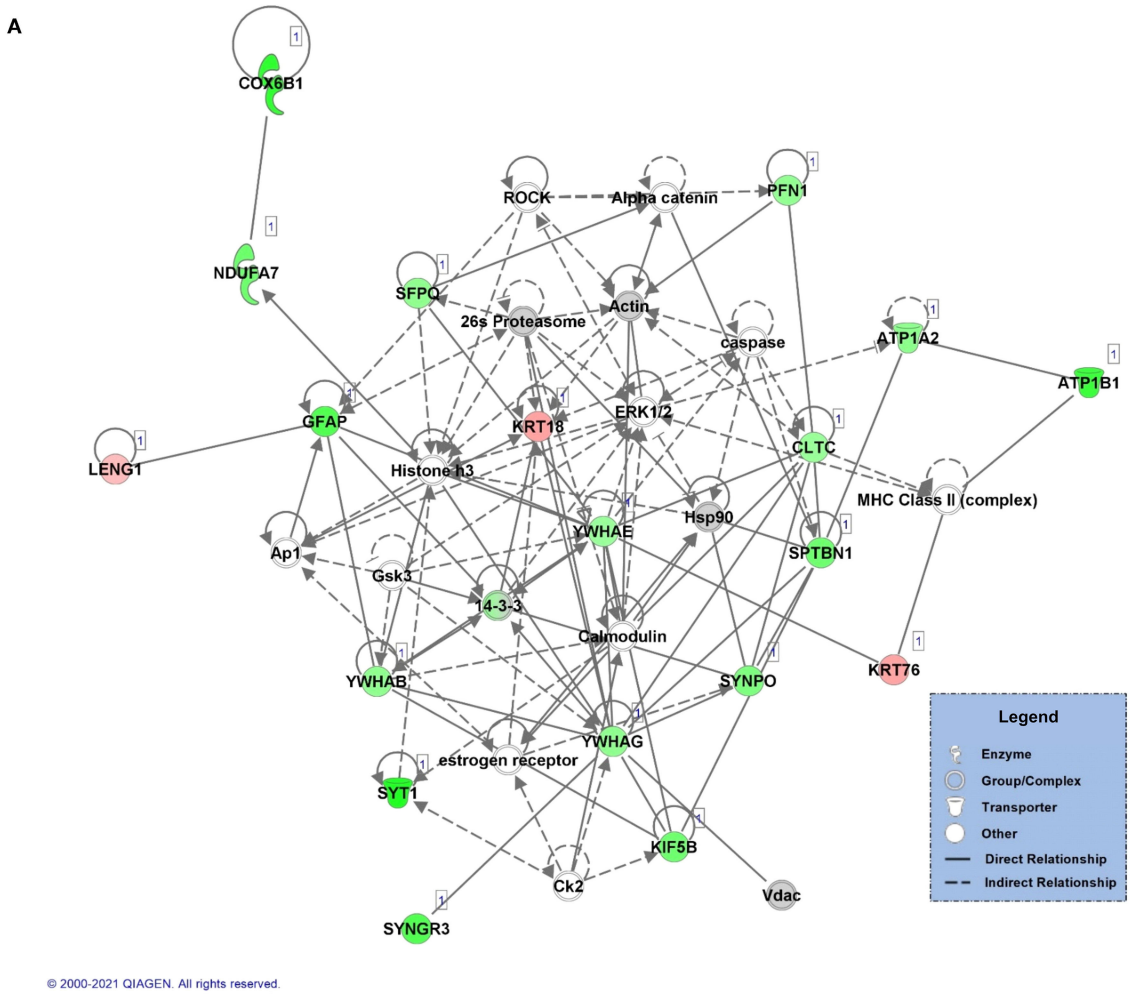


FIGURE 3 | Pathway and network analysis of proteins regulated by K48 polyubiquitin in the amygdala of females. **(A)** Network of associated proteins with high fold change ratio of K48 polyubiquitin targeting in females was created using IPA. The proteins with lower levels in K48-purified samples 1 h after training are shown in green and those that had enhanced levels are shown in red. Greater fold-changes have brighter colors. Solid lines indicate a direct relationship, while dotted lines refer to indirect relationships between the proteins. **(B)** The canonical pathway associated with proteins differentially polyubiquitinated (K48) in the female amygdala detected by IPA.



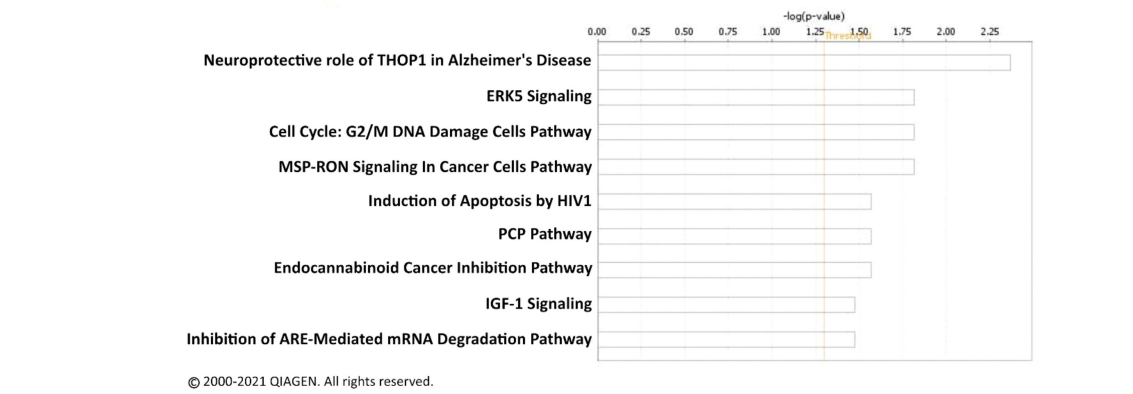
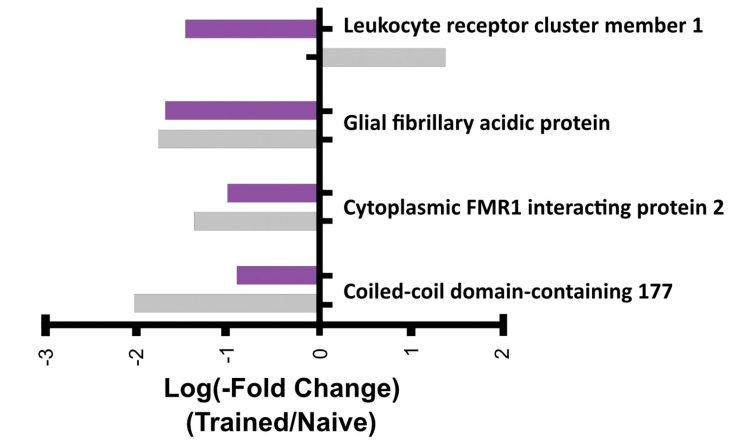
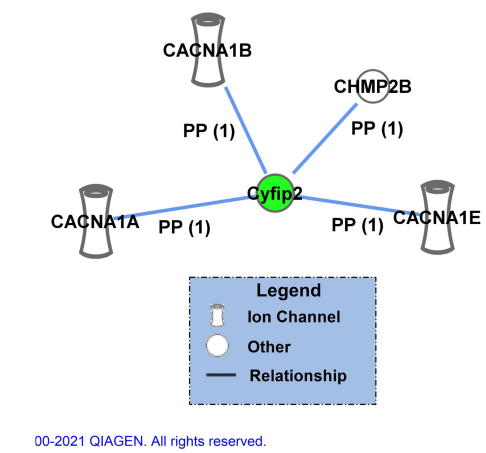


FIGURE 4 | Pathway and network analysis of proteins regulated by K48 polyubiquitin in the amygdala of males. **(A)** Network of associated proteins with a high fold change ratio of K48 polyubiquitin targeting in males was created using IPA. The proteins with lower levels in K48-purified samples 1 h after training are shown in green and those that had enhanced levels are shown in red. Greater fold-changes have brighter colors. Solid lines indicate a direct relationship while dotted lines refer to indirect relationships between the proteins. **(B)** The top canonical pathways associated with the altered K48 polyubiquitin targets after the training in the male amygdala and their corresponding score (-log[p value]). A white bar indicates that there is no clear signal for activation or inhibition.





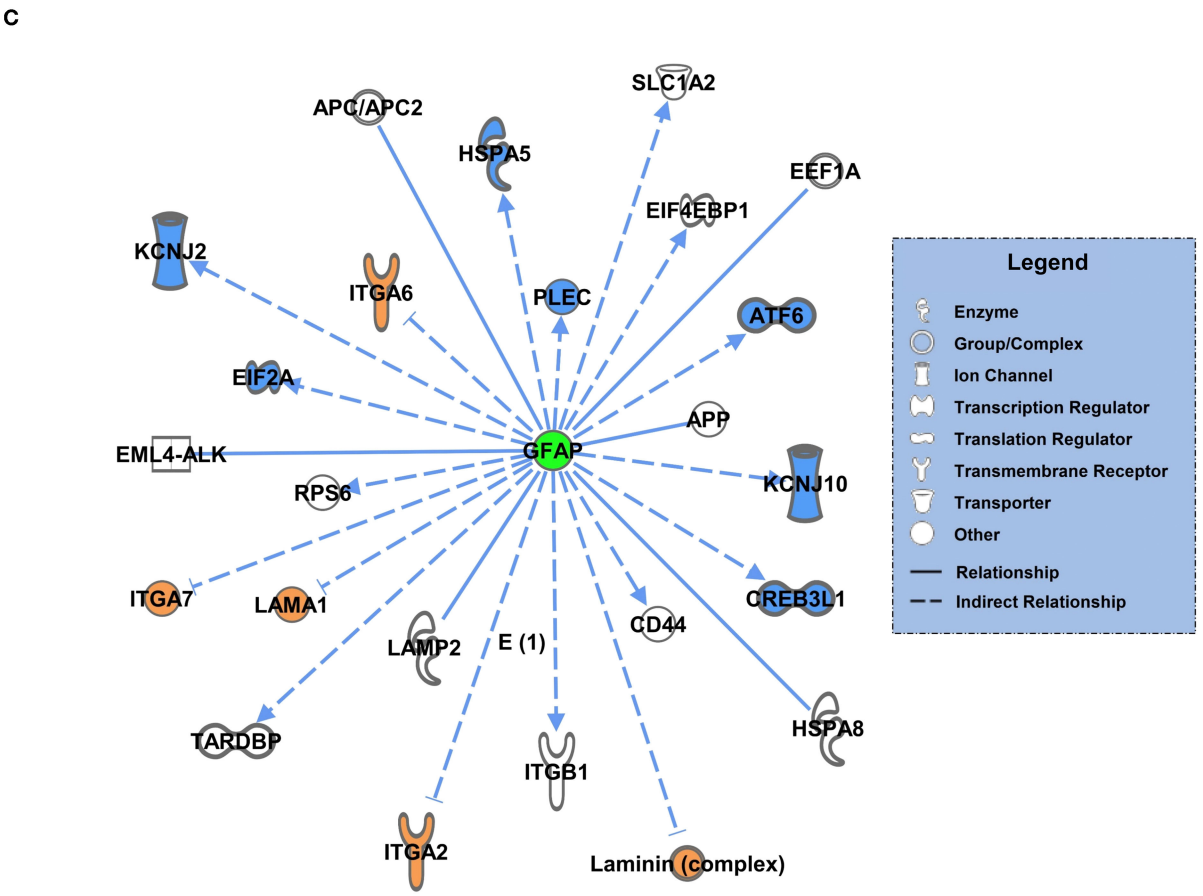


FIGURE 5 | Sex-independent K48 polyubiquitin targets in the amygdala during fear memory formation **(A)** Proteins found to be significantly altered in both female and male K48-purified samples following fear conditioning, with corresponding log value (-fold change). **(B,C)** Using IPA, the downstream proteins of Cyfip2 **(B)** and GFAP **(C)** were identified. Green denotes a decrease in protein abundance in the K48-purified samples, which reflects reduced protein expression levels and leads to decreased (shown in blue) or increased (shown in orange) function/levels of the effector protein. Direct relationships are shown with solid lines and the indirect relationship is indicated by a dotted line.

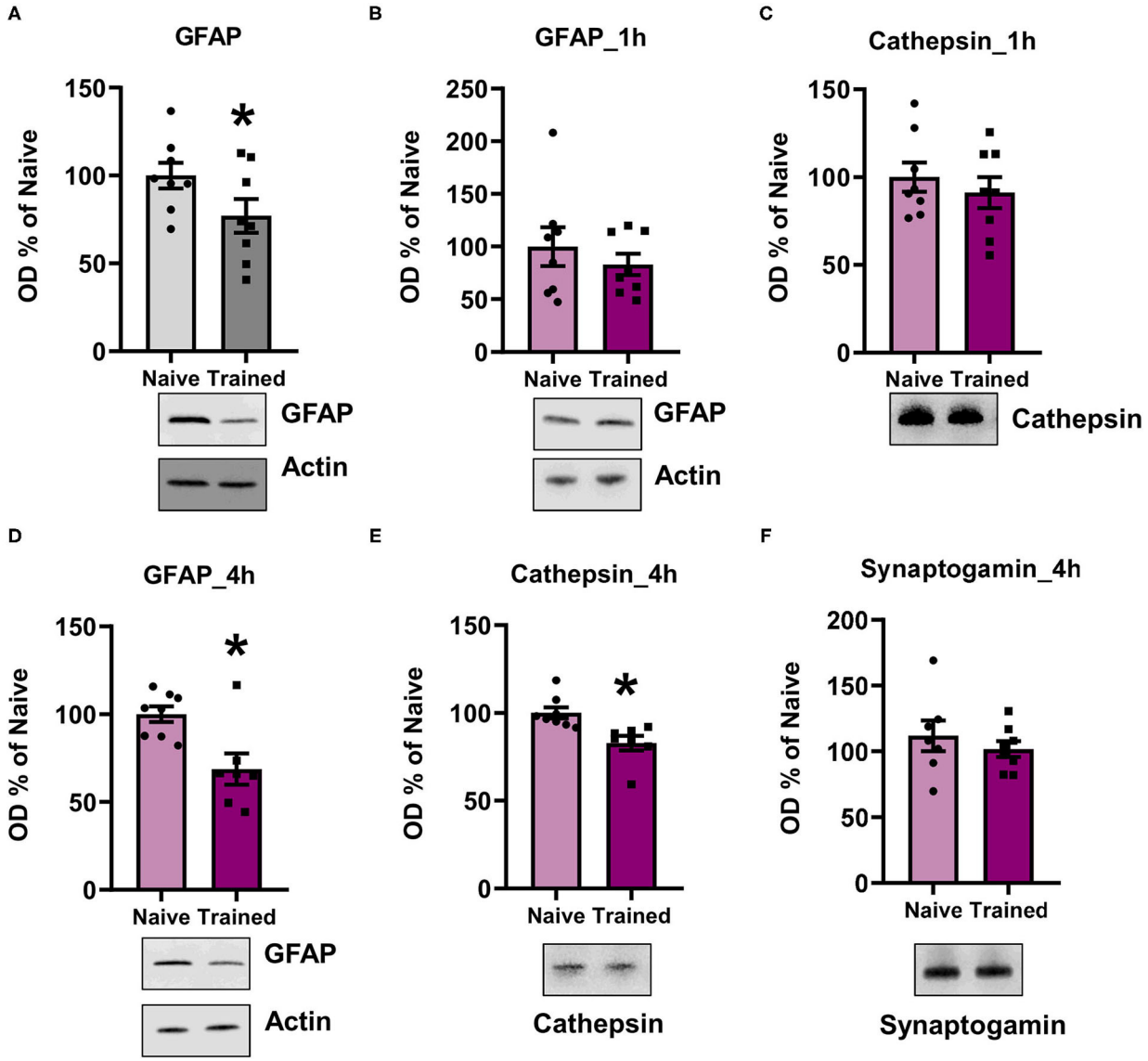


FIGURE 6 | K48 polyubiquitination targets substrates for degradation in both males and females during fear memory formation. **(A,B)** In males **(A)** but not females **(B)**, GFAP levels were decreased in the basolateral amygdala (BLA) 1 h after contextual fear conditioning ($n = 8$ per group per sex). **(C)** Cathespin-D levels did not change in the BLA of females 1 h after contextual fear conditioning. **(D–F)** In females, GFAP **(D)** and Cathespin-D **(E)**, but not synaptogamin **(F)**, levels were decreased in the BLA 4 h after contextual fear conditioning ($n = 7$–8 per group). *$P < 0.05$ from Naïve.

synaptogamin ($t_{13} = 0.8033$, $p = 0.2181$; **Figure 6F**), which was not indicated as a target in females in our proteomic analysis, in the trained animals compared to the naïve controls. These results confirm that the decreased fold-changes in TUBE enriched samples were due to K48 polyubiquitin targeting substrates for degradation by the proteasome, which is consistent with its canonical function.

DISCUSSION

Although the ubiquitin-proteasome system has been consistently implicated as a critical regulator of fear memory formation in several brain regions, including the amygdala, the specific proteins being targeted by the protein degradation process have yet to be elucidated (Lopez-Salon et al., 2001; Jarome et al., 2011; Rosenberg et al., 2016; Cullen et al., 2017; Orsi et al., 2019; Dulka et al., 2020; Devulapalli et al., 2021). Additionally, our group has recently demonstrated that there is a sex-specific role for ubiquitin-proteasome signaling in fear memory formation in the amygdala (Devulapalli et al., 2021), though little is known about the functional significance of protein degradation to fear memory consolidation and how this varies between males and females. Here, we performed the first unbiased proteomic analysis of K48 polyubiquitin protein targets in the

amygdala of male and female rats following fear conditioning. To the best of our knowledge, this study provides the first proteome-wide insight into protein targets of degradation-specific K48 polyubiquitination during fear memory formation in both male and female rodents. Importantly, we provide strong evidence that sex differences exist in the protein targets of the degradation process in the amygdala following fear conditioning. Additionally, although in males and females the major cellular pathways targeted by K48 polyubiquitination overlapped in some cases, the specific proteins did not. Together, these results provide critical information that fills the gap in knowledge concerning the proteins being targeted by protein degradation during fear memory formation in both male and female rodents.

In this study, LC/MS was conducted to identify and quantify levels of individual proteins that had increased or decreased abundance in K48-purified amygdala samples following fear conditioning. This altered abundance indicates changes in K48 targeting of the specific proteins following fear conditioning, which we defined reduced abundance as indication of active degradation of the substrate. However, it should be noted that another interpretation could exist as it is possible that the target protein could have lost the K48 polyubiquitin mark through deubiquitination, without undergoing degradation by the proteasome. Our western blot data suggests that this is unlikely, though, as we confirmed that several proteins identified as targets of K48 polyubiquitin in our LC/MS analysis had reduced expression following fear conditioning. These data indicate that K48 polyubiquitination was likely targeting these substrates for degradation during the memory consolidation process, consistent with its canonical function in the UPS. Interestingly, one of the main targets that overlapped in K48 targeting in both sexes was GFAP, a critical component regulating astrocyte morphology. Specifically, GFAP has known roles as an intermediate filament for structural support of mature astrocytes (Eng et al., 2000) and studies suggest that astrocytes undergo morphological changes following activity (Choi et al., 2016). Therefore, K48 polyubiquitination-mediated degradation of GFAP after fear conditioning may be critically involved in astrocyte plasticity that occurs during memory formation, though the cell-type specific role of protein degradation to memory formation has never been directly tested.

The proteomic analysis revealed 11 proteins with significantly enhanced levels following K48 purification in the amygdala of males and females, with no overlap in protein targets between sexes. Conversely, 106 proteins had decreased abundance in K48-purified samples collected from the amygdala following fear conditioning, of which only 3 proteins overlapped between sexes. This minimal overlap supports an important sex-specific role for protein degradation in fear memory formation. In our previous study, we reported that protein degradation *via* K48 polyubiquitination was not increased in the amygdala of females following learning, though it was increased in males (Devulapalli et al., 2021). These results were obtained through western blot analysis, which, unlike LC/MS, quantified the global level of K48 polyubiquitination in the nuclear fraction and was not specific or sensitive enough to identify the changes in K48 polyubiquitin levels of individual proteins in the female amygdala. Importantly, the larger deficit between the number of proteins with increased as opposed to decreased levels following K48 purification in females and the minimal overlap in protein targets between sexes may explain why our previously reported western blot analysis was unable to detect changes in K48 polyubiquitination following fear conditioning in the female amygdala. Based on these findings, it is clear that females do engage protein degradation in the amygdala during fear memory formation. The current findings provide a greater context for understanding sex-differences in the role of degradation-specific K48 polyubiquitination during fear memory formation in the amygdala.

To better understand the role of the proteins being targeted by K48 polyubiquitination, pathway analysis was conducted. The most notable pathways identified in males were the neuroprotective role of THOP1, DNA damage pathways, and ERK5 signaling, all of which have been previously implicated in memory formation. For example, THOP1 knockout mice have impaired memory retention (Santos et al., 2019), and our group and others have previously reported that DNA double-stranded breaks occur and are necessary for memory formation and modification in the hippocampus (Madabhushi et al., 2015; Navabpour et al., 2020), though this has yet to be reported in the amygdala. Furthermore, ERK/MAPK signaling has long been implicated in memory consolidation in the amygdala and other brain regions (Chwang et al., 2006; Ehrlich and Josselyn, 2016). Collectively, the pathways identified in our study as targets of learning-related changes in protein degradation in the male amygdala support previous reports of specific molecular processes and signaling pathways involved in fear memory formation. Alternatively, in females, transcriptional regulatory network in embryonic stem cells was the only significant pathway identified. Although the pathway is broad, several important genes related to embryonic stem cells have also been implicated in memory formation, such as WNT and SHH (Maguschak and Ressler, 2011; Hung et al., 2014), and other factors, such as SOX2, have been implicated in glia and neuron functioning (Mercurio et al., 2019). Importantly, though the protein targets and pathways identified in males and females contain little overlap, there was likely a shared role for structural remodeling, which is based on the many overlapping non-ubiquitinated proteins related to the cytoskeleton that were identified in our network analyses between sexes. Collectively, these data suggest that while the protein targets of K48 polyubiquitination appear to be largely different between males and females, there may be a shared role for structural remodeling between sexes, though more research will be needed to confirm this observation.

The findings presented here provide novel insight into the specific proteins that are being targeted by K48 polyubiquitination in the amygdala of male and female rats following fear conditioning. However, there is still much to be discovered about the specific role of K48 polyubiquitination-related signaling during fear memory formation. One limitation of our study was that we did not directly manipulate K48 polyubiquitination and test the effects on memory and target substrate stability. Since K48 is a linkage site on the ubiquitin protein, which has multiple (4) coding genes, it is difficult to

specifically manipulate endogenous K48 polyubiquitination without altering other lysine-linkage sites. In fact, due to these difficulties, to date, there is only indirect evidence for degradation-specific K48 polyubiquitination in fear memory formation, which comes from proteasome manipulations (Lopez-Salon et al., 2001; Artinian et al., 2008; Lee et al., 2008; Jarome et al., 2011; Rodriguez-Ortiz et al., 2011; Reis et al., 2013; Felsenberg et al., 2014; Figueiredo et al., 2015; Furini et al., 2015; Rosenberg et al., 2016; Devulapalli et al., 2021). However, such manipulations are not specific to K48 and actually cause an artificial accumulation of many forms of polyubiquitinated proteins, which can deplete the free ubiquitin pool. As a result, while the evidence obtained through proteasome manipulation is valuable, other lysine-linkage sites besides K48 that do and do not act as a degradation mark would be affected by the manipulations of the proteasome, making interpretation of results difficult. This may explain why SHANK, MOV10 and IkB were not identified as significant targets of K48 in either sex during fear memory formation in our dataset, despite previous evidence demonstrating proteasome-mediated degradation of these proteins following learning (Lopez-Salon et al., 2001; Yeh et al., 2002; Lee et al., 2008; Jarome et al., 2011). Furthermore, this could have occurred due to these putative degradation targets being marked by a polyubiquitin chain carrying another linkage site, such as K11 (Xu et al., 2009; Meyer and Rape, 2014; Meza Gutierrez et al., 2018; Musaus et al., 2020). Unfortunately, until more precise technology is developed, the specific role of K48 polyubiquitination in fear memory formation in any brain region will remain equivocal. Identifying a method for specifically manipulating lysine-specific ubiquitin linkage sites will be key for elucidating the roles of K48 polyubiquitination during fear memory formation, as well as whether other ubiquitin linkage sites have similar roles and targets between the sexes following fear conditioning.

Another limitation could be the time of tissue collection. In the present study, amygdala tissue was only collected 1 h after fear conditioning, which has been consistently reported by our group as the earliest peak in protein degradation after training (Jarome et al., 2011, 2013; Reis et al., 2013; Orsi et al., 2019; Devulapalli et al., 2021). However, later time points, such as 4 h, maintain significant levels of protein degradation in the amygdala (Jarome et al., 2013) and other brain regions (Lopez-Salon et al., 2001; Rosenberg et al., 2016). Additionally, protein homeostasis is necessary as protein synthesis and degradation are both required for long-term memory (Fonseca et al., 2006; Cajigas et al., 2010). Therefore, it is possible then that unique proteins may be targeted by K48 polyubiquitination at different time points following fear conditioning, as protein degradation remains at a significant level for several hours. Thus, identifying targets of K48 polyubiquitination at different time points after fear conditioning may identify new proteins involved in memory consolidation and help elucidate the potential roles of K48 polyubiquitination in this process. Despite this, the findings presented here still provide critical insight into the sex-specific K48 polyubiquitin protein targets in the amygdala during fear memory formation. Future studies should use this K48-TUBE-LC/MS approach to examine how the protein degradation targets change across time during the memory consolidation process.

In the present study associative control animals were not used due to our previous reports which demonstrate that exposing rats to the context and shock alone or in a non-associative manner (immediate shock training) does not engage protein degradation in the amygdala, as measured by changes in functional proteasome activity and K48 and proteasome-specific protein polyubiquitination (Jarome et al., 2011, 2013; Orsi et al., 2019). However, these previous studies were only conducted using male rodents, so it is unclear if shock or contextual stimuli alone would induce protein degradation changes in the amygdala of female rats. Therefore, it is possible that some of the K48 polyubiquitin protein targets identified in the amygdala of females could be engaged as a result of context or shock alone, independent of associative learning. While in females we cannot completely dissociate those proteins that are targeted by K48 polyubiquitination in response to the context-shock association compared to either stimuli independently, our findings still indicate an important sex effect in the proteins being targeted for protein degradation within the amygdala following fear conditioning. Further validation of the learning-specificity of these K48 polyubiquitin targets in females will need to be addressed in future studies.

In conclusion, we present the first detailed analysis of sex differences in the target substrates of the proteasome-mediated protein degradation process in the amygdala during fear memory formation. These results provide the first evidence suggesting potential sex-specific functions of the K48 polyubiquitination-mediated protein degradation process during fear memory formation, which could have important implications for understanding the etiology of sex-related differences in the formation of fear memories that may underlie post-traumatic stress disorder. However, it should be noted that as we were unable to confirm that the animals used in the present study presented behaviors characteristic of PTSD, we cannot conclude that these identified targets of protein degradation are pathological in nature. Instead, our findings may have more direct implications in sex-dependent protein degradation processes underlying learning and memory in general, which could significantly advance our understanding of how ubiquitin proteasome activity contributes to memory formation in cells.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://www.ebi.ac.uk/pride/archive/, PXD027544.

ETHICS STATEMENT

The animal study was reviewed and approved by Virginia Polytechnic Institute and State University Institutional Animal Care and Use Committee (protocol #18-019).

AUTHOR CONTRIBUTIONS

KF, MM, and TJ designed the experiments. KF, MM, and KM conducted the experiments. WR and RH performed mass

spectrometry. SN performed analysis of mass spectrometry data. KF, SN, MM, and TJ wrote the manuscript. All authors edited, reviewed, and approved the manuscript for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2021.716284/full#supplementary-material

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Mitochondrial Extracellular Vesicles – Origins and Roles

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Extracellular vesicles (EVs) have emerged in the last decade as critical cell-to-cell communication devices used to carry nucleic acids and proteins between cells. EV cargo includes plasma membrane and endosomal proteins, but EVs also contain material from other cellular compartments, including mitochondria. Within cells, mitochondria are responsible for a large range of metabolic reactions, but they can also produce damaging levels of reactive oxygen species and induce inflammation when damaged. Consistent with this, recent evidence suggests that EV-mediated transfer of mitochondrial content alters metabolic and inflammatory responses of recipient cells. As EV mitochondrial content is also altered in some pathologies, this could have important implications for their diagnosis and treatment. In this review, we will discuss the nature and roles of mitochondrial EVs, with a special emphasis on the nervous system.

Keywords: mitochondria, extracellular vesicle, metabolism, inflammation, mitochondrial quality control

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INTRODUCTION

Mitochondria play a crucial role in maintaining metabolic homeostasis. This includes ATP production, but also a range of biosynthetic and catabolic pathways required for cellular homeostasis. In addition, mitochondria regulate cell death and the production of reactive oxygen species (ROS) (Friedman and Nunnari, 2014; Murphy et al., 2016). To perform these tasks, mitochondria require proteins that are encoded by both nuclear and mitochondrial DNA (mtDNA) (West and Shadel, 2017; Russell et al., 2020). Mutations in mitochondrial genes encoded by either genome are associated with neurological or muscular pathologies (Vafai and Mootha, 2012). Similarly, damaged or otherwise dysfunctional mitochondria can cause cellular damage through increased mitochondrial ROS production (West and Shadel, 2017). These events can also lead to the release of mtDNA to the cytosol where it acts as a damage-associated molecular pattern (DAMP) that triggers inflammation (West and Shadel, 2017). Given the potentially deleterious consequences of these events, cells have evolved a number of quality control mechanisms, including mitophagy and mitochondria-derived vesicles (MDVs), that promote the lysosomal degradation of damaged mitochondrial components. Failure of these quality control mechanisms and accumulation of dysfunctional mitochondria result in many pathologies ranging from cardiovascular diseases to cancer (Srinivasan et al., 2017; Johnson et al., 2021). However, the nervous

system is particularly sensitive to alterations in mitochondrial quality control mechanisms and alterations in these are particularly prominent in neurodegenerative diseases. Intriguingly, recent studies have shown that mitochondrial content can also be found outside of cells, either as free mtDNA, functional mitochondria or within extracellular vesicles (EVs), where it could participate in the etiology of these diseases (Yousefi et al., 2009; Keshari et al., 2012; Lindqvist et al., 2018; Singel et al., 2019).

EXTRACELLULAR VESICLES

Most cells release an array of molecules and vesicles into their environment that serve to communicate with other cells or get rid of unwanted cellular material (Devhare and Ray, 2018; Mathieu et al., 2019). This secreted material includes EVs, lipid bilayer-delimited vesicles that carry proteins, DNA and RNA molecules. As such, EVs have been implicated in a large range of physiological and pathological processes, including immune responses, cancer progression, and neurodegeneration (Shanmughapriya et al., 2020; Ma et al., 2021; She et al., 2021). EVs can be divided into different types based on their size and origin: exosomes are 30–150 nm in size and derived from intracellular vesicles (multivesicular bodies, a form of late endosome); microvesicles directly bud off the plasma membrane and are usually between 100 and 1000 nm; while apoptotic bodies are generated from dying cells and are generally between 100 and 5000 nm (Devhare and Ray, 2018; Théry et al., 2018). This simple classification however does not take into account that EVs from different origins can have similar sizes and densities, or that distinct vesicles within the same class of EVs express diverse surface markers and transport distinct cargoes (Kowal et al., 2016; Théry et al., 2018). As a result, while some common themes have emerged, there is a great heterogeneity in the structure and functions reported for EVs.

In recent years, the discovery of mitochondrial content within EVs has led to the identification of a number of biological functions of extracellular mitochondrial content, including outsourcing mitochondrial degradation, activating inflammation, and modulating metabolism. In the following sections, we will discuss the nature and roles of extracellular mitochondrial content, as well as the major gaps in our understanding of extracellular mitochondrial content, with a focus on the nervous system whenever the data is available.

EVIDENCE FOR THE RELEASE OF MITOCHONDRIAL MATERIAL FROM CELLS

A number of studies have shown that mitochondria or mitochondrial components (proteins, mtDNA, and cardiolipin) are secreted by cells. This extracellular mitochondrial content was found in media from cells in culture, as well as in biological fluids (plasma and cerebrospinal fluid) under both physiological and pathological conditions (Chou et al., 2017; Al Amir Dache et al., 2020). Among this, several studies have specifically showed extracellular mitochondrial content within the nervous system. This includes astrocytes and microglia under basal conditions (Guescini et al., 2010) and in cellular models of amyotrophic lateral sclerosis (SOD1) and Huntington's disease (Htt) (Joshi et al., 2019), neural stem cells (Peruzzotti-Jametti et al., 2021), glioblastoma (Guescini et al., 2010), as well as animal models of subarachnoid hemorrhage (Chou et al., 2017) and Down syndrome (D'Acunzo et al., 2021).

Three types of extracellular mitochondrial content have been reported: free mtDNA, functional mitochondria, and mitochondrial content within EVs. Some cells including neutrophils release free mtDNA that function to stimulate inflammation and fight pathogens (Yousefi et al., 2009; Keshari et al., 2012). Elevated extracellular mtDNA was also reported in rheumatoid arthritis (Collins et al., 2004) and depressive patients (Lindqvist et al., 2018), suggesting that mtDNA release is associated with several pathological conditions. On the other hand, most studies looking at extracellular mitochondrial components found evidence that it was contained within membrane-bound vesicles, either as cell-free functional mitochondria or mitochondrial content packed within EVs (**Table 1**). The first evidence of functional cell-free extracellular mitochondria came from the study of platelets, where it was shown that their activation in a pro-inflammatory setting (LPS stimulation) led to the release of free and encapsulated active mitochondria (Boudreau et al., 2014). These were defined by their ability to be stained with mitochondrial dyes (mitotracker or JC-1), their oxygen consumption and their ultrastructure. Subsequent studies showed evidence for the presence of cell-free mitochondria associated with cell lines, microglia and biological fluids (Joshi et al., 2019; Puhm et al., 2019; Al Amir Dache et al., 2020; Choong et al., 2020; Levoux et al., 2021; Peruzzotti-Jametti et al., 2021).

In contrast, other studies are more consistent with specific mitochondrial components being released within EVs. In these studies, specific mitochondrial proteins were excluded from EVs and EM analysis did not show vesicles with typical mitochondrial structure (Torralba et al., 2018; D'Acunzo et al., 2021; Todkar et al., 2021). Nevertheless, the excluded proteins varied across the studied [i.e., TOM20 is present only in Todkar et al. (2021); VDAC is excluded from Torralba et al. (2018) but not D'Acunzo et al. (2021) suggesting the presence of different types of mitochondrial EVs].

Several factors can help explain the differences in mitochondrial EV content. First, a number of studies reporting functional extracellular mitochondria used immune-related cells (platelets and microglia) in a pro-inflammatory (e.g., LPS) setting, while the studies reporting selective release of mitochondrial content mainly used resting non-immune cell lines. This suggests that cellular conditions greatly affect the nature and amount of extracellular mitochondrial material. In fact, both LPS and the Complex III inhibitor Antimycin A affect the release of mitochondrial content (Bernimoulin et al., 2009; Boudreau et al., 2014; Joshi et al., 2019; Choong et al., 2020; Crewe et al., 2021; Peruzzotti-Jametti et al., 2021; Todkar et al., 2021) although the resulting effects are not always consistent.

TABLE 1 | Nature and properties of mitochondrial EVs.

EV source	Disease link	Type of EV	Effect on target cells	References
Microglia, astrocytes (LPS)	Neurodegeneration	Mitochondria, lEVs (<10 k × *g*)	Pro-inflammatory effect	Joshi et al., 2019
THP1 (LPS)		Mitochondria, lEVs (<18 k × *g*)	Pro-inflammatory effect	Puhm et al., 2019
Platelets (LPS)	Rheumatoid arthritis	Mitochondria, EVs (>100 nm), MTR^+, $JC1^+$	Pro-inflammatory effect	Boudreau et al., 2014
Neural stem cells		EVs (>1 k × *g*), MTR^+, $JC1^+$	Stimulates OXPHOS, inhibits the pro-inflammatory activity of LPS-activated macrophages	Peruzzotti-Jametti et al., 2021
Platelets	Wound healing	EVs (trans-well cultures)	Stimulates pro-angiogenic properties of MSCs causes metabolic remodeling	Levoux et al., 2021
MSCs		sEVs + lEVs	In macrophages, enhances their bioenergetics, blocks their activation	Phinney et al., 2015
T cells		sEVs (>10 k × *g*)	Induces antiviral responses in dendritic cells	Torralba et al., 2018
Plasma, breast cancer cells	Cancer	sEVs (>12 k × *g*)	Exit from metabolic dormancy	Sansone et al., 2017
Bronchoalveolar lavage Myeloid-derived regulatory cells	Asthma	sEVs (>10 k × *g*), MTR^+ Exosomes kit isolation	Transfer to T cells, increasing ROS	Hough et al., 2018
Adipocytes	Heart ischemia/reperfusion	sEVs (>10 k × *g*)	Increases ROS in cardiomyocytes, protective role	Crewe et al., 2021
MEFs, U2OS		sEVs + lEVs	Oxidized mitochondrial content is excluded from EVs, preventing DAMP-induced inflammation in RAW cells	Todkar et al., 2021
PC12, fibroblasts, cerebrospinal fluid	Parkinson's disease	EVs (<60 k × *g*), $JC1^+$	Mitochondria quality control, not tested on recipient cells	Choong et al., 2020
Cerebrospinal fluid	Subarachnoid hemorrhage	lEVs (14 k × rpm), $JC1^+$	Not tested on target cells, correlates with better outcome	Chou et al., 2017
Brain	Down syndrome	sEVs (>10 k × *g*)		D'Acunzo et al., 2021
Melanoma cells, plasma	Melanoma	sEVs + lEVs		Jang et al., 2019
Plasma, cancer cells	Cancer	sEVs (<16 k × *g*), MTR^+		Al Amir Dache et al., 2020

EV type classification was normalized to reflect the MISEV2018 guidelines (Théry et al., 2018) and the centrifugation speed noted when available. sEVs, small EVs; lEVs, large EVs; MTR, mitotracker.

Beside the use of different systems, a major cause of the discrepancies in reported extracellular mitochondrial content is likely the variations in isolation protocols. In fact, while studies reporting intact active mitochondria mainly analyzed larger vesicles isolated at lower centrifugation speeds (<20 k × *g*), studies reporting selective mitochondrial content have analyzed much smaller vesicles (100 k × *g*), sometimes at the exclusion of the smaller (<10 k × *g*) vesicles (Torralba et al., 2018; D'Acunzo et al., 2021). This is a crucial point as most mitochondrial EV content has been reported to fractionate in larger vesicles in a thorough proteomic EV analysis (Kowal et al., 2016). A more complete analysis of mitochondrial EVs in accordance with the MISEV2018 guidelines (Théry et al., 2018) will thus be required to identify the exact nature of mitochondrial EV content under different conditions. This includes using more sensitive fractionation [i.e., OptiPrep (Kowal et al., 2016)] along with recognized markers for the different fractions (Théry et al., 2018) and the quantification of a larger number of proteins (outer membrane, inner membrane, and matrix). It will also be important to use membrane potential-sensitive dyes (TMRM, JC-1) along with proper controls (depolarization with CCCP). Given the complexity of other types of EVs, it is likely that several biochemically and functionally distinct types of mitochondrial EVs will emerge, with each their own triggers and regulation. In support of this, two studies found complementary mitochondrial EVs, one including ETC component but excluding mtDNA-associated proteins (D'Acunzo et al., 2021), the other containing

the mtDNA-associated protein TFAM but excluding ETC components (Torralba et al., 2018).

MECHANISMS REGULATING MITOCHONDRIA EXTRACELLULAR VESICLES

Another important step in the proper characterization of mitochondrial EVs is the identification of the mechanism(s) through which the release occurs. Based on the size of extracellular mitochondria and some mitochondrial EVs, it was originally suggested that these are released as microvesicles, directly though plasma membrane blebbing. However, live cell imaging of mesenchymal stem cells (MSCs) suggested that mitochondrial content is first incorporated into LC3-positive autophagosomes before being released (Phinney et al., 2015), more consistent with an exosomal/endolysosomal mechanism.

An endosomal origin is also suggested by recent studies linking extracellular mitochondrial content to MDVs (Crewe et al., 2021; Todkar et al., 2021; Vasam et al., 2021). MDVs are small vesicles that deliver mitochondrial content to other organelles including peroxisomes and late endosomes/lysosomes, the latter serving to degrade damaged mitochondrial components (Sugiura et al., 2014). We recently demonstrated that in resting cells, the inclusion of mitochondrial content inside EVs requires MDV formation (Todkar et al., 2021). This conclusion is also supported by a recent study in adipocytes (Crewe et al., 2021) and a proteomic analysis of MDVs (Vasam et al., 2021). This process is regulated by the Parkinson's disease (PD) associated protein Parkin, which plays a key role in mitochondrial quality control by promoting both the autophagic degradation (mitophagy) of damaged mitochondria and the generation of MDVs containing oxidized mitochondrial components destined for lysosomal degradation. Parkin activation thus targets damaged mitochondrial content to lysosomes for degradation, therefore preventing their inclusion into EVs (Todkar et al., 2021). Consistent with this, inhibition of lysosomal activity increases the release of mitochondrial EVs (Crewe et al., 2021). Interestingly, in a distinct study, Parkin overexpression was shown to prevent the release of extracellular mitochondria while its mutation in patient fibroblasts stimulated it (Choong et al., 2020), further supporting a role for the Pink1/Parkin pathway in the regulation of mitochondrial EVs. Interestingly, a similar Parkin-regulated MDV pathway controls the presentation of mitochondrial antigens to the immune system, while deletion of the Parkin activator Pink1 causes PD symptoms in mice with a gut infection (Matheoud et al., 2016, 2019). As Parkin-dependent sorting of oxidized mitochondrial content prevents the release of pro-inflammatory mitochondrial content (Todkar et al., 2021), both pathways support the role of inflammation in the etiology of PD. Whether the two pathways act together or independently however remains to be determined.

Overall, emerging evidence indicates that cells package and release some of their mitochondrial content in the extracellular milieu. While this is likely to involve several functionally distinct vesicles and mechanisms depending on the cellular context, MDVs likely plays a crucial role in this process. Nevertheless, while the mechanism regulating mitochondrial EVs are still emerging, a larger body of work has started to address their roles in both physiological and pathological contexts.

FUNCTIONAL ROLES OF EXTRACELLULAR MITOCHONDRIAL CONTENT

Extracellular mitochondrial content has been proposed to have different roles depending on the cells involved and the stimulus that triggered its release (**Table 1**). In the central nervous system, oligodendrocytes transfer material to be degraded to microglia, allowing efficient removal of cellular material without risking immune activation. In this setting, EVs containing oligodendrocyte-specific proteins are selectively endocytosed by microglial cells and sent to lysosomes for degradation (Fitzner et al., 2011). Similarly, damaged axonal mitochondria within the optic nerve can be transferred to astrocytes for lysosomal degradation (Davis et al., 2014), although in this case, the role of EVs was not assessed. A role for EVs in this process is nevertheless supported by the observation that MSCs use EVs to export mitochondria to macrophages to outsource mitophagy (Phinney et al., 2015). However, others have shown that damaged mitochondrial content (oxidized or mitochondria lacking mtDNA) is retained within the donor cell (Mahrouf-Yorgov et al., 2017; Todkar et al., 2021). While this could be down to differences in the nature of the EVs and the recipient cells, accumulating evidence suggests that the main roles of mitochondrial EVs could instead be related to alterations in cellular metabolism and inflammation.

Cells, especially MSCs, have been known for a long time to be able to transfer mitochondrial content to cells lacking mtDNA, thereby rescuing their metabolic activity (Spees et al., 2006; Dong et al., 2017; Peruzzotti-Jametti et al., 2021). However, the nature and metabolic functions of physiological mitochondrial transfer are still emerging. Several recent studies have shown that macrophages endocytose mitochondrial EVs released by other cells, which stimulates their mitochondrial activity (Hough et al., 2018; Joshi et al., 2019; Puhm et al., 2019; Peruzzotti-Jametti et al., 2021). This occurred in a number of *in vitro* and *in vivo* setting, including a model of experimental autoimmune encephalomyelitis where astrocytes also endocytosed mitochondria (Peruzzotti-Jametti et al., 2021). Similarly, endocytosis of mitochondrial EVs stimulates the mitochondrial activity of other cell types including MSCs (Levoux et al., 2021) and brain microvasculature (Liu et al., 2019), the latter leading to reduced infarct size following a stroke (Middle Cerebral Artery Occlusion in rats). Altogether, these results indicate a role for mitochondrial transfer in regulating metabolism at both the level of cells and the organism (Brestoff et al., 2021).

Free extracellular mitochondria and mitochondrial EVs have also been shown to regulate inflammation, although the effect can be either anti- or pro-inflammatory depending on the context. Extracellular mitochondria were originally associated

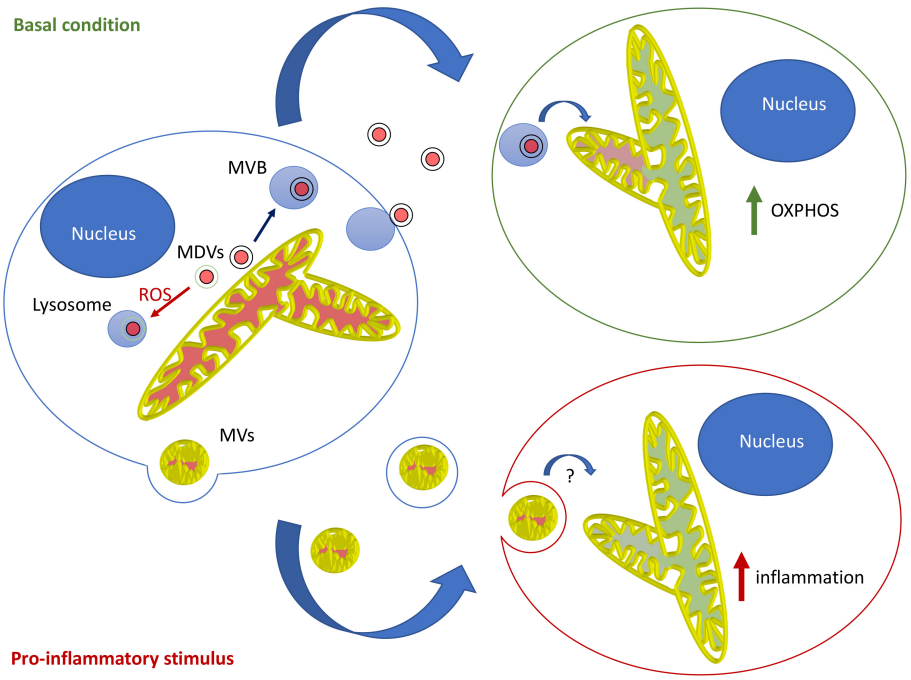

FIGURE 1 | Model for the roles and mechanisms of secretion of mitochondrial EVs. Under basal conditions, MDVs are required to package mitochondrial components into mitochondrial EVs, which serve to increase OXPHOS in recipient cells. In this setting, oxidized mitochondrial components are not released into EVs but rather sent to lysosomes for degradation through a different type of MDV (Parkin-dependent). On the other hand, pro-inflammatory stimuli like LPS cause the release of free mitochondria or mitochondria packaged within microvesicles that further stimulate inflammation.

with increased inflammation in setting that were already pro-inflammatory. For example, LPS stimulation of immune cells, including platelets, stimulates the release of free and encapsulated mitochondria that further drive inflammation (Boudreau et al., 2014). This was also demonstrated in models where expression of neurotoxic proteins associated with neurodegenerative diseases in microglia stimulated mitochondria-dependent activation of astrocytes and neuronal death (Joshi et al., 2019). Nevertheless, immune stimulation by mitochondrial content can occur in the absence of inflammation. In this case, T lymphocytes have been shown to activate the antiviral state of antigen presenting cells (dendritic cells) by transferring mtDNA (Torralba et al., 2018).

In contrast, other studies have reported that mitochondrial transfer to macrophages has anti-inflammatory roles (Phinney et al., 2015; Peruzzotti-Jametti et al., 2021). Importantly, while the pro-inflammatory role of extracellular mitochondria is activated by pro-inflammatory treatments (i.e., LPS), the anti-inflammatory activity is promoted by the transfer of mitochondrial content from resting cells. In this case, the anti-inflammatory activity was directly linked to mitochondrial EV-driven increase in OXPHOS in the recipient macrophages. This is likely down to the metabolic differences between pro-inflammatory (M1) and anti-inflammatory (M2) macrophages. Upon stimulation with a pro-inflammatory signal, naïve macrophages differentiate toward an M1, glycolytic phenotype. In contrast, differentiation toward a M2 fate promotes mitochondrial OXPHOS. Thus, the hypothesis is that by stimulating OXPHOS in M1 macrophages, mitochondrial EVs will downregulate their pro-inflammatory phenotype. This is indeed what was observed when LPS-activated macrophages were exposed to EVs isolated from neural stem cells (Peruzzotti-Jametti et al., 2021). In addition, release of highly immunogenic oxidized mitochondrial content is actively repressed by cells (Todkar et al., 2021), preventing inflammatory responses and avoiding the transfer of non-functional material that could hamper metabolic fine-tuning of distant cells.

Overall, the current evidence suggests that in most contexts, mitochondrial EVs serve to modulate the metabolism of distant cells and prevent unwanted immune activation. This likely involves MDV-dependent loading of mitochondrial content into EVs, loading that is blocked by mitochondrial damage in a Parkin-dependent manner. In contrast, the pro-inflammatory role of mitochondrial EVs probably requires microvesicle-like EVs and is likely restricted to specific cells and circumstances. Importantly, this model supports the notion that different types of mitochondrial EVs fulfill different roles, with MDV/endosome-derived EVs regulating metabolism and LPS-induced, microvesicle-like EVs promoting inflammation under specific conditions (**Figure 1**).

CONCLUSION

As the field of EVs has been expanding, it is now clear that a variety of cells, including neurons, astrocytes, and microglia

actively release mitochondrial content in their environment. However, the exact nature and roles of these mitochondrial EVs remain to be fully defined. In that respect, a better characterization of mitochondrial EVs released under a range of physiological and pathological conditions will certainly be crucial. In addition, while the presence of mitochondrial EVs has clearly been established in the nervous system, their roles remain elusive since only a very limited number of studies have started to address this question. As the brain is metabolically highly active and astrocytes are a key partner in the regulation of neuronal function, it will be important to determine if the metabolic changes occurring in macrophages and MSCs following mitochondrial transfer also occur between neurons, astrocytes and microglia. This is certainly plausible as astrocytes and microglia do release active mitochondrial EVs (Joshi et al., 2019).

A better understanding of the mechanisms underlying mitochondrial EV transfer between cells could also lead to new diagnostic tools and treatments. For example, mitochondrial EVs could represent an important biomarker for disease diagnosis, especially for neurodegenerative disorders. In addition, mitochondrial EVs could serve as a therapeutic tool not only in neurodegenerative diseases, but also for stroke and other brain pathologies, for example by the use of MSCs.

AUTHOR CONTRIBUTIONS

Both authors listed have made a substantial, direct and intellectual contribution to the work and approved it for publication.

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Transcriptome of the Krushinsky-Molodkina Audiogenic Rat Strain and Identification of Possible Audiogenic Epilepsy-Associated Genes

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Audiogenic epilepsy (AE), inherent to several rodent strains is widely studied as a model of generalized convulsive epilepsy. The molecular mechanisms that determine the manifestation of AE are not well understood. In the present work, we compared transcriptomes from the *corpora quadrigemina* in the midbrain zone, which are crucial for AE development, to identify genes associated with the AE phenotype. Three rat strains without sound exposure were compared: Krushinsky-Molodkina (KM) strain (100% AE-prone); Wistar outbred rat strain (non-AE prone) and "0" strain (partially AE-prone), selected from F2 KM × Wistar hybrids for their lack of AE. The findings showed that the KM strain gene expression profile exhibited a number of characteristics that differed from those of the Wistar and "0" strain profiles. In particular, the KM rats showed increased expression of a number of genes involved in the positive regulation of the MAPK signaling cascade and genes involved in the positive regulation of apoptotic processes. Another characteristic of the KM strain which differed from that of the Wistar and "0" rats was a multi-fold increase in the expression level of the *Ttr* gene and a significant decrease in the expression of the *Msh3* gene. Decreased expression of a number of oxidative phosphorylation-related genes and a few other genes was also identified in the KM strain. Our data confirm the complex multigenic nature of AE inheritance in rodents. A comparison with data obtained from other independently selected AE-prone rodent strains suggests some common causes for the formation of the audiogenic phenotype.

Keywords: audiogenic epilepsy, Wistar rats, KM rat strain, transcriptomic analysis (RNA-seq), *Ttr*, *Msh3*, MAPK signaling cascade

INTRODUCTION

Epilepsy is one of most common neurological diseases and up to 30% of all epilepsy cases are characterized by high pharmacoresistance. Many drugs used to treat epilepsy are characterized by severe side effects (Ornoy, 2009; Perucca and Gilliam, 2012; Chen et al., 2017). Accordingly, there is a need to develop new antiepileptic drugs, which requires the availability of valid laboratory models

of epilepsy that permit investigation into the molecular mechanisms underlying epileptogenesis and evaluation of the effectiveness of new anticonvulsants. Several genetic models of epilepsy have been described, including rodent model strains. The development of convulsive seizures in response to intense sound (audiogenic epilepsy, AE) is induced in rodent models of epilepsy. In contrast to chemically induced or electroshock-provoked seizures, animals with the AE phenotype recover quickly after seizures and can be used as "self-controls" in long-term experiments with repeated provocation of seizures in the same animal (for reviews of different epilepsy models, see Bizière and Chambon, 1987; Kupferberg, 2001; Löscher, 2017). It should be noted that the so called refractory epilepsy is also present in the list of epilepsy forms in humans, most of these cases being also pharmacoresistant. The partial or generalized seizures in these cases occur in response to sensory stimulation (tactile, optical or acoustical, or even as a reaction to the certain mood of a patient). These cases are rather rare (about 6%) and not well analyzed, and there are only sparse indications concerning their genetic base (Xue and Ritaccio, 2006; Maguire, 2012; Taylor et al., 2013; Dinopoulos et al., 2014; Dewhurst et al., 2015; Sala-Padró et al., 2015; Atalar et al., 2020; Geenen et al., 2021). As for animal models the AE rodent strains used have certain benefits in comparison to chemical and electroshock models and they are widely used, as they permit to make chronical observations (Garcia-Cairasco et al., 2017). The genetic basis of AE development in rodents has attracted the attention of many groups (Ribak et al., 1988; Garcia-Cairasco, 2002; Poletaeva et al., 2017; Damasceno et al., 2018, 2020; Díaz-Casado et al., 2020). The data currently available generally support previously postulated hypotheses of polygenic inheritance of AE (Poletaeva et al., 2017), which is also characteristic of many forms of human epilepsy (Koeleman, 2018; Leu et al., 2020).

In the course of tonic audiogenic convulsion development (starting with the activation of acoustic nuclei in the medulla), the intense excitation process activates a specific brainstem neuronal network, in which inferior and superior colliculi (IC + SC) comprising the *corpora quadrigemina* play key roles. This activation was demonstrated in AE-prone rodent strains selected independently: GEPR (genetic epilepsy-prone rats, United States), WAR (Wistar audiogenic rats, Brazil), KM (Krushinsky-Molodkina, Russia), and in AE-prone GASH/Sal hamster strain (Faingold et al., 1992; Ribak et al., 1993; Faingold, 1999, 2002; Faingold and Casebeer, 1999; Garcia-Cairasco, 2002; Merrill et al., 2003; Doretto et al., 2009; Solius et al., 2016, 2019; Prieto-Martín et al., 2017; Ribak, 2017; Revishchin et al., 2018). It is now widely accepted that at the biochemical and physiological levels, AE proneness is associated with anomalies in brain GABAergic and glutamatergic systems, particularly in the *corpora quadrigemina* (Ribak et al., 1993; Korotkov et al., 2015; Solius et al., 2016; Chernigovskaya et al., 2019). The specific morphology of these brain stem structures was described in detail (Solius et al., 2016, 2019; Ribak, 2017; Revishchin et al., 2018), as well as the increased activity of the MAPK/ERK pathway in the striatum and hippocampus of KM rats (Dorofeeva et al., 2017; Chernigovskaya et al., 2019). Notably, in KM and GEPR rat strains, dopamine, norepinephrine, and serotonin brain levels significantly differed from those of control non-AE-prone animals (Poletaeva et al., 2017) and of histamine and adenosine system rats as well (Feng and Faingold, 2000; Vinogradova et al., 2007; Midzyanovskaya et al., 2016).

In this work, we investigated the profile of gene expression in the *corpora quadrigemina* consisting of two structures, inferior and superior colliculi (IC and SC) in three rat strains not exposed to sound (i.e., the "background" profile) to identify genetic traits associated with a hereditary predisposition to AE. It is known that an audiogenic seizure begins after an intense acoustic "startle reaction" with a phase of "wild running", which is accompanied by clonic-tonic convulsions (Poletaeva et al., 2017). It was described previously (using different AE models) that the initial "ignition" of the seizure generating process is localized in IC, while the further generation of the wild run stage depends on the involvement of SC neuronal network. Both of these structures carrying several genetic anomalies, found in the best described AE rat models (Ribak et al., 1993; Faingold, 1999, 2002; Faingold and Randall, 1999; Doretto et al., 2009; N'Gouemo et al., 2009, 2010; Solius et al., 2016; Coleman et al., 2017; García et al., 2017; Ribak, 2017; Chernigovskaya et al., 2018; Revishchin et al., 2018). Nevertheless, the results of these and other works show that, although the beginning of the impulse in SC precedes that in IC, both of these brain stem structures are activated during the initial behavioral reactions in response to the sound stimulus. For example, in experimental work with damage of the deep layers of the SC (DLSC) in DBA/2 mice, there was a weakening of audiogenic convulsive seizures, but not a cessation of wild running (Willott and Lu, 1980; Tsutsui et al., 1992; Ribak et al., 1994). SC damage, including deep layers, in GEPR-3 and GEPR-9 rats did not stop the wild running phase, but weakened the severity of clonic-tonic convulsive reactions (Merrill et al., 2003). This work showed that the initial behavioral episodes (wild running or myoclonic tremors) are the only behavioral components of seizure in GEPR that persist after massive SC lesions. The results demonstrate that, although the beginning of the impulse in SC precedes that in IC, both of these brain stem structures are activated during the initial behavioral reactions in response to the sound stimulus. To date, it has been established that the progression from wild running to the occurrence of convulsive reactions is slowed down by injuries at both the IC and SC levels. The interruption of the connection between IC and SC reduced the severity of sound-induced seizures in sound-sensitive Wistar and GEPR-9 rats (Tsutsui et al., 1992; Ribak et al., 1994; Merrill et al., 2003). In addition, the involvement of IC in the initiation of the wild running phase is also demonstrated by the fact that unilateral electrical stimulation of the rostral part of the external IC core (ICx) in a normal (non-AE-prone) rat causes a wild running behavior component (Ribak et al., 1994; Chakravarty and Faingold, 1996). These data show that pathways from the IC to the SC play a role in the spread of the audiogenic seizures, although the details of "intra-quadrigeminal" interactions for norm and AE pathology are not well known. Thus we concluded that the separation of IC and SC structures for our initial connectome analysis could damage the internal connections between these nuclei (as the axons from IC could contain the RNA molecules, which play

certain role in case of sound exposure, i.e., are important for the subsequent excitation of SC). Thus the separation of these two paired structures could affect the results, making them less convincing. This was the ground for our choice to monitor the molecular events in these two structures cumulatively.

We used the inbred Krushinsky-Molodkina (KM) strain, which was selected for AE proneness in the late 1940s from the Wistar outbred population. The KM strain has a history of more than 40 generations of brother-sister inbreeding. Almost 100% of the individuals in this strain develop a characteristic epileptiform seizure 3–4 s after the onset of sound exposure (120 dB intensity), which is first manifested with a "wild run" phase, quickly followed by clonic and tonic seizures (Poletaeva et al., 2017). For controls, we used non-AE-prone rats of an outbred Wistar strain and "0" strain rats, based on an initial F2 (KM × Wistar) hybrid population, first selected in 1998 for their non-AE-prone phenotype. This selection aimed to create a non-AE-prone strain with a genetic background closer to that of KM rats than that of current Wistar rats (Fedotova et al., 2012; Poletaeva et al., 2014, 2017). In the "0" strain population, the proportion of rats without AE ranges from 30 to 60%. According to lab protocol these animals had been exposed to sound three times at the age of 3 months and respective proportions of AE-non-prone animals were found, the exact proportions varies in generations. Thus, by comparing the transcriptomes of these three strains, we were able to identify the peculiarities of gene expression associated with both the development of the AE phenotype (as a result of the selection of KM rats from the Wistar rat population) and the respective changes (i.e., the type of "compensation") in the initial AE phenotype exhibited in the KM rats as a result of "0" strain selection.

Previously, limited data were obtained for transcriptomes of the following other audiogenic rodent strains: WAR, selected from Wistar rats in Brazil (independent of the KM rat population), and AE-prone GASH/Sal, a hamster strain (Damasceno et al., 2018, 2020; Díaz-Casado et al., 2020; Díaz-Rodríguez et al., 2020). Thus, the gene expression profile of the *corpora quadrigemina* in KM rats may be compared with that of other AE-prone animals.

In the present study, we were able to identify characteristic features in the *corpora quadrigemina* transcriptome in KM rats and differentiate them from those in the Wistar strain and, to a lesser extent, from those associated with the transcriptome in the "0" strain. The results obtained revealed a number of gene expression profile features that may be associated with AE. These changes included those at the transcription level of the *Ttr* gene, which is involved in the regulation of the expression of GABAergic receptors (Zhou et al., 2019), and in the *Msh3* gene, which encodes a component of the DNA repair system (Park et al., 2013). Moreover, KM rats showed increased expression of several genes, the products of which are involved in the positive regulation of the MAPK signaling cascade, as well as in phosphorylation of ERK1/2, which may be involved in the development of the AE-prone phenotype (Nateri et al., 2007; Glazova et al., 2015; Dorofeeva et al., 2017). Several genetic traits that are plausibly connected to the development of AE are universal, as they were discovered in two distant rodent species (rats and hamsters), while others are inherent for definitive strains that were bred independently (i.e., KM and WAR rats) (Damasceno et al., 2020). In general, the data obtained reveal the complex polygenic nature of AE-prone rodents.

MATERIALS AND METHODS

Animals

The brain tissues of 4-month-old male rats grouped into three genotypes (Wistar, KM, and "0") were used. The animals were maintained in standard plastic cages (T4) with *ad libitum* food (Lab Feed) and water and a natural light-dark schedule. The manifestation of AE seizures was tested in a transparent plastic sound-attenuating chamber ("Open Science") using a standard classroom bell (sound intensity of 120 dB). For the KM rats, the maximum intensity of the tonic convulsions in response to sound (score of "4," according laboratory classification) was recorded (Poletaeva et al., 2017).

Herein, we used Wistar rats and rats of selected "0" strain, as they demonstrate no or moderate seizures in response to sound. According to lab protocol at the age of 3 months KM rats were sound exposed to verify their seizure reaction (tested once only). All "0" strain animals were exposed three times at the same age as KMs. This "triple" test was needed in order to verify the real lack of their reaction to sound. This strain is still under selection and non-proneness of these animals is not 100%. In the previous selection generations only 30–60% of "0" strain animals had no AE seizures. The lab protocol for genetic selection is triple AE testing for "0" rats in order to be sure that there were no minor AE signs (Fedotova et al., 2012). Wistar rats were tested for AE 4 days before sacrificing, while KM and "0" rats were also exposed to sound at the same time in order to equalize the physiological status of all three groups. The 4 day interval was considered to be enough for negative reactions to wane, and there were no changes noted in animal behavior before sacrificing. The strain "0" rats are under selection for AE non-proneness F2 (KM × Wistar) hybrids being their initial population. In F2 and in further generations animals differed in their reaction to sound from the total lack of audiogenic seizures to clonic-tonic fit. In F41 selection generation the animals tested demonstrated only wild run AE stage.

In 1940–1950s KM rats (as well as later Wistar Audiogenic Rat strain from Brazil, Garcia-Cairasco et al., 2017) were selected from Wistar outbred population. About 15% of Wistar rats usually demonstrate the moderate AE proneness, wild run stage and (rarely) clonic convulsions. In our experiment Wistar rats were tested for sound sensitivity in the same way as KM rats. The few animals that showed the "wild run" phase were excluded from the further experiment. Thus, neither "0," nor Wistar rats, used in this study, were AE-prone. At the same time Wistar and "0" rats had distinct acoustic startle-response, indicating that they were not deaf.

The inferior and superior colliculi (IC + SC) of *corpora quadrigemina* were isolated from animals 4 days after sound exposures, in order not to compromise gene expression profile with seizure-mediated transcriptional changes, as we consider that 2 day interval [acquired in Damasceno et al. (2018)] are not enough for elimination the plausible metabolic consequences of seizure induction and/or of sound exposure (in cases when no

seizure occurs). Euthanasia was carried out by rapid guillotine decapitation ("Open Science"). After euthanasia, the brains were quickly extracted in the cold and the fraction of the brain stem which contained IC + SC was isolated. The isolated brain tissue samples were frozen and stored in liquid nitrogen.

The experimental procedures were performed in accordance with requirements of Declaration 2010/63/EC.

RNA Extraction, Preparation of RNA-Seq Libraries and Sequencing

For RNA extraction, IC + SC sections were isolated from the brains of 4-month-old KM (n = 3), Wistar (n = 4), and 0 (n = 4) rats. Total RNA was extracted using Extract RNA reagent (Evrogen). The concentration of RNA was measured with a Qubit fluorometer (Invitrogen). The quality of the RNA was determined with an Agilent BioAnalyzer 2100 using an RNA 600 nano kit. The RNA integrity number (RIN) of all the RNA samples was not less than 8. Poly(A)-enriched libraries were prepared for RNA-seq using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina according to the manufacturer's protocol. Seventy-five-bp single-end sequencing was conducted on an Illumina NextSeq 500 platform.

RNA-Seq Analysis

As a result of deep sequencing, we obtained 15–22 million reads of RNA for each library. Preprocessing of these data was performed using a PPLine script that includes Trimmomatic for the adapter, length and quality trimming, STAR for mapping reads to the rat genome (release Rnor_6.0), and HTSeq-count for calculation of the read counts aligned on genes (Dobin et al., 2013; Bolger et al., 2014; Anders et al., 2015; Krasnov et al., 2015). A differential expression analysis was performed with the edgeR package using a quasi-likelihood negative binomial generalized log-linear model (QLF test) (Robinson et al., 2010). Gene Ontology and KEGG enrichment analyses were performed using the topGO (v.2.42.0) and clusterProfiler packages (Yu et al., 2012; Alexa et al., 2020). For GSEA analysis only differential expressed genes ($p < 0.05$) were used. The differential expression analysis, visualization, and GSEA were performed using RStudio program and ggplot2 package, as well as custom scripts written in Python (Wickham, 2009).[1] A Venn diagram was generated using FunRich (Pathan et al., 2015).

Compensation coefficients of the differentially expressed genes in the "0" rat samples compared to those in the KM rats were calculated using the equation (mean (logCPM) KM – mean (logCPM) 0)/(mean (logCPM) KM – mean (logCPM) W). The density distribution of genes was estimated in an R environment using kernel density estimation (KDE).

The RNA-seq data reported in this study can be accessed using GEO accession number GSE173885.

Immunoblotting

Samples from six animals of each strain were used for the immunoblotting series. Total protein extracts of the IC + SC tissue samples were prepared using the ReadyPrep protein extraction kit (Total Protein) (Bio-Rad) according to the manufacturer's recommendations upon addition of phosphatase inhibitor cocktail (Sigma). Thereafter, the protein samples were homogenized in SDS-PAGE buffer (65 mM Tris–HCl, pH 6.8; 10% glycerol; 2% SDS; 1% DTT; and 0.01% bromophenol blue) and heated to 100°C for 5 min. Electrophoresis was performed in 10% PAAG, and the proteins were transferred to a Hybond ECL membrane (GE Healthcare) by the semidry technique with buffer containing 25 mM Tris, 0.2 M glycine, 20% methanol, and 0.01% SDS with a current power of 1.5–2 mA/cm^2 for 1.5 h. After protein transfer, the membrane was blocked by 5% ECL blocking agent (GE Healthcare) in TBST (20 mM Tris–HCl, pH 7.6; 0.14 M NaCl; and 0.01% Tween 20) for 1 h. After blocking, the membrane was incubated for 12 h with primary anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) polyclonal rabbit antibodies (9101, Cell Signaling) to detect phospho-ERK1/2 and anti-p44/42 MAPK (ERK1/2) polyclonal rabbit antibodies (9102, Cell Signaling) to detect total ERK1/2 at the dilutions recommended by the manufacturer. Next, the membranes were washed four times for 10 min each time with TBST and incubated for 1 h with horseradish peroxidase-conjugated secondary anti-rabbit antibodies (A0545, Sigma-Aldrich) at the dilution recommended by the manufacturer. To remove bound primary and secondary antibodies and reprobe the proteins, the membranes were washed with Restore PLUS Western blot stripping buffer (Thermo Fisher Scientific). For signal detection, the SuperSignal West Pico PLUS chemiluminescent substrate (Thermo Fisher Scientific) was used. The signal intensities were measured with a gel-documenting system, ChemiDoc MP, and the Quantity One 1-D analysis software program (Bio-Rad). The optical densities of the bands were analyzed in the quantized image with the ImageJ program. Phospho-ERK1/2 signals were normalized to the signals obtained for total ERK1/2.

Quantitative PCR

Four-month-old KM (n = 9) and Wistar (n = 10), and "0" (n = 9) rats were used sampled for PCR analysis. Total RNA extraction from the *corpora quadrigemina* was performed using RNAzol RT (Molecular Research Center, United States). The concentration of isolated total RNA was measured with a NanoDrop spectrophotometer (Thermo Fisher Scientific, United States). cDNA was prepared from 1 μg of total RNA using random primers and MMLV reverse transcriptase (Evrogen, Russia). The analysis was performed on an ABI PRISM® 7500 sequence detection system (Applied Biosystems, United States). Detection of amplification products was carried out using SYBR Green 1 in the presence of ROX reference dye (Evrogen, Russia) according to the manufacturer's recommendations. Expression levels of genes of interest were normalized to the expression of the *Ywhaz* housekeeping gene (Schwarz et al., 2020) and calculated using the 2^{-dCt} equation. The sequences of the primers used are shown in **Supplementary Table 1**.

Statistical Analysis

Non-parametric ANOVA (Kruskal-Wallis test with *post hoc* analysis) was used to compare qPCR results and Western

[1] https://ggplot2.tidyverse.org

blot signals, respectively. p-values < 0.05 were considered statistically significant.

RESULTS

The Observed Inter-Strain Differences in Gene Expression Profiles Indicate the Multifactorial Nature of Audiogenic Seizures

As mentioned above, aiming to reveal the molecular mechanisms of AE, the gene expression profiles of KM rat *corpora quadrigemina* were compared with those of Wistar strain (W) rats, which do not exhibit AE, and "0" strain rats selected from KM × Wistar hybrids for the absence of AE phenotype (**Figure 1A**).

Differential gene expression analysis revealed 1488 genes [DEGs, $p < 0.05$, quasi-likelihood F-test (QLF test)] in the transcriptome of the KM samples compared to the transcriptomes of age-matched W rats. The transcriptome of "0" rat samples showed fewer differences from that of the KM rat samples (494 DEGs, $p < 0.05$, QLF test; **Supplementary Table 2**). This result (fewer differences in the KM rats vs the "0" rats, compared to those found in KM vs W) is in accordance with the possible role of two factors. The first factor is related to the relatively recent appearance of a non-AE-prone phenotype in the course of "0" rat strain selection (fewer than 40 generations), and the second factor is related to the influence of the KM rat genotype on that of the "0" rat strain, which revealed the "hidden" AE proneness of the "0" rats (i.e., proneness not evident in the normal state of these animals). At the initial stages of "0" strain rat selection, several backcrosses to the KM parental rat strain had been performed (Fedotova et al., 2012) in order to obtain animals with a genetic background more similar to that of KM rats as currently existing Wistar rats differ from KM rats, due to the history of long separate breeding. This "hidden" AE proneness was revealed first, as not 100% of the "0" rats were non-AE-prone, and then, as expected, the AE seizures in the "0" rats developed after induction with a subthreshold dose of penthylenetetrazole (Fedotova et al., 2016; Poletaeva et al., 2017). In accordance with this finding, we used only the rats in the "0" rat strain group that did not respond to sound (see "Materials and Methods" section).

To annotate functional gene expression differences in the three rat genotypes, a comparative analysis of genes with altered expression levels was performed with KM, W, and "0" strain rats (the first strain being highly AE-prone, 100% of animals from two other groups were non-seizure prone). The results are discussed in terms of the possible involvement of these genes in neurological processes (Gene Ontology) and molecular networks (KEGG pathway enrichment).

The Venn diagram (**Figure 1B**) demonstrates the number of DEGs in the KM rat IC + SC brain tissue samples compared with those in the W and "0" rat tissue samples. Genes with expression levels that differed between the KM and W rats are involved in various processes, including cell migration, cytokine production, ribosome biogenesis, glutathione metabolism, regulation of PI3K signaling, DNA repair and the MAPK cascade (**Figure 1**). These results are not surprising, as KM and W rats were bred separately for more than two hundred generations, starting at the end of the 1940s. Selection of the "0" rat strain from KM × W hybrid rats resulted in differences that affected the expression of genes participating in dopamine biosynthesis, nerve impulse transmission, type I interferon signaling and regulation of the JAK-STAT cascade (**Figure 1**). Interestingly, genes common to the two groups that were compared (KM vs W rats and KM vs "0" rats) are involved in the regulation of the MAPK cascade and apoptotic process (**Figure 1**).

Analysis of enrichment of DEGs in KEGG pathways showed that differences in the gene expression profile included the downregulation of ribosome biogenesis, glutathione metabolism, the mTOR pathway, oxidative phosphorylation, glutathione metabolism, serotonergic synapse, and the synaptic vesicle cycle in the KM rats compared with the W rats (**Figure 2**). The upregulated pathways in the KM rats included the PI3K-Akt, Rap1, Ras, and MAPK signaling pathways (**Figure 2**). The comparison of KM and "0" rat samples revealed the upregulation of aldosterone synthesis, thyroid hormone and Notch signaling pathways in the KM rats (**Figure 2**). Finally, the transforming growth factor β (TGFβ) signaling pathway was upregulated in the KM rats compared with both the W and "0" rats (**Figure 2**).

These data indicate that breeding of the KM strain induced significant gene expression profile changes in comparison with that of the Wistar rats, and the latter can be regarded comprising the initial population. The selection for increased sound reactivity among successively bred KM strain rats, including seizure provocation, resulted in "shifts" toward a presumably pathological state, which affected different processes. This selection affected synaptic plasticity, cytokine production, the DNA repair system and key neurochemical pathways, including signal transmission *via* different kinases (MAPK and PI3K). It is reasonable to suggest that the "0" strain non-AE-prone phenotype is probably the result of changes in MAP kinase activity, dopamine synthesis and type I interferon signaling, which may be crucial for decreasing AE proneness.

The Mitigation of ERK1/2 Activity and Changes in the Expression of Genes Involved in the MAP Kinase Pathway Are Characteristics of the Non-AE-prone Phenotype

Extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2) are essential components of the classical pathway of MAP kinase, and they are believed to be among the factors that mediate epileptiform seizures, at least in *Fmr1*-KO mice and KM rats (Nateri et al., 2007; Curia et al., 2013; Glazova et al., 2015; Dorofeeva et al., 2017). Different regional distributions of ERK1/2 in KM and W rats and higher levels of phosphorylated ERK1/2 (i.e., the active form) in KM rats were recently demonstrated (Dorofeeva et al., 2017; Chernigovskaya et al., 2019). In accordance with these data, the differential gene expression pattern of MAPK pathway participants in this study was shared by comparisons of two pairs (KM vs W rats and KM vs "0" rats)

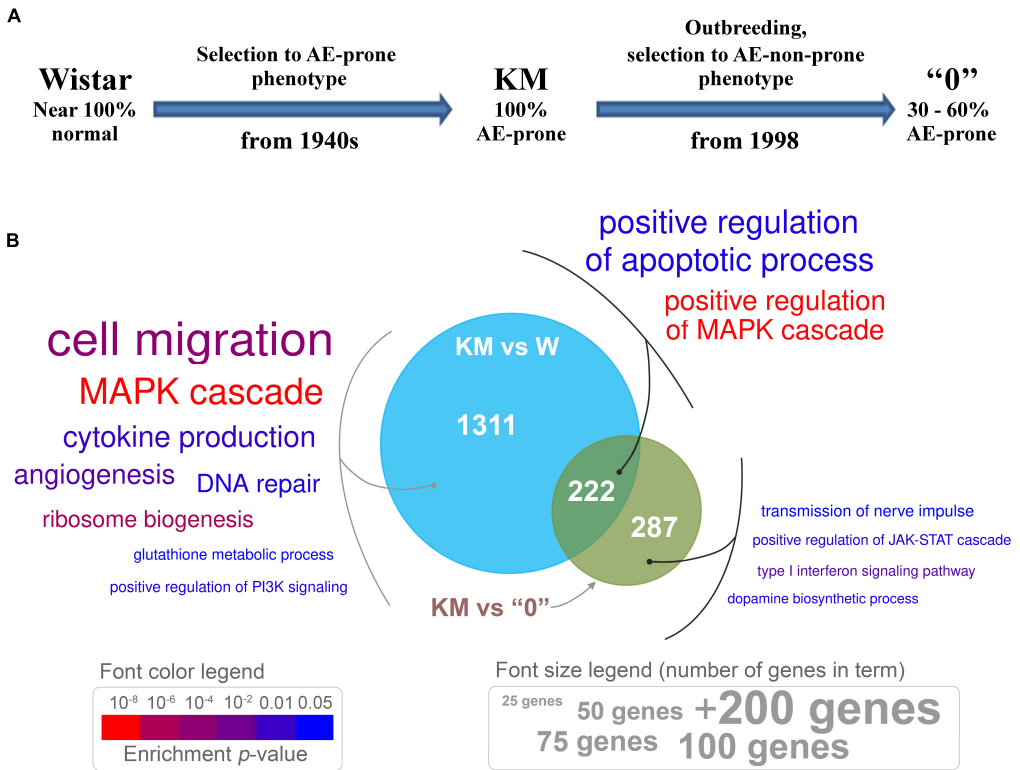

FIGURE 1 | (A) The sequential procedure for deriving the Krushinsky-Molodkina (KM) and "0" strains from the Wistar strain. **(B)** Venn diagram depicting differentially expressed genes (DEGs) between KM samples and those from W and "0" rats, which exhibit distinct genotypes. Genes with differential expression, $p < 0.05$, and expression changes greater than 1.5-fold (either up- or downregulated) were used for enrichment analysis. Enriched Gene Ontology (GO) terms are presented in colored text. The font size is proportional to the number of genes in a term (see the "font size legend"). Font color indicates the enrichment test *p*-value (see the "font color legend").

(**Figure 1**). This observation indicates that expression changes in components of the MAP kinase cascade distinguish KM rats from both W and "0" rats (**Figure 1**).

The detailed analysis of genes involved in the KM-specific MAPK cascade revealed the upregulation (in comparison with the expression in W rats) of classical MAP kinase pathway components, including the following genes: receptor tyrosine kinase (*RTK*) and RTK ligand, epidermal growth factor (*EGF*), nucleotide exchange factor RasGRP, and cyclic AMP-dependent transcription factor ATF-4, also known as CREB (**Figure 3A**). The KM rat gene expression profile was also characterized and compared to that of the W rats, and higher expression levels of interleukin 1 receptor (*IL1R*) and myeloid differentiation primary response-88 (*MYD88*) genes, as well as growth arrest and DNA damage (*GADD45*) genes and downstream agents of the p38 MAP kinase pathway were found. The latter observation suggests that neural cells of KM rats are highly susceptible to stress signals due to cytokine stimulation and DNA damage (**Figure 3A**; Mikhailov et al., 2005). Importantly, the expression changes of the aforementioned genes in "0", when compared to KM samples, demonstrated the opposite expression pattern (Spearman's correlation = −0.625, $p < 0.05$), which suggests that MAPK signaling features characteristic of KM rats are "compensated" in some manner in "0" rats (**Figures 3A,B**). This observation was supported by Western blot analysis, through which we determined the quantity of the phosphorylated (active) forms of ERK1/2 in W, KM and "0" rat samples. The results showed the increased levels of phosphorylated ERK1 and ERK2 observed in the KM rats samples compared with those in the W rat samples (increased by 40%, $p < 0.05$, Kruskal-Wallis test with *post hoc* analysis), and these levels were lower in "0" rats, resembling the levels intrinsic to W rats (**Figure 3C**).

Considering these results, we hypothesized that this "compensation" of the expression levels of genes implicated in the MAPK cascade is one of the factors contributing to the acquisition of the non-AE-prone phenotype in "0" rats.

Non-AE-proneness Is Accompanied by the "Reversion" of the Expression Pattern of More Than One-Half of the Genes, Resembling That of the Progenitor State

The emergence of differences in genetic patterns (and thus in gene expression profiles) of KM and "0" rats was analyzed according to the strain selection history, which followed two stages. In the first selection stage, the AE-prone phenotype was acquired by KM rats. This development proceeded through

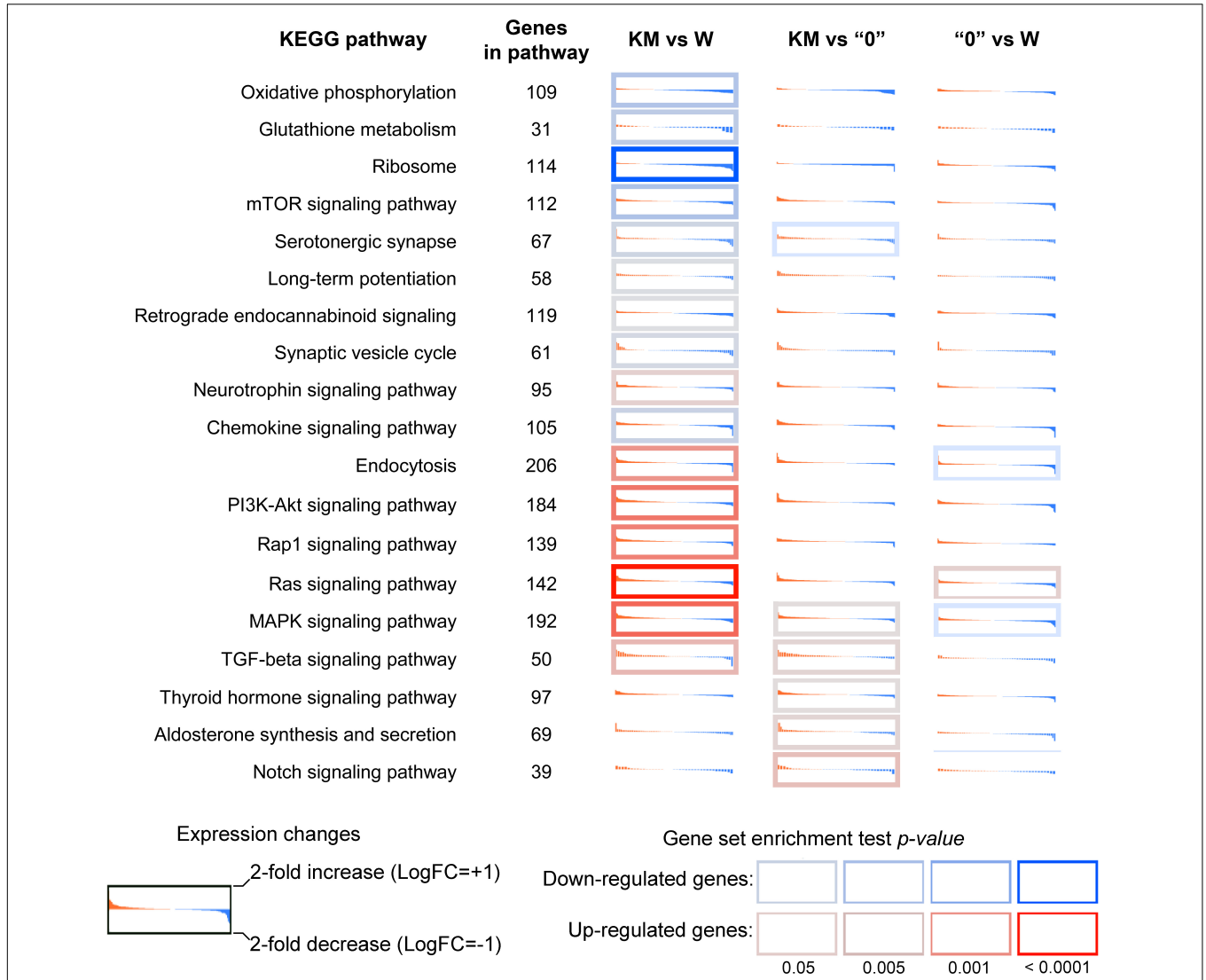

FIGURE 2 | Differential expression profiles of genes participating in the most affected KEGG pathways in the KM, W, and "0" rats. For each KEGG term, genes were identified (irrespective of *p*-value) and sorted by decreasing log2-fold change. Red-to-blue sub-plots illustrate expression level changes (log-scale, with values decreasing across the plot). The log2 expression level fold change (LogFC) range extends from −1 (i.e., 2-fold downregulation; blue) to +1 (i.e., 2-fold overexpression; red). The cell border indicates the gene set enrichment (GSEA) *p*-value for a pathway. A red border indicates that a KEGG pathway is enriched with upregulated genes; a blue border indicates that a KEGG pathway is enriched with downregulated genes. The *p*-value was calculated for up- and downregulated genes using Fisher's exact test.

several intermediate stages, which reflect the gradual increase in AE proneness (both penetrance and expressivity) during dozens of generational selections and resulted in nearly 100% AE proneness in the current KM strain. The second stage involved the selection of non-AE-prone animals from KM × W rat hybrids. Given the multifactorial nature of the AE-prone phenotype, it is logical to assume that selection for the non-AE-prone phenotype among rats in the W × KM hybrid population resulted in the selection of gene alleles with an expression pattern characteristic of non-AE-prone Wistar rats. To test this assumption, we compared the expression levels of genes with the greatest significant differences between the KM and W rats and between the KM and "0" rats ($p < 0.01$). This comparative analysis showed negative correlations of log2-fold changes (Spearman's correlation = −0.711, $p < 0.01$), with over 70% of the DEGs exhibiting an opposite direction of expression, indicating that the pathophysiological genetic peculiarities in the KM rats had been lost in the course of strain "0" selection (**Figure 4A**).

To quantify this putative "compensation" effect in the gene expression profile in the "0" strain rats, we calculated the compensation coefficient as described in the "Materials and Methods" section. According to the formula used for the calculation, in cases when the compensation coefficient was close to 100% (i.e., in the "0" rat samples) almost complete "compensation" was observed for genes that showed different

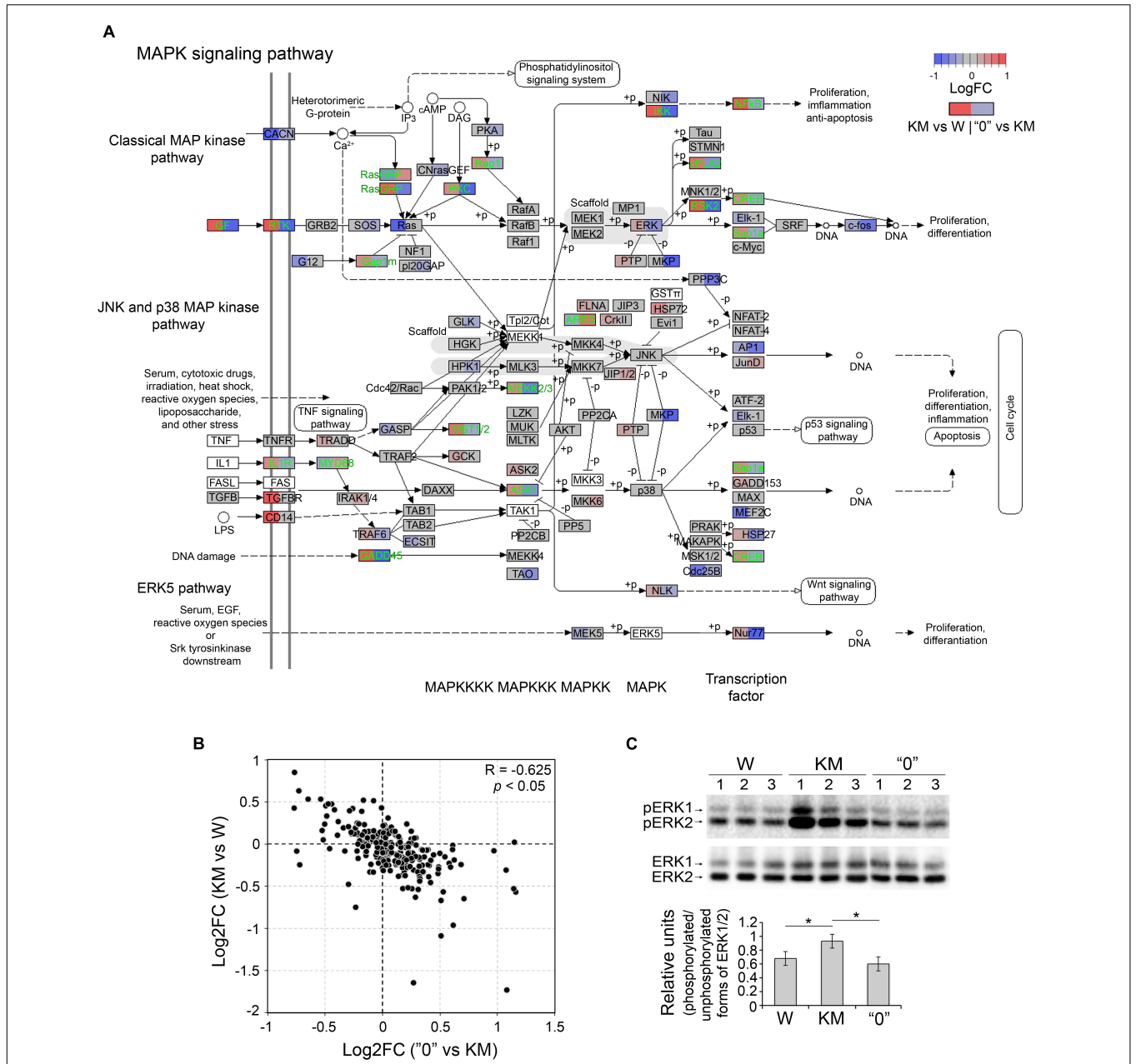

FIGURE 3 | The expression of MAPK pathway and ERK1/2 genes was alleviated in "0" rats. **(A)** Differential expression of genes participating in the MAPK pathway (rno04010). Gene expression changes are shown in color (log2-scale; LogFC; red – upregulation, blue – downregulation, gray – no change, white background – the absence of expression). Each gene block is divided into two sections (the left block shows differential expression between KM and W rats; right – between "0" and KM samples). Genes that demonstrate opposite changes between two pairs of comparisons ("compensated" genes) are shown in green font. **(B)** Scatter plot depicting the pairwise comparison of log2-fold changes between the KM vs W and "0" vs KM groups. R – Spearman's correlation coefficient. **(C)** Western blot analysis of phosphorylated and unphosphorylated forms of ERK1/2 (*top*) and the calculation of the optical density of the signals (*bottom*). For semiquantitative estimation, the optical density of phosphorylated ERK1/2 was subjected to two levels of normalization: first between samples and second on the unphosphorylated form of ERK1/2. *$p < 0.05$, Kruskal-Wallis test with *post hoc* analysis.

expression patterns in the KM in comparison with the W rats. Higher values of the compensation coefficient corresponded to overcompensation, and negative values corresponded to cases for which no compensation or enhancement of the KM phenotype was evident. Applying this approach, we showed that the expression levels of a large fraction of DEGs observed in the comparison of the KM and W rat samples were compensated (the compensation coefficient was between 1 and 100 for as many as 70% of the DEGs, with the most-significant changes indicated by $p < 0.01$; **Figure 4B** and **Supplementary Figure 1**). These data

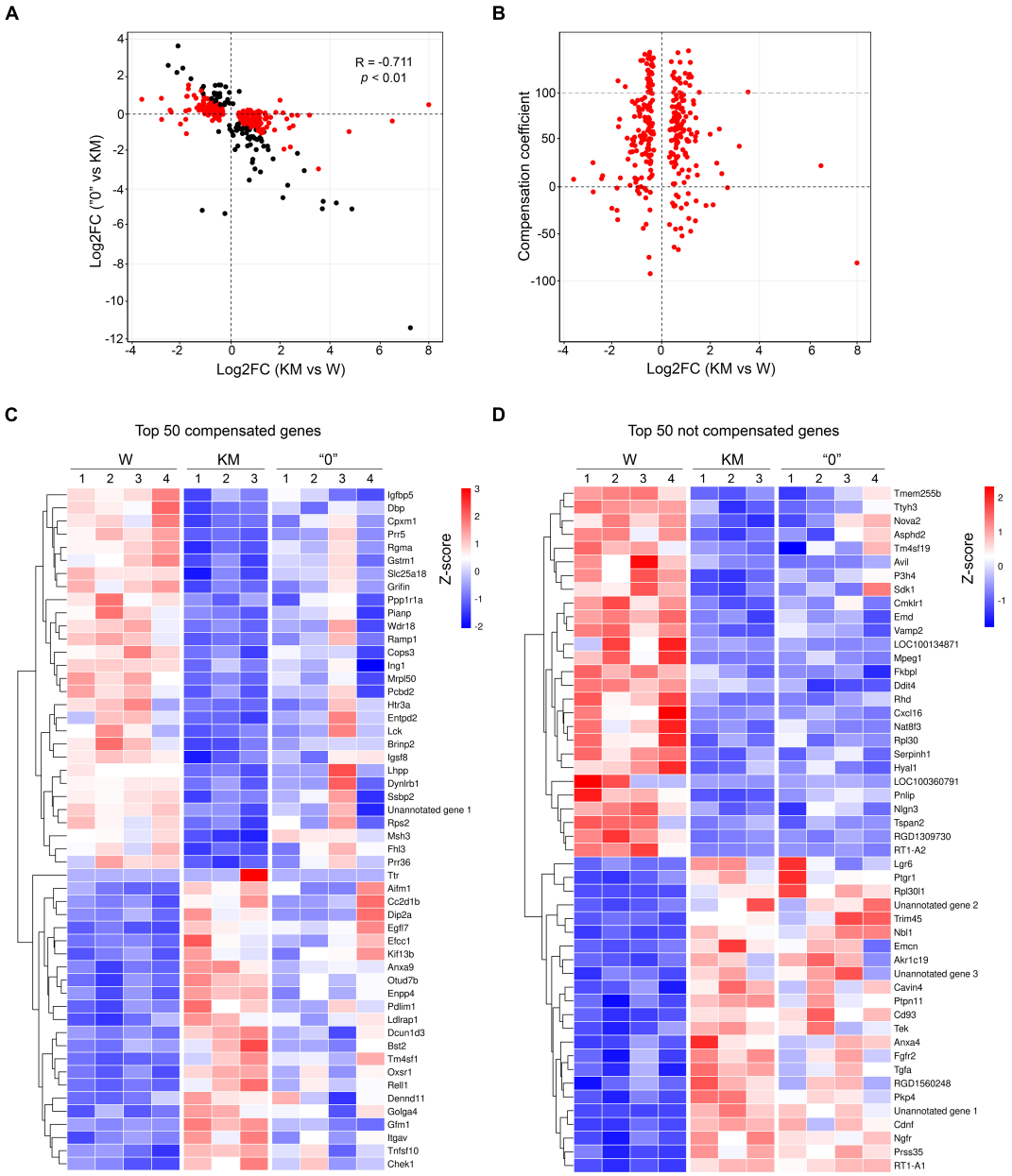

FIGURE 4 | The expression levels of most genes in the "0" rat samples tended to be reverted to the levels in the W rats. **(A)** The log2-fold change of gene expression that shows the significant difference in the expression levels between the KM and W rat transcriptomes (X-axis) and between the "0" and KM rat transcriptomes (Y-axis). Positive values correspond to activation in the KM or "0" rats. One point represents one gene, and only genes exhibiting significant changes ($p < 0.01$, QLF test) are shown. Genes with differences in expression between the KM and W transcriptomes are shown in red, and those with differences only between the "0" and KM transcriptomes are shown in black. **(B)** Dependence of the compensation coefficient (Y-axis) on the log2-fold change in the KM and W rat comparison (X-axis). The compensation coefficient was calculated as a percentage of mean (logCPM) KM – mean (logCPM) 0)/(mean (logCPM) KM – mean (logCPM) W. **(C)** Heat map of the top 50 compensated genes (>50 of the compensation coefficient value) displayed in line from the highest *p*-value at the top to the lowest *p*-value at the bottom. **(D)** Heat map of the top 50 genes exhibiting the least or no compensatory expression (<50 of compensation coefficient value) displayed in line from the highest *p*-value at the top to the lowest *p*-value at the bottom. The expression values of the genes presented in panels **(C,D)** are Z-transformed.

also indicate that the gene expression profile in the "0" rat strain was substantially "reversed" (i.e., "returned") to that representing original expression levels, which were observed in the *corpora quadrigemina* of the W rats. Importantly, these "compensation data" were revealed for both upregulated and downregulated expression patterns, which distinguished the KM rat samples from the W rat samples) (**Figure 4C**). However, at least 15% of the DEGs that were predominantly downregulated in the KM rats (compared with the W rats) were not compensated in the "0" rats (**Figure 4D**). This finding may signify that the "0" rat

strain carries part of the KM rat genetic background (the "0" rat strain can be induced to AE proneness with subthreshold penthylenetetrazole injection, as explained above).

To validate the gene expression patterns revealed by RNA-seq, we performed qPCR analysis for six selected genes exhibiting opposite expression differences between the KM and "0" rat samples in comparison with the pattern of differences in the KM and W rat sample pairing. These genes evaluated were acyl-CoA synthetase medium-chain family member 5 (*Acsm5*); calcium voltage-gated channel auxiliary subunit gamma 4 (*Cacng4*); potassium voltage-gated channel subfamily E regulatory subunits 2 and 5 (*Kcne2* and *Kcne5*); mutS homolog 3 (*Msh3*), a component of the DNA mismatch repair system; and the transthyretin (*Ttr*) gene, which encodes the protein that transports thyroxin and the retinol-binding protein complex (Fishel, 1998; Park et al., 2013; Sharma et al., 2019). This analysis confirmed the differences in KM rat gene expression compared with W rat gene expression, as well as the trend in the "0" rat strain of gene expression level "reversion" to the levels observed in the W rats ($p < 0.05$, Kruskal-Wallis test with *post hoc* analysis, **Figure 5**).

In addition, in the comparison between KM and W rats, a decrease in the expression level of genes encoding components of the 1st, 3rd, and 4th complexes of the respiratory chain and of the F-ATPase genes was observed. In the "0" rats, compensation for the expression of the genes encoding 1st and 4th complexes and ATP synthase was detected, but not for the 3rd complex (**Supplementary Figure 2**).

Taken together, these results indicated that the selection of the "0" rat strain resulted in the large "reversion" in the gene expression profile toward that intrinsic to the progenitor W rats.

Thus, the genes that tended to "compensate" for the expression level in the "0" rat strain (see above) can presumably be considered genes involved in the development of the AE phenotype in the KM strain. Among these genes, we focused primarily on the genes involved in human epilepsy (i.e., their mutations and/or changes in the expression level of these genes in humans increase the risk of epilepsy). The DEGs in other rodent model strains with an AE phenotype were also of interest in this analysis, in particular, insulin-like growth factor-binding protein 5 (IGFBP5), which has been shown to associate with four and a half LIM domains protein 2 (FHL2), which tunes neuronal functioning (including seizure proneness) via androgen production and respective receptors (Reddy, 2004; Jamali et al., 2006). These genes also included glutathione S-transferases (GSTs), which conjugate electrophilic compounds and thus may play neuroprotective roles by removing exogenous and endogenous oxidants. Carriers of GSTM1-null genotypes in European and Asian populations have been found to show significant risk of developing epilepsy, emphasizing the involvement of the antioxidant system in seizure susceptibility (Ercegovac et al., 2015; Prabha et al., 2016). In our study, we found downregulation of *Igfbp5* and *Gstm1* gene expression in the KM rats. The abnormal pattern of the brain antioxidant system in KM rat strain has been previously demonstrated (Medvedev et al., 1992). The changes detected in the expression of the *Msh3* and *Ttr* genes in our study are also of interest because mutations or changes in the expression levels of these genes had been previously detected in other AE-prone rodent strains (WAR rats and GASH/Sal hamsters) (Damasceno et al., 2020; Díaz-Rodríguez et al., 2020).

DISCUSSION

Analysis of the transcriptome in audiogenic rat strain (KM) and its comparison with the transcriptome of non-AE-prone rats in the original Wistar population and with "0" rats subjected to "reverse" selection allowed us to identify candidate genes for which expression changes are presumably the cause of AE phenotype acquisition. This approach met with certain difficulty, as some of the differences detected (1488 DEGs in the KM vs W rats and 494 DEGs in the KM vs "0" rats) may have been the result of changes in the genetic background of the strains, which was noted in both the KM and Wistar rats due to random changes occurring during the separate breeding of KM and W rat strains over many generations. We assumed that seizure proneness (including AE proneness) emerged during the early stages of brain development. If this supposition is accurate, then an analysis of the adult animal brain transcriptome would not lead to the detection of key changes in gene expression that are critical for AE phenotype development from the early ontogeny to the age of 3 months. A previous analysis of AE development in KM rat ontogenesis showed that AE seizures are reliably (at full intensity) expressed at the age of 3 months (Fedotova and Semiokhina, 2002; Poletaeva et al., 2017). Similar dynamics were described for GEPR AE expression (Thompson et al., 1991). In accordance with these observations, we used 4-month-old rats in experiments (see "Materials and Methods" section). Notably, a direct comparison of gene expression patterns in rats and humans is not appropriate because of the immense differences in brain structures. Nevertheless, the comparison of our transcriptomic data for the set of rat strains (W, KM, and "0" strains) with data obtained for other rodent AE-prone animals, such as WAR rats and GASH/Sal hamsters (Damasceno et al., 2020; Díaz-Rodríguez et al., 2020), allowed us to identify patterns that are apparently causally related to the development of AE. The observed partial reversion of the gene expression profile in the "0" rats (partially non-AE-prone but derived from KM rats) toward the expression profile of the W rats and the ability to compare our results with other AE rodent models allowed us to reduce the number of genes of interest.

Considering our data, we concluded that the most characteristic feature of the KM rat IC + SC transcriptome is ∼10-fold decrease in the expression of the *Msh3* gene (MutS homolog) in comparison with that in the W and "0" rats (**Figure 5**). Along these lines, it was previously shown that the GASH/Sal AE-prone hamster strain carries a mutation in the *Msh3* gene potentially capable of disrupting Msh2/Msh3 protein heterodimerization (Díaz-Casado et al., 2020). These proteins were shown to form the MutSß complex, which participates in DNA repair by recognizing insertion-deletion loops of 1–15 nucleotides and DNA with 3′ single-stranded overhangs (Gupta et al., 2011); the authors suggested that the increase

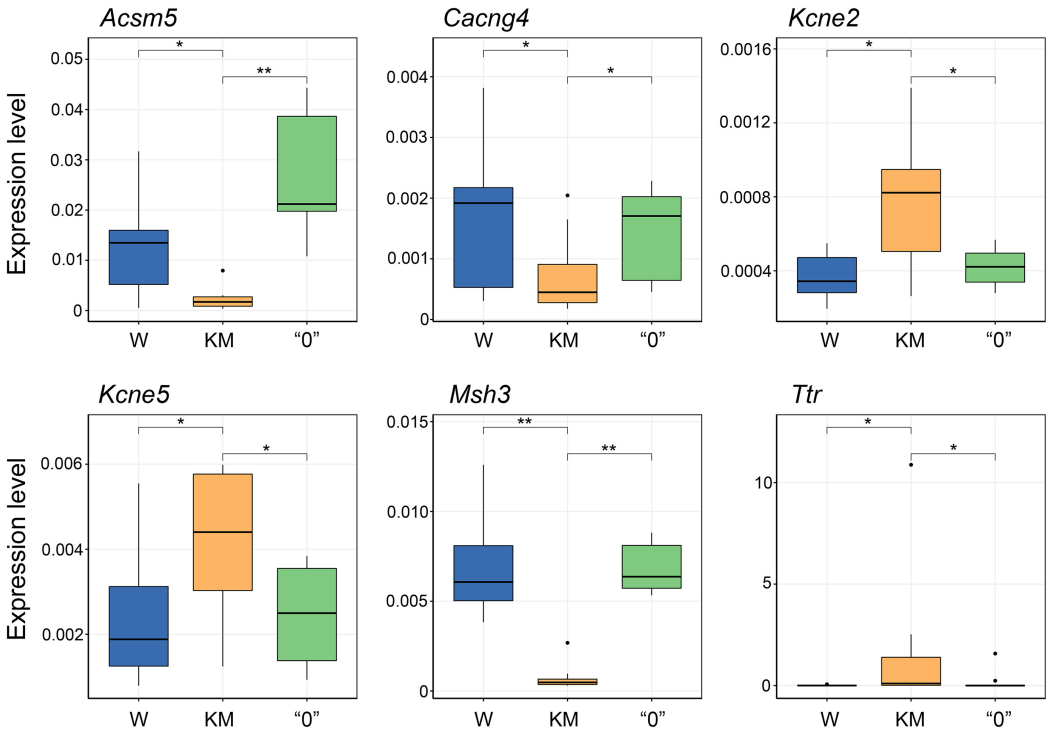

FIGURE 5 | qPCR validation of gene expression changes initially observed through RNA-seq analysis. Expression levels determined by the 2^{-dCt} equation, including normalization of the genes of interest levels to a housekeeping gene (*Ywhaz*) for each group of samples. Expression levels were estimated for no fewer than 6 rats. $^{*}p < 0.05$, $^{**}p < 0.01$, Kruskal-Wallis test with *post hoc* analysis.

in *Msh3* expression level observed in GASH/Sal hamsters is probably due to changes in compensatory gene expression (Díaz-Casado et al., 2020). Disruption of the MutSß complex in mice with Msh2 deficiency was reported to induce a decrease in the expression levels of cytochrome *c* oxidase subunit 2 (CoxII), the ATP synthase β-subunit, and superoxide dismutase genes (in comparison with those in WT animals) (Francisconi et al., 2006). This genotypic change increased animals' susceptibility to kainic acid (KA)-induced seizures. A single KA injection increased seizure proneness and hippocampal neuronal death, while systemic KA injections increased the mortality rate together with an increase in tonic-clonic seizure severity (Francisconi et al., 2006). The data previously reported also showed that disruption to the DNA repair system can cause mitochondriopathy in the brain, which presumably leads to predisposition for convulsions. As mentioned above, the gene expression profile of the KM rats compared to the W rats in our study demonstrated a decrease in *Acsm5* gene expression (**Figure 5**) and in a number of genes encoding components of the 1st (NADH:ubiquinone oxidoreductase), 3rd (coenzyme Q:cytochrome *c* oxidoreductase), and 4th (cytochrome *c* oxidase) respiratory complexes and F_0-ATP synthase. Characteristically, this decrease was partially compensated in the "0" rats (**Supplementary Figure 2**). It was previously shown that KM rats demonstrated reduced ATP production and increased H_2O_2 levels in the brain and liver compared with W rats (Venediktova et al., 2017). In our study, KM rat mitochondrial disorders also induced an increase in the expression of a number of genes that positively regulate apoptosis (**Figure 4**). An increased apoptosis rate in the inferior colliculi in KM rats compared to W was previously described (Chernigovskaya et al., 2018). This increase in apoptosis rate may be associated with abnormal brain development in KM rats, which in turn may be the cause of epileptogenesis, this effect was experimentally demonstrated in inferior colliculi and hippocampus of KM rats (Chernigovskaya et al., 2018; Kulikov et al., 2020). The delay in IC development was also demonstrated, in line with apoptosis, (Kulikov et al., 2020).

In addition to mitochondrial disorders, the reduced expression of the *Msh3* gene and an increase in DNA repair disorders may elevate the frequency of mutations and hence contribute to the rapid selection of the audiogenic phenotype. Similarly, it was previously shown that the 101/HY mouse strain, which is characterized by intense AE, is hypersensitive to chemical mutagens (these mice carry a mutation in the locus controlling DNA repair after chemical mutagenesis) (Boiarshinova et al., 2009). The mechanisms of reducing the expression of the *Msh3* gene in KM rats need further study.

An important link in the process of AE development is the increase in MAPK/ERK1/2 activity. We showed the overexpression of a number of MAPK cascade genes and increased phospho-ERK1/2 levels in KM rats (**Figures 1**, **3**) in comparison to both W and "0" strain rats. A decrease

in the expression level of some MAPK cascade genes in "0" rats can be considered as a compensatory effect, leading to a decrease in sensitivity to loud sound. The proapoptotic (and presumably proinflammatory) responses in the KM rats may have been connected to the increased transcription of a number of genes coding the upstream components of the JNK/p38 cascade (IL1R, TGFBR, and CD14) that are critical for these reactions (Verstrepen et al., 2008). In KM rats the apoptotic events were described in Chernigovskaya et al. (2018). The upregulation of inflammatory pathways in neuronal tissue was shown to have a crucial effect in the development of epilepsy (Rana and Musto, 2018). To our knowledge no current data on the KM strain inflammation system exist. In addition, KM rats have been shown to exhibit a significant increase in the expression of *GADD45* genes, which are involved in the activation of p38 and induction of apoptosis in response to DNA damage, which may be associated with impaired mitochondrial function, increased H_2O_2 production and inflammation (Liebermann and Hoffman, 2002). In our study, KM rats exhibited an increase in the transcription of growth factor genes and genes encoding the receptor tyrosine kinases PKC and RasGRP, which may have led to the increase in the activation of ERK1/2. On the other hand, the *Ras* transcription level was found to decreased in the KM rats, which may have compensated for the increase in upstream components in the system (**Figure 4**). Notably, the increase in the phosphorylation level of ERK1/2 in the KM rats (in comparison with the W and "0" rats) was directly shown in the present work. Previously, elevated levels of phospho-ERK1/2 were demonstrated in the inferior colliculi, hippocampus and striatum of KM rats (Dorofeeva et al., 2017; Chernigovskaya et al., 2018, 2019). We assume that activation of ERK1/2 may be a key mechanism for the development of AE. ERK1/2 activity contributes to a decrease in the seizure threshold via mechanisms involving enhanced glutamatergic synaptic transmission, which is induced *via* the expression of glutamate excretion (VGLUT2) and subsequent stimulation of NMDA receptors, as well as by an increase in NMDA receptor number (Nateri et al., 2007; Doyle et al., 2010; Gangarossa et al., 2011, 2019). On the other hand, it was previously demonstrated that activation of glutamate via NMDA and AMPA and kainate receptors during seizures leads to an increase in ERK1/2 activity, which causes suppression of the functional activity and sensitivity of GABAA receptors in the granular cells of the dentate gyrus in the hippocampus, making the entire hippocampus susceptible to seizures and status epilepticus development (Kapur, 2018). A correlation between changes in NMDA and ERK1/2 kinase levels was previously shown by exploring the pharmacological glutamate receptor NR2B subunit inhibitor ifenprodil, which blocks ERK-induced seizures (Nateri et al., 2007). It was also demonstrated that inhibition of ERK1/2 by intraperitoneal injections of SL327 (a selective MEK1/2 inhibitor) blocked AE seizures in KM rats (Glazova et al., 2015).

Another characteristic feature of the KM strain that is lacking in the W and "0" strains is increased expression of the *Ttr* gene (**Figure 5**), which was previously shown to encode a protein called transthyretin (Soprano et al., 1985). Mutations in this gene in humans cause family amyloid polyneuropathy characterized by amyloid formation, slowly progressive dementia, seizures, ataxia, and subarachnoid hemorrhage (Ando et al., 2013; Franco et al., 2016). Several studies reported the neuroprotective role of transthyretin in Alzheimer's disease and in cases of cobalt-induced seizures in animals (Stein et al., 2004; Kajiwara et al., 2008; Li and Buxbaum, 2011). These findings suggested that overexpression of the *Ttr* gene in KM rats may be a compensatory mechanism. On the other hand, transthyretin regulates the activity of GABAA receptors, which play an important role in the control of predisposition to seizures (Zhou et al., 2019). Importantly, high levels of *Ttr* expression and transthyretin protein and mutations in the *Ttr* gene were demonstrated in GASH/Sal hamsters and in WAR rats. To this end deficiency of GABAA receptor-mediated neurotransmission were previously shown in GASH/Sal hamster and WAR rat models (Damasceno et al., 2020; Díaz-Casado et al., 2020).

Finally, we found significant differences in the expression of several other genes in KM rats (vs W rats) that were compensated in the "0" rats and, according to available data, may be associated with the development of AE. We have shown that in KM rats, compared with W and "0" rats, there was a decrease in the transcription of the *Igfbp5* and *Gstm1* genes, the mutations of which were shown to increase the risk of developing epilepsy in humans (Reddy, 2004; Jamali et al., 2006; Ercegovac et al., 2015; Prabha et al., 2016). The *Gstm1* gene encodes glutathione S-transferase, which is an important component of the antioxidant defense system in the brain. Notably, disturbances in the antioxidant system in the KM rat brain, along with effectiveness of the respective therapy, were previously demonstrated (Fedotova et al., 1990; Medvedev et al., 1992). In addition, in our study, changes in the expression of genes coding K-channel regulatory subunits Kcne2 and Kcne5, as well as Cacng4, which regulates the activity of L-type Ca-channels, were found in KM rats, and these changes were compensated in the "0" strain (**Figure 5**). In some cases, epilepsy is considered "channelopathy" because it involves K- and Ca-channel anomalies in animal models and human epilepsy (Biervert et al., 1998; Lenzen et al., 2005; Uehara et al., 2008; Poolos and Johnston, 2012; Snowball et al., 2019), although no detailed analysis of the roles of these subunits in human epilepsy has been reported. Finally, changes in dopamine synthesis and type I interferon signaling genes expression, detected in "0" strain relative to KM may be crucial for compensation of AE proneness (Rana and Musto, 2018; Akyuz et al., 2021). However, there were no significant changes in dopamine receptors in KM and "0" strains relative to Wistar strain.

In summary, in rodents with AE phenotypes with different genetic origins, similar patterns of the expression of certain genes were found. In particular, an increase in *Ttr* transcription and mutations of the *Msh3* gene were previously described (Damasceno et al., 2020; Díaz-Casado et al., 2020). These gene expression peculiarities were associated with distinct pathological

traits in brain function. On the other hand, other studies showed no changes in the brains of KM rats, concerning the expression of other genes characteristic of WAR and/or GASH/Sal models (*Npy*, *Egr3*, and *Rgs2*) while authors (Damasceno et al., 2020) associated these genes with a predisposition to AE in these animals. Additionally, in KM rats (as in rats of other AE-prone strains), no changes in the expression level of the *Vlgr1* gene were reported, while mutation of this gene caused the development of AE in Frings mice (Klein et al., 2005). In general, the data obtained confirm the polygenic inheritance of AE, as previously demonstrated by a diallel crossing experiment (Poletaeva et al., 2017). A few peculiarities of the gene expression profile in AE-prone animals relative to controls – AE-resistant strains – are common to two rodent species (rat and hamster), while other features are inherently "individual" to certain epileptic rat strain models. The expression of certain genes in the brains of animals with audiogenic epilepsy differs even among different strains of AE-prone rats, which may be associated with peculiarities of the genetic "background" of the original populations together with events during the selection process, among other causes. Notably, a number of gene expression characteristic traits in the brains of animals with AE, found both in this study and previously in the WAR strain, were confirmed in the GASH/Sal hamster strain (Damasceno et al., 2020; Díaz-Casado et al., 2020). This finding probably indicates that these genes represent certain "nodes" in brain pathology common to different AE genotypes.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/search/all/?term=GSE173885.

ETHICS STATEMENT

The animal study was reviewed and approved by the Commission on Biomedical Ethics of the FSBI V. V. Zakusov Research Institute of Pharmacology (protocol No. 1 of January 31, 2020). The organization and execution of experimental work were carried out in accordance with the order of the European Convention for the Protection of Vertebrate Animals (Strasbourg 1986) and the Order of the Ministry of Health of the Russian Federation April 1, 2016, No 199N.

AUTHOR CONTRIBUTIONS

LC, SF, and AR: RNA-seq, Q-RT-PCR, and bioinformatic analysis. AD and DG: western blot analysis. IF and IP: working with animals. SF, ME, SL, and DG: experiment design and writing a manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2021.738930/full#supplementary-material

Supplementary Figure 1 | Distribution of compensation coefficient for differentially expressed genes in the KM and W rats. The histogram shows the compensation coefficients for expression changes in the "0" rats. Density distribution was estimated using kernel density estimation (KDE). The compensation coefficient was calculated as a percentage of mean (logCPM) KM – mean (logCPM) 0)/(mean (logCPM) KM – mean (logCPM) W.

Supplementary Figure 2 | The expression of genes involved in the oxidative phosphorylation pathway was partly attenuated in the "0" rats. **(A)** Differential expression of genes participating in the oxidative phosphorylation pathway (KEGG rno00190). The illustration of the pathway is shown with slight modifications. Gene expression changes are shown in color (log2-scale; LogFC; red – upregulation, blue – downregulation, gray – no change, white background – the absence of expression). Each gene block is divided into two sections (the left block shows differentially expressed genes in the KM and W rat samples; the right block shows the differentially expressed genes in the "0" and KM rat samples). **(B)** Scatter plot depicting the pairwise comparison of log2-fold changes between the KM vs W rat and "0" vs KM rat groups. R – Spearman's correlation coefficient.

Supplementary Table 1 | Primers for Q-RT-PCR used in the work.

Supplementary Table 2 | Differentially expressed genes (DEG) between all groups of comparison.

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REVIEW
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Lipid Metabolism Influence on Neurodegenerative Disease Progression: Is the Vehicle as Important as the Cargo?

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Many neurodegenerative diseases are characterized by abnormal protein aggregates, including the two most common neurodegenerative diseases Alzheimer's disease (AD) and Parkinson's disease (PD). In the global search to prevent and treat diseases, most research has been focused on the early stages of the diseases, including how these pathogenic protein aggregates are initially formed. We argue, however, that an equally important aspect of disease etiology is the characteristic *spread* of protein aggregates throughout the nervous system, a key process in disease progression. Growing evidence suggests that both alterations in lipid metabolism and dysregulation of extracellular vesicles (EVs) accelerate the spread of protein aggregation and progression of neurodegeneration, both in neurons and potentially in surrounding glia. We will review how these two pathways are intertwined and accelerate the progression of AD and PD. Understanding how lipid metabolism, EV biogenesis, and EV uptake regulate the spread of pathogenic protein aggregation could reveal novel therapeutic targets to slow or halt neurodegenerative disease progression.

Keywords: lipid metabolism, protein aggregation and propagation, extracellular vesicle, Parkinson's disease, Alzheimer's disease, glia, glucocerebrosidase (GBA), ceramide

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INTRODUCTION

Age-related neurodegenerative diseases are a growing medical burden in our aging population. Alzheimer's disease (AD), the most common neurodegenerative disease, affects 11.3% of people ages 65 or older, and Parkinson's disease (PD), the second most common neurodegenerative disease, affects approximately 1% of people ages 60 or older. Therapies for these diseases are limited to symptomatic treatments only, and there is an urgent need for treatments that could halt or slow the underlying neurodegenerative processes.Many neurodegenerative diseases, including AD and PD, have characteristic neuropathologic findings of pathologic bodies that include proteins specific to each disease. In AD, amyloid β-peptides (Aβ) formed by proteolytic cleavage of the amyloid precursor protein (APP) by secretases aggregate into extracellular amyloid plaques, and hyperphosphorylated tau accumulates intracellularly to form neurofibrillary tangles (NFTs; Masters et al., 1985; Grundke-Iqbal et al., 1986). PD and dementia with Lewy Bodies (DLB) are characterized by Lewy bodies (LBs) and Lewy neurites (LN) that include oligomerized α-synuclein (α-syn), as well as lipid membranes and organelles (Spillantini et al., 1997; Shahmoradian et al., 2019).

While we will focus on AD and PD-related pathology in this review, several other neurodegenerative diseases such as other α-synucleinopathies and tauopathies, Huntington's disease, Amyotrophic lateral sclerosis (ALS), and Prion disease are also characterized by neuronal inclusions with specific pathologic protein aggregates. Understanding the mechanisms underlying protein aggregation and spread in AD and PD could hopefully be applied to other aggregate-prone neurodegenerative conditions to develop disease-modifying therapies.

Each neurodegenerative pathologic aggregate has a distinctive temporo-spatial pattern of spread throughout the nervous system that is characteristic of the neurodegenerative disease and correlates with clinical progression (Bancher et al., 1993). AD disease onset is associated with the formation of Aβ plaques and NFTs in the entorhinal cortex, with predictable spread to the hippocampus and eventually throughout the neocortex as the disease progresses (Braak and Braak, 1991; Braak et al., 2006; Nath et al., 2012). In PD, LBs and LNs first appear in the lower brainstem and olfactory neurons, ascending through the midbrain and limbic system to eventually spread throughout the neocortex by the end-stage of the disease (Braak et al., 2003). Understanding the mechanisms regulating the spread of these neurodegeneration-associated aggregates, which are closely associated with clinical disease progression, could reveal novel pathways that could be therapeutically altered to slow or halt disease progression.

A major clue into the mechanism of protein aggregate spread emerged from early investigations of GBA-associated PD. *Glucosylceramidase Beta* (*GBA*) encodes a lysosomal enzyme glucocerebrosidase (GCase), that hydrolyzes glucosylceramide into glucose and ceramide. Recessive mutations in *GBA* cause Gaucher's disease, the most common lysosomal storage disease [*GBA* (OMIM 606463)]. However, in the early 1990s, clinicians caring for Gaucher's patients noted that there seemed to be an unusually high prevalence of PD in 1st degree relatives, far greater than expected within the general population (Neudorfer et al., 1996). Through further clinical and genome- wide association studies, *GBA* mutations are now recognized to increase the risk of developing PD by at least 5-fold compared to non-carriers making *GBA* mutations the strongest genetic risk factor for PD (Sidransky et al., 2009; Nalls et al., 2013). Examination of longitudinal cohorts of PD patients later revealed that *GBA* mutations not only increase the risk of developing PD but also lead to a more malignant clinical course. PD patients carrying *GBA* mutations have an increased risk of developing cognitive deficits and faster progression of both cognitive and motor function decline (Winder-Rhodes et al., 2013; Brockmann et al., 2015; Davis et al., 2016a,b). This observation was further supported by a transgenic α-syn^{A53T} mouse model that had an earlier onset and faster progression of degenerative symptoms in a heterozygous GBA knockout background (Tayebi et al., 2017). These findings suggest that *GBA* may influence not only the development of Lewy pathology in PD but also the spread of Lewy pathology throughout the brain, manifested by accelerated disease progression.

Increasing evidence suggests that extracellular vesicles (EVs) such as exosomes and synaptic vesicles (SVs) play a major role in the propagation of neurodegeneration-associated protein aggregates throughout the brain. Crucially, examination of *GBA*-PD patient tissues as well as modeling *GBA*-associated neurodegeneration has revealed that GBA activity regulates EVs, which may explain its role in accelerating protein aggregate spread (Papadopoulos et al., 2018; Thomas et al., 2018; Jewett et al., 2021). As *GBA* encodes an enzyme important for ceramide metabolism, investigations into the lipid metabolism alterations due to *GBA* mutations have led to interesting hypotheses into new mechanisms by which lipid metabolism influences neurodegenerative disease progression. Lipid and in particular ceramide metabolism alterations have also been reported in AD (Han et al., 2002; Wang et al., 2008; Mielke et al., 2010, 2012; Czubowicz et al., 2019), supporting a role for ceramide in promoting aggregation of Aβ associated with lipid rafts and spread of tau and amyloid beta (Aβ) *via* exosomes, as well as alterations in lipid metabolism associated with disease risk and progression. In this review, we will next examine the evidence supporting the role of lipid metabolites in influencing exosome biogenesis, uptake by recipient cells, and rate of disease progression. Together, these suggest that the vehicle, not just the cargo of EVs, is important for the pathogenesis of neurodegenerative diseases.

LIPID METABOLISM IN NEURODEGENERATION

Lipids are crucial for the normal development and function of the central nervous system (CNS). Sphingolipids and cholesterol are the most abundant class of lipids that are found in the CNS, mainly residing in myelin (O'brien and Sampson, 1965; Kishimoto et al., 1969; Giussani et al., 2021). Lipids are not only the building blocks for membranes, but lipid composition, degree of unsaturation, and length of fatty acyl tails can dictate fluidity of membranes, lipid raft membrane microdomains, and influence vesicle fusion and secretion (Mencarelli and Martinez-Martinez, 2013). Sphingosine, a metabolite of ceramide, is also bioactive and can regulate multiple cellular functions, including signaling, apoptosis, mitochondrial function, immune function, and metabolism (Mencarelli and Martinez-Martinez, 2013; Wang and Bieberich, 2018).

Lipids are increasingly implicated in neurodegenerative diseases. Identification of a growing number of genes involved in lipid metabolism through Mendelian inheritance, genome-wide association studies (GWAS), and transcriptomic studies have been implicated in AD, PD, and other neurodegenerative diseases. The *Apolipoprotein epsilon4* allele is the most common genetic risk factor for AD and is important for transporting cholesterol into the brain (Mahley, 1988; Huang and Mahley, 2014). Numerous lipid metabolism-related risk genes have been identified through GWAS studies for AD, recently reviewed by Chew et al. (2020). A growing number of causative genes and risk loci for PD have also been implicated in lipid metabolism, including *PLA2G6/PARK14, SCARB2, SMPD1, SREBF1, DGKQ*, which were recently reviewed (Alecu and Bennett, 2019). And, as reviewed above, *GBA* is a critical gene in ceramide metabolism,

and mutations in *GBA* are the most penetrant genetic risk factor for PD.

Lipidomic analysis of tissues from AD patients and AD animal models have revealed alterations in the levels of numerous lipids, including fatty acids, glycerolipids, glycerophospholipids, sphingolipids, and cholesterol (Mesa-Herrera et al., 2019; Chew et al., 2020). Phospholipid deficiencies such as the level of ethanolamine plasmalogen relative to phosphatidylethanolamine levels were observed in postmortem brain tissue of AD patients (Ginsberg et al., 1995). Ceramide has been of particular interest given the paradigm of the ceramide-sphingosine-1-P (S-1-P) rheostat where the balance of these two closely linked and regulated lipids may determine cell death vs. survival (reviewed in Hait et al., 2006; Taniguchi and Okazaki, 2020). While ceramide has been shown to have pro-apoptotic, autophagic, and inflammatory effects, S-1-P has a pro-survival effect mediated by G-protein coupled receptor signaling. Ceramide is also implicated in AD pathogenesis by promoting aggregation of Aβ through interaction *via* lipid rafts, which are enriched in cholesterol and sphingolipids, and ceramide-enriched exosome membranes (Czubowicz et al., 2019). Higher ceramide levels have been reported in several studies analyzing tissue from AD patients and rodent models of AD. Brain tissue from AD patients with mild to moderate symptoms associated with higher total ceramide levels compared to age-matched controls (Han et al., 2002). Ceramide levels were also increased in cerebrospinal fluid (CSF) of AD patients compared to ALS patients and controls, and immunohistochemistry of frontal cortex revealed increased ceramide in astrocytes (Satoi et al., 2005). Analysis of plasma from a small group of individuals with AD, mild cognitive impairment, and controls found that higher baseline levels of the long-chain ceramides C22:0 and C24:0 were predictive of cognitive decline and hippocampal atrophy (Mielke et al., 2010). Analysis of serum in a longitudinal study of 99 women during their 8th decade again found higher baseline levels of long-chain ceramides to be associated with increased risk of AD (Mielke et al., 2012). Serum ceramide levels were increased in both AD and DLB patients (Savica et al., 2016). Ceramides have also been found to be increased in brain tissue from rodent models of AD (Alessenko et al., 2004; Wang et al., 2008).

Significant alterations in lipid metabolism are also observed in PD. While it is not unexpected that *GBA* carriers with PD may have lipid metabolism abnormalities, even sporadic PD patients without *GBA* mutations were observed to have reduced GCase activity in postmortem tissue (Murphy et al., 2014). Furthermore, the level of reduction in *GBA* enzyme activity inversely correlated with α-syn expression, although high molecular weight α-syn oligomers were not assessed in this study (Murphy et al., 2014). Subsequent studies have confirmed that even sporadic PD patients without *GBA* mutations have reduced GCase enzyme activity, albeit to a lesser extent than GBA carriers with PD (Alcalay et al., 2015). Sporadic PD patients also have altered levels of glucosylceramide and ceramide, the substrate and product of GCase enzyme activity (Mielke et al., 2013; Guedes et al., 2017), further supporting the observation that GBA enzymatic dysfunction is not a prerequisite for ceramide accumulation. EVs isolated from PD patient brain tissue were found to have increased levels of membranous ceramides that increased binding affinity for α-syn, leading to increased α-syn accumulation, aggregation, and propagation (Kurzawa-Akanbi et al., 2021). These studies suggest that reduced GCase activity and the resulting alterations in lipid metabolism are critical in the pathogenic process causing PD. Finally, reducing the substrate of GCase in a mouse model of *GBA* PD through inhibition of glucosylceramide synthase reduced insoluble α-syn oligomerization and accumulation of ubiquitinated proteins (Sardi et al., 2017), providing compelling evidence that ameliorating altered lipid metabolism in PD can reduce the underlying pathology.

Ceramides have multiple bioactive influences, and it remains unclear whether they may have a neuroprotective or neurotoxic influence. Since *GBA* mutations lead to accumulation of glucosylceramide and possible relative reduction in ceramide, it has been hypothesized that reduced ceramide may also contribute to the pathogenesis in *GBA*-related PD. In support of this hypothesis, GCase-deficient HEK293 cells treated with exogenous C18-Ceramide or the acid ceramidase Carmofur reversed impairment in secretory autophagy and reduced α-syn levels (Kim et al., 2018). Similar findings were observed in iPSC-derived dopaminergic neurons heterozygous for a *GBA* mutation, where treatment with Carmofur resulted in reduced ubiquitinated proteins and oxidized α-syn species (Kim et al., 2018). Treatment of our *Drosophila GBA* deficient model with Carmofur also led to a reduction in accumulated insoluble ubiquitinated proteins (unpublished).

Altered metabolism of ceramide and glucosylceramide leads to additional downstream lipid alterations with as yet unclear pathogenic effects. In a double mutant transgenic mouse model heterozygous for *GBA* and expressing wild-type human α-syn through its endogenous promoter, glucosylsphingosine was significantly increased, while glucosylceramide, ceramide, and sphingosine levels were similar to wild-type *GBA* controls without human α-syn expression (Ikuno et al., 2019). This is likely due to the deacylation of glucosylceramide by lysosomal acid ceramidase, resulting in glucosylsphingosine, which also accumulates in Gaucher's disease (Ferraz et al., 2016). Glucosylsphingosine can exit the lysosome, where it can be hydrolyzed by non-lysosomal GBA2 into ceramide, sphingosine, and sphingosine-1-phosphate (Abed Rabbo et al., 2021), resulting in complex alterations of downstream lipids that remain poorly understood.

Phospholipid bis(monoacylglycerol)phosphate (BMP) species are also lipids of interest in neurodegeneration, as they are enriched in late endosome-lysosomes. Inhibiting Vps34, a lipid kinase important for the synthesis of phosphatidylinositol-3-phosphate (PI3P), which was found to be reduced in the AD brain and is an important regulator of endosomal trafficking (Morel et al., 2013), resulting in increased secretion of exosomes by cultured neurons and in a conditional knockout mouse model (Miranda et al., 2018). The increased exosome biogenesis occurred in the context of lysosomal stress, where C-terminal APP fragments were selectively sorted as exosome cargo. The resulting exosomes had a unique enrichment in BMPs in addition to ceramides and sphingomyelin (Miranda

et al., 2018). Interestingly, specific urine BMPs were found to be associated with *LRRK2* G2019S mutation carriers with PD and cognitive decline (Alcalay et al., 2020). *LRRK2*, the most common genetic cause of PD, is also implicated in endolysosomal function (Erb and Moore, 2020), suggesting BMPs could be exploited as a biomarker for lipid alterations associated with neurodegeneration involving endolysosomal impairment.

Another way that lipid metabolism alterations may promote neurodegenerative pathology is through direct interaction with aggregate-prone proteins. Significant evidence supports this to be true with α-syn. Sharon et al. (2003) demonstrated that α-syn oligomerizes into insoluble aggregates in the presence of polyunsaturated fatty acids, but not saturated fatty acids, and the length of the fatty acyl chain also influences oligomerization. In an *in vitro* system using small unilamellar vesicles, α-syn adopted an amyloid-like conformation under conditions where it binds to the lipid bilayer (Galvagnion et al., 2015). In AD, studies have shown that Aβ fibrillization is accelerated and binding is enhanced when incubated with artificially engineered phospholipid vesicles due to the vesicles' high membrane curvature (∼30 nm diameter; Sugiura et al., 2015). However, it has not been reported whether the extent of the Aβ aggregation observed in this *in vitro* study is comparable to *in vivo* exosomes or SVs.

EV BIOGENESIS AND LIPID COMPOSITION

Intercellular communication by way of small lipid-bound vesicles, known as EVs, are essential and evolutionarily conserved throughout all multicellular organisms (Lawson et al., 2017). EVs are released from many cell types, including neurons, into the extracellular space and carry proteins, lipids, and genetic cargo (Thery et al., 2002; Zhang et al., 2015; Lawson et al., 2017). EVs are composed of a lipid bilayer that not only protects the encapsulated products but also influences extracellular trafficking and membrane fusion to recipient cells (Skotland et al., 2017).There are varying types of EVs, but they can be roughly categorized as either exosomes (50–100 nm in size) or microvesicles (100–1,000 nm in size; Stahl et al., 2019). Of the two subtypes, exosomes and their similar but smaller counterpart (∼40 nm), synaptic vesicles (SVs), are the most well studied and are shown to participate in the spread of aggregated proteins involved in neurodegenerative diseases such as AD and PD (Coleman and Hill, 2015; Skotland et al., 2017; Vogel et al., 2020; Gagliardi et al., 2021).

Lipids are essential for the biogenesis and formation of exosomes as they form the lipid bilayer of the vesicles. Biogenesis of exosomes initiates when the cytoplasmic membrane invaginates extracellular proteins and buds inward to generate early endosomes. Early endosomes then go through another round of inward budding, leading to the formation of intraluminal vesicles (ILVs) and late endosomes. Late endosomes encompassing ILVs are recognized as multivesicular bodies (MVBs). The MVBs either fuse with lysosomes for lysosomal degradation or fuse with the plasma membrane for exocytosis generation of exosomes (**Figure 1**). Canonical endolysosomal trafficking is mediated by Endosomal Sorting Complexes Required for Transport (ESCRT) complexes and small Rab GTPases (Weeratunga et al., 2020). Regulation of how ESCRT machinery sorts endocytic cargo for recycling and degradation continues to be an active area of discovery and has revealed lipid mediators such as phosphoinositides and lipid transporters (Shen et al., 2011; Stalder and Gershlick, 2020). In addition to the ESCRT-mediated exosome biogenesis pathway, there is also an ESCRT-independent biogenesis pathway that relies on neutral sphingomyelinase (nSMase; Trajkovic et al., 2008; Menck et al., 2017). Neutral sphingomyelinase hydrolyzes membrane lipid sphingomyelin, generating ceramide and phosphocholine. Sphingomyelin hydrolysis is also an important pathway for larger microparticle EV biogenesis in both astrocytes and glia (Bianco et al., 2009).

SVs also use endosomal intermediates but differ from exosomes in that fusion with the presynaptic plasma membrane for neurotransmitter release relies on calcium-triggered fusion mediated by synapse-specific machinery (Sudhof and Rizo, 2011; Wu et al., 2014). Endocytosis of SVs can occur through four suggested pathways: (1) "kiss-and-run" endocytosis; (2) clathrin-mediated endocytosis; (3) ultrafast endocytosis; and (4) bulk endocytosis. These endocytic pathways are reviewed in detail in Janas et al. (2016; **Figure 2**).

Exosomes and SVs have many overlapping features, but also have distinctive protein markers, size, and lipid compositions. Both types of vesicles have been shown to aid the pathogenic spread of neurodegenerative disorders *via* the endocytic pathway, which is where these vesicles are formed. Both exosomes and synaptic vesicles contain similar cholesterol levels (44% mol) that help maintain the arrangement of phospholipids in the membrane's bilayer and thereby help maintain the rigidity of the vesicle's membranes (Wang et al., 2020). While both exosomes and synaptic vesicles are equally highly enriched in cholesterol, exosomes are also highly enriched in sphingolipids in the form of sphingomyelin (SM), which is crucial for the structure and rigidity of exosomes. SM d18:1/16:0, SMd18:1/24:0, and SMd18:1/24:1 constitute approximately 35%, 20%, and 20%, respectively of the total SM species in exosomes and play a pivotal role in exosome biogenesis by promoting the budding of exosomes (Skotland et al., 2017). On the other hand, synaptic vesicle membranes have comparatively higher levels of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositols (Janas et al., 2016).

EVs AS A VEHICLE MEDIATING NEURODEGENERATIVE DISEASE PROGRESSION

Recent studies increasingly suggest that EVs play a critical role in the progression of neurodegenerative diseases. There is mounting evidence supporting upregulation of exosome biogenesis as an alternative strategy for cells when the lysosomal function is impaired. Although upregulation of exosome biogenesis may be a mechanism for the cell to get rid of unwanted proteins, these exosomes appear to also be vehicles

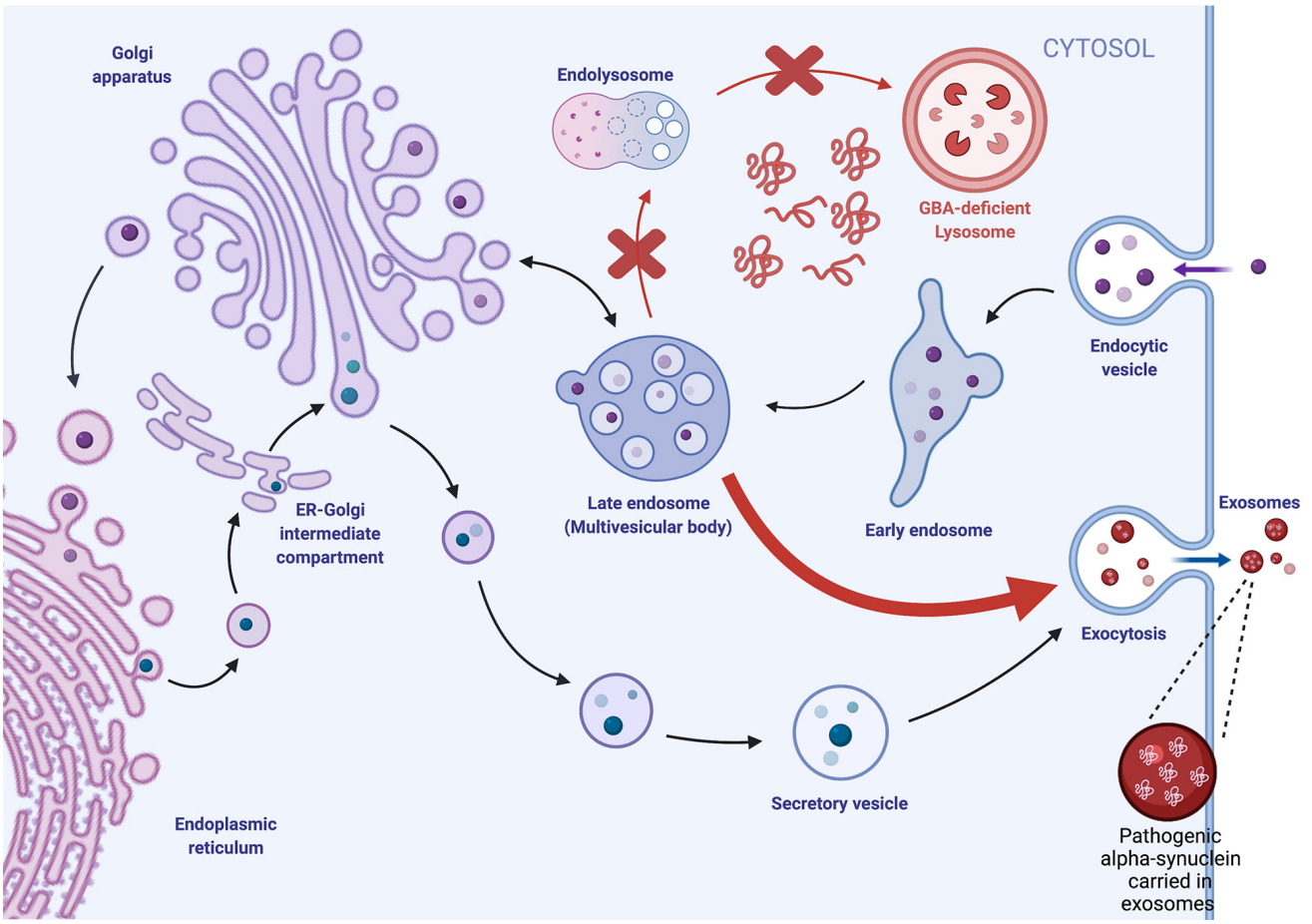

FIGURE 1 | Exosome biogenesis is dysregulated by lysosomal GBA deficiencies. Extracellular proteins are endocytosed and trafficked intracellularly *via* endocytes, where they undergo multiple rounds of inward budding to generate intraluminal vesicles (ILVs) within late endosomes/multivesicular bodies (MVBs). MVBs can fuse with the lysosome for degradation or fuse with the plasma membrane, where ILVs are released as extracellular vesicles (EVs). We hypothesize that GBA deficiency impairs lysosomal degradation, leading to the accumulation of intracellular protein aggregates and increased trafficking of the MVB to the plasma membrane for EV biogenesis.

for propagating pathogenic protein aggregation, ultimately promoting disease progression.

Aβ fragments and tau have been shown to be secreted through exosomes, which mediate cell to cell transfer of these proteins (Saman et al., 2012; Asai et al., 2015; Wang et al., 2017; Laulagnier et al., 2018; Sardar Sinha et al., 2018). In N2a neuroblastoma cells expressing the APP Swedish mutation, C-terminal fragments of APP resulting from cleavage by β- and gamma-secretases were enriched within secreted exosomes and selectively internalized by recipient neurons (Laulagnier et al., 2018). Exosomes isolated from human AD brain tissues were internalized by co-cultured neuroblastoma cells, resulting in increased cytotoxicity compared to exosomes from control brain tissue (Sardar Sinha et al., 2018). Furthermore, inhibiting exosome formation by knocking down ESCRT proteins TSG101 and VPS4A, which are essential for exosome formation, decreased the number of secreted exosomes and inhibited the spread of Aβ (Sardar Sinha et al., 2018). An ESCRT-independent exosome biogenesis pathway has also been identified, which relies on neutral sphingomyelinase (nSMase; Menck et al., 2017). Neutral sphingomyelinase hydrolyzes membrane lipid sphingomyelin, generating ceramide. This lipid-dependent exosome pathway is relevant to neurodegeneration, as inhibition of neutral sphingomyelinase 2 (nSMase2) with the inhibitor GW4869 in 5XFAD mice significantly reduced exosome production, brain ceramide levels, and Aβ plaque pathology (Dinkins et al., 2016). Together, these studies support a role for exosomes in propagating AD pathology.

Exosomes are also implicated in the propagation of Lewy pathology in PD. α-syn is normally membrane-bound and associated with synaptic vesicles. In disease contexts, it has been observed to be packaged both within exosomes and associated with the outer membrane of exosomes. Neuroblastoma SH-SY5Y cells expressing wildtype α-syn were found to secrete α-syn monomers and oligomers extracellularly in association with exosomes in a calcium-dependent manner (Emmanouilidou et al., 2010) as well as when the lysosomal function was inhibited (Alvarez-Erviti et al., 2011). Several subsequent studies have

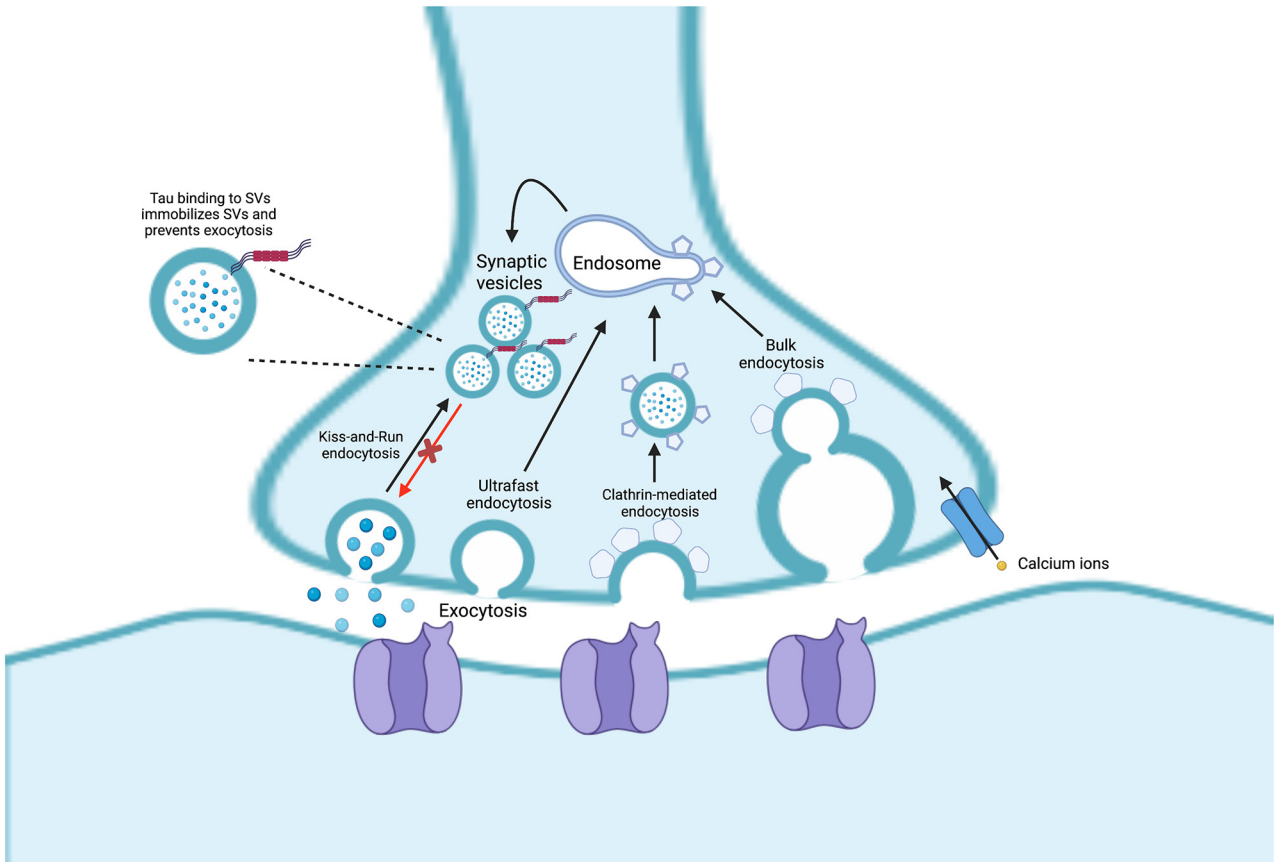

FIGURE 2 | Endocytosis of synaptic vesicles (SVs) can occur through four pathways: (1) "kiss-and-run" endocytosis; (2) ultrafast endocytosis; (3) clathrin-mediated endocytosis; and (4) bulk endocytosis. In Alzheimer's disease (AD), pathogenic tau can bind to the SVs which can lead them to be less mobile, inducing SV clustering and reducing neurotransmission.

confirmed the association of α-syn with secreted EVs, reviewed in Emmanouilidou and Vekrellis (2016). Internalization of α-syn-containing exosomes by normal SH-SY5Y cells resulted in increased α-syn within the recipient cells, demonstrating that exosomes can be a vehicle for transferring and propagating pathogenic proteins to other cells (Emmanouilidou et al., 2010; Alvarez-Erviti et al., 2011). Similar findings were observed in human neuroglioma and rat primary cortical neuron cultures overexpressing human wild-type α-syn, where inhibiting autophagy increased production of EVs containing α-syn as well as proteins associated with autophagy such as p62, LC3II, and LAMP2A (Minakaki et al., 2018). Exosomes isolated from CSF of PD patients and injected into a reporter cell line for α-syn oligomerization resulted in increased oligomerization (Stuendl et al., 2016). Subsequent studies have replicated these findings *in vivo*, where exosomes isolated from CSF or brain tissue of PD and DLB patients were injected into mouse brains, resulting in α-syn oligomerization around the site of injection of EVs (Ngolab et al., 2017; Minakaki et al., 2018). These studies further indicate that EVs can mediate cell to cell transfer of α-syn pathology.

GBA mutations appear to have a similar effect in impairing lysosome function, resulting in increased production of EVs carrying pathogenic proteins such as α-syn that propagate pathogenic aggregates in recipient cells. Our work using a *Drosophila* model of GBA deficiency not only revealed increased protein aggregation but also upregulation of proteins associated with EV biogenesis and dysregulation of EVs, including increased levels of EV-intrinsic proteins and Ref(2)p, the *Drosophila* homolog of p62 (Davis et al., 2016c; Thomas et al., 2018). A similar finding was observed in *synuclein* A53T mice treated with Conduritol B epoxide (CBE) to inhibit GCase enzyme activity, resulting in an increase in α-syn-containing exosomes (Papadopoulos et al., 2018). Furthermore, tissue-specific restoration of wildtype *GBA* in *GBA* mutant flies revealed non-cell autonomous rescue of protein aggregation in distant tissues including the brain (Jewett et al., 2021). This non-cell autonomous suppression of protein aggregation was accompanied by normalization of the dysregulation in EVs observed in *GBA* mutant flies (Jewett et al., 2021). Together, these results suggest that GBA deficiency accelerates PD pathogenesis through the propagation of protein aggregates by dysregulating exosome biogenesis. This mechanism could support why *GBA* mutations are observed to associate with faster progression of PD, as the upregulation in exosome biogenesis may accelerate the spread of Lewy pathology.

Synapses are crucial sites for chemical communication between neurons and play a vital role in the dynamic functions of the brain. *In vivo* studies in mice and humans indicate that tau protein aggregation spreads readily to trans-synaptic regions of the brain, although exosomes at the synaptic cleft, not synaptic vesicles, may be mediating propagation of tau aggregates (Liu et al., 2012; de Calignon et al., 2012; Vogel et al., 2020; Miyoshi et al., 2021). In AD, synaptic dysfunction is considered to be an early pathological event, preceding Aβ deposits in the brain (Marsh and Alifragis, 2018). Though accumulation of hyperphosphorylated tau in NFTs is a hallmark of AD, soluble, non-aggregated forms of tau appear to be the main toxic element inducing early synaptic deficits (Ahmed et al., 2014; Zhou et al., 2017). In studies using fly and rodent models and post-mortem human brain, tau has been shown to associate with SVs either through protein binding or through direct membrane interactions (Zhou et al., 2017). Tau binding to SVs leads them to be crosslinked and less mobile, thereby inducing SV clustering and attenuating neurotransmission (McInnes et al., 2018).

LIPID ALTERATIONS INFLUENCING EXOSOMES AND PROGRESSION OF NEURODEGENERATION

Lipid metabolism alterations influence multiple aspects of EV biogenesis, including the vesicular trafficking steps leading to exosome release, exosome morphology, and the selective cargo associated with EVs. Lipid composition strongly dictates the curvature of the lipid membrane, and because EVs are so small, the surface area and curvature of the inner membrane can differ significantly from the outer membrane (Skotland et al., 2017). EV membranes are enriched in lipids with monounsaturated fatty acyl groups compared to plasma membranes, likely due in part to the high curvature of EV membranes (Skotland et al., 2017). Unsaturation of fatty acyl chains of ceramides allows for denser packing of ceramide molecules and reduced fluidity of membranes. The length of fatty acyl chains can also influence membrane curvature, with shorter fatty acyl chains positively affecting the curvature in lipid monolayers (Skotland et al., 2017).

Alterations in lipid density and membrane curvature led to various α-syn structural changes and alterations in binding affinity, suggesting that the curvature of the membrane may be a key influence in driving interactions promoting α-syn oligomerization (Middleton and Rhoades, 2010; Pranke et al., 2011). The binding of α-syn to negatively charged lipids within the membrane was also observed to induce conformational changes of EV membranes, increasing vesicle permeability (Hannestad et al., 2020). These studies suggest a bidirectional interaction between the exosome membrane and α-syn that may further enhance neurotoxic effects.

The lipid composition of exosomes also modulates α-syn aggregation, exosome release, and cargo sorting. α-syn has increased affinity to anionic lipids and membranes with increased fluidity (Kjaer et al., 2009; Galvagnion et al., 2016). Systematic analyses of alterations in membrane composition of spherical unilamellar vesicles on *in vitro* α-syn aggregation revealed that gangliosides GM1 and GM3 enrichment accelerated the aggregation of α-syn (Grey et al., 2015), indicating that lipid composition can have a significant influence on α-syn pathology. The reduction of ceramide, which is critical for ESCRT-independent exosome release, with inhibition of neutral sphingomyelinase 2 (nSMase2) reduced the spread of α-syn among co-cultured SH-SY5Y cells (Sackmann et al., 2019). A recent study of brain tissue and CSF of patients with PD or DLB with and without *GBA* mutations found that there was a significant increase in several ceramide species in brain tissue and EVs isolated from CSF from all disease patients compared to control, independent of *GBA* genotype (Kurzawa-Akanbi et al., 2021). Interestingly, proteomic analysis revealed the presence of α-syn, β-syn, gamma-syn, and tau in both disease and control EVs, with no significant difference in levels between either group. However, wild-type α-syn monomers were observed to bind much more readily to disease EVs than control EVs, and this interaction resulted in fibrillization of α-syn, which was absent in the presence of control EVs (Kurzawa-Akanbi et al., 2021). Although it was surprising that the presence of disease rather than *GBA* genotype was associated with α-syn aggregation, lipid alterations, and α-syn-EV interactions, this study interrogated post-mortem tissues from presumably end-stage disease, where the effect of GBA genotype may not be as strong as early and mid-stage disease. These studies suggest that the lipid composition of EVs may be more influential in the progression of neurodegeneration than the EV cargo itself.

In AD, Aβ associates with exosomes as well, although there is conflicting evidence on whether this association promotes or slows the progression of disease (Rajendran et al., 2006). A reduction in the expression of TSG101 and RAB35 and exosome production was observed in a mouse model ApoE4, suggesting that ApoE4 disrupts exosome biogenesis, leading to endolysosomal deficits and Aβ accumulation (Peng et al., 2019). Exosomes were also reduced in human postmortem brain samples of ApoE4 carriers compared to *ApoE3* carriers (Peng et al., 2019). However, the loss of nSMase2 in 5XFAD mice, a model of Aβ aggregation, resulted in decreased ceramide levels and brain exosomes, reducing plaque burden and tau phosphorylation as well as improving cognition on a fear-based conditioning task (Dinkins et al., 2016). It is possible that while overall production of exosomes may be decreased in AD, a subpopulation of exosomes dependent on ceramide metabolism for biogenesis is responsible for the propagation of AD pathology, and further reduction of ceramide levels and exosome production may be neuroprotective. In addition, alterations in lipid composition of EV membranes may have cell-specific effects, as an increase in gangliosides in exosomes from neuronal cells deficient in sphingomyelin synthase 2 (SMS2) resulted in increased uptake and lysosomal degradation of Aβ into co-cultured microglia (Yuyama et al., 2012).

GLIAL ROLE IN PROPAGATION OF NEURODEGENERATIVE PATHOLOGY

Much of the focus thus far in understanding the propagation of neurodegenerative protein aggregate pathology has been on

neuron-to-neuron transfer. However, the field is beginning to turn its attention to glial cells directly interacting with neurons. Increasing evidence shows that both astrocytes and microglia can uptake neuronal exosomes as well as release exosomes containing neurodegenerative-related proteins and lipids. We will first assess evidence for exosome uptake by glia, which are the most abundant cell type within the brain. We will then review evidence for whether recipient non-neuronal cells are playing a neuroprotective or toxic role to neighboring neurons in their ability to process and propagate aggregate-prone proteins.

Astrocytes are the most abundant glial cell type in the CNS and are essential in supporting and maintaining a homeostatic environment for neurons *via* mechanisms such as glutamate synthesis, vasomodulation, and glycogen storage and metabolism (Phelps, 1972; Stobart and Anderson, 2013; Nortley and Attwell, 2017; Barber and Raben, 2019). Astrocytes support neuronal lipid metabolism by synthesizing lipids associated with synaptic vesicle formation and engulfing oxidized lipids from highly metabolic neurons (Barber and Raben, 2019). Astrocytes also synthesize lipoproteins that mediate cholesterol efflux by neurons and are a reservoir for fatty acids and glycogen that are metabolized by neurons (Gu et al., 2010; Chen et al., 2013). Astrocytes have also been shown to endocytose fatty acids from hyperactive neurons and accumulate lipid droplets, along with shuttling fatty acids to the mitochondria for oxidative phosphorylation and performing lipolysis, thereby preventing neuronal lipotoxicity (Qi et al., 2021).

More recently, astrocytes have been implicated in the propagation of pathogenic protein aggregates. While astrocytes do not express α-syn they have been observed to uptake cytoplasmic pre-aggregate species and oligomeric fibrils of α-syn more readily than neurons (Lee et al., 2010; Gustafsson et al., 2017; Loria et al., 2017; di Domenico et al., 2019; Tsunemi et al., 2020) They have also been shown to uptake Aβ protofibrils and aggregates (Mulder et al., 2014; Sollvander et al., 2016; Nilson et al., 2017) and tau monomeric species, pre–formed tau fibrils, and phosphorylated tau aggregates (Martini-Stoica et al., 2018; Perea et al., 2019; Mate De Gerando et al., 2021). Furthermore, astrocytes uptake Aβ more readily when packaged within EVs (Dinkins et al., 2016), as well as α-syn associated with EVs (Tsunemi et al., 2020). In post-mortem AD brains, astrocytes were found to localize around amyloid-plaques and hyperphosphorylated tau in hippocampal regions (Richetin et al., 2020). Similar findings were observed in an AD mouse model, where astrocytes surrounded Aβ aggregates (Dinkins et al., 2016). This evidence suggests a protective role of astrocytes where in addition to internalizing free extracellular pathogenic proteins associated with neurodegeneration, astrocytes can also endocytose pathogenic proteins associated with EVs, and may do this more efficiently than neurons. These studies have led to the hypothesis that astrocytic uptake of secreted extracellular aggregate-prone proteins assists in preventing the uptake of proteins that otherwise would be endocytosed by neurons and seed further neuronal spread of pathology.

Astrocytes' role in neurodegenerative contexts is further assessed by studies examining neurodegenerative genetic perturbations. Expressing α-syn with the familial PD mutation A53T in astrocytes of mice caused widespread astrogliosis in multiple brain regions and increased levels of α-syn aggregation (Gu et al., 2010). Dysregulation of the blood-brain barrier, as evidenced by altered Aquaporin 4 and Glut1 expression, and reduced expression of excitatory amino acid transporters was also observed, suggesting impairment in astrocytic function in maintaining neuronal excitation homeostasis (Gu et al., 2010). Astrocytes derived from iPSCs from patients with *ATP13A2* mutations causing familial parkinsonism had reduced endocytosis of neuronal EV-associated α-syn (Tsunemi et al., 2020). When *ATP13A2* mutant astrocytes were co-cultured with control neurons, there was an increase in neuronal intracellular α-syn, indicating that astrocytes normally have a neuroprotective role in reducing neuronal pathology.

However, once endocytosed, astrocytes may not effectively degrade aggregate-prone proteins, leading to increased neurotoxic effects. In iPSC-derived astrocytes co-cultured with neurons, astrocytes showed preferential endocytosis of neuronal secreted α-syn, but α-syn subsequently accumulated within astrocytes and co-localized with LAMP-2 over several days, suggesting that lysosomal degradation was impaired (di Domenico et al., 2019; **Figure 3A**). Similarly, when primary mouse neurons, astrocytes, and oligodendrocytes were co-cultured and exposed to recombinant labeled α-syn oligomers, astrocytes rapidly took up most of the α-syn oligomers compared to neurons and oligodendrocytes (Lindstrom et al., 2017). However, α-syn oligomers remained detectable and co-localized with LAMP-1 for over 12 days after uptake, and astrocytes developed evidence of mitochondrial damage (Lindstrom et al., 2017). In addition, death of co-cultured neurons was observed 12 days after exposure to the α-syn oligomers, coinciding with the impairments in endolysosomal trafficking and mitochondrial defects in the astrocytes, suggesting that astrocytes may be producing non-cell autonomous neurotoxic signals in response to the endocytosed α-syn oligomers. Rat primary astrocytes were also observed to uptake α-syn oligomers, this time purified from postmortem brain tissue of PD patients, more efficiently than rat primary cortical neurons (Cavaliere et al., 2017). Cavaliere et al. (2017) cultured neurons and astrocytes in a microfluidic chamber system to test whether α-syn oligomers exposed to neurons or astrocytes could be transferred to neurons or astrocytes. Consistent with other studies, they found that α-syn oligomers endocytosed by astrocytes led to increased neuronal cell death compared to direct uptake of α-syn oligomers by neurons. These studies suggest that endocytosis of α-syn may cause astrocytes to become neurotoxic reactive astrocytes.

Astrocytes can also uptake tau pathology, which can be enhanced by lysosomal function. Mate De Gerando et al. (2021) confirmed that tau can be transferred between neurons and astrocytes *in vivo* in a mouse model by expressing a pro-aggregating tau peptide in neurons and human wild-type tau in astrocytes in the hippocampus. Using an antibody that only detects the aggregates formed by co-expression of both forms of tau, positive tau-aggregate staining was observed in

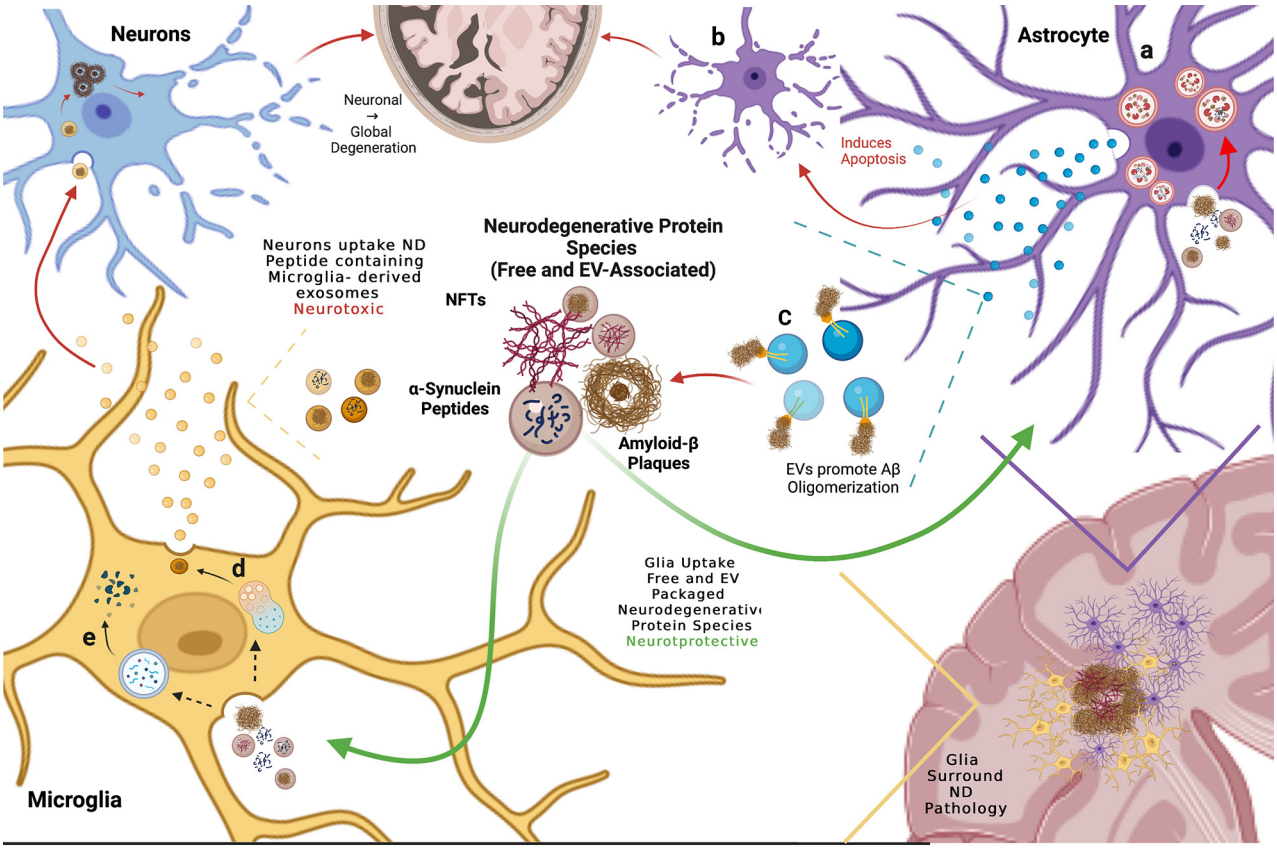

FIGURE 3 | Glial cells mediate propagation of neurodegenerative protein species by up-taking both free and extra-cellular vesicle (EV)-associated proteins, thereby preventing neuronal uptake and spread of disease. **(A)** Astrocytes endocytose free and EV-associated aggregate prone proteins including α-syn and Aβ and process them in the lysosome but subsequent lysosomal dysfunction results in debris-burdened enlarged lysosomes that do not degrade the engulfed material. **(B)** When exposed to Aβ, astrocytes release EVs that induce apoptosis in neighboring astrocytes. **(C)** Astrocytes also release EVs enriched in glycosphingolipids (GSLs) that bind monomeric Aβ, promoting oligomerization and plaque formation. **(D)** Microglia uptake both free and EV-associated aggregate-prone proteins and process them through the endolysosomal system where they are repackaged into EVs that are released and engulfed by neighboring neurons, presenting evidence of neurotoxic propagation from glia to neurons. **(E)** Microglia degrade endocytosed pathogenic proteins *via* phagocytosis, clearing aggregate prone proteins.

both cell types, indicating transfer of tau species between neurons and astrocytes. Martini-Stoica et al. (2018) overexpressed Transcription Factor EB (TEFB), a master regulator of lysosomal biogenesis, in astrocytes and found that this not only increased trafficking of pre-formed fibrillary tau fibrils to the lysosome but also increased uptake of these fibrils. Increased expression of TFEB in astrocytes in the PS19 tauopathy mouse model reduced tau pathology and decreased levels of astrocyte gliosis, although increased expression of astrocytic TFEB in a more rapidly progressive tauopathy mouse model did not reduce tau pathology. These studies suggest that healthy astrocytes can process tau oligomers effectively *via* endolysosomal trafficking to reduce the spread of tau pathology and gliosis.

However, astrocytes may also release exosomes that mediate the spread of pathogenic protein aggregates. Astrocytes localized near Aβ peptides and aggregates in whole mouse brains upregulated ceramide production, resulting in increased biogenesis of ceramide (specifically C18:0, C24:0)-enriched exosomes that induced an apoptotic response in neighboring astrocytes (Wang et al., 2012; Dinkins et al., 2016; **Figure 3B**). The astrocyte-derived exosomes released in response to Aβ exposure also contained phosphorylated-tau (p-tau), a precursor for neurofibrillary tau tangles (NFTs; Chiarini et al., 2017). This suggests that astrocytes recycle endocytosed pathogenic cargo, re-releasing them as astrocytic EVs and potentially facilitating the spread of disease.

Astrocyte-derived exosomes may have distinctive lipid composition compared to neuronal-derived exosomes which could affect protein aggregation and internalization by recipient cells. In addition to enrichment in ceramides (Wang et al., 2012), the ganglioside GM1 was found in the outer layer of exosomes released by astrocytes in the aggressive Alzheimer's mouse 5XFAD model. Of note, GSLs are more abundant in neuronal exosomes relative to glial exosomes, which have been implicated in the binding of monomeric Aβ (Yuyama et al., 2014). Exosomes isolated

from primary astrocytes were observed to induce aggregation of monomeric $A\beta_{1-42}$ peptide 20-fold greater than amyloid alone, suggesting that astrocytic exosomes facilitate the formation of Aβ plaques within the AD brain (Dinkins et al., 2014). Adding an anti-ceramide antibody reduced Aβ aggregation, suggesting that the ceramide enrichment of astrocyte-derived exosomes is required for monomeric Aβ binding and oligomerization (Dinkins et al., 2016). Therefore, the efficacy of astrocyte-derived exosomes as a vehicle for Aβ plaque formation and spread appears to be dependent on lipid composition.

In conclusion, evidence suggests that astrocytes reduce the neuronal spread of pathology by readily taking up free extracellular or EV-associated neurodegenerative proteins due to more efficient endolysosomal trafficking compared to neurons. However, it remains unclear whether astrocytic uptake of neuronal exosomes and pathogenic proteins is ultimately neuroprotective or neurotoxic. These glial cells may eventually accumulate pathogenic proteins, leading to the release of astrocytic exosomes that further propagate pathogenic aggregate pathology and conversion to reactive cytotoxic astrocytes (**Figure 3**). Further work will need to address the possible neuroprotective vs. neurotoxic roles of astrocytes in the propagation of pathogenic proteins in neurodegeneration.

There is increasing recognition of microglia in the clearance of pathogenic proteins in multiple neurodegenerative conditions. Microglia are the primary immune cells of the CNS that become active under conditions of injury, infection, or even neurodegeneration. When active they are phagocytic, assuming a macrophage-like phenotype where they become mobile and endocytose surrounding debris (Neumann et al., 2009). They have been observed to migrate to newly formed amyloid plaques within 24 h of formation in an aggressive AD mouse model and prevent the continued formation of those plaques (Meyer-Luehmann et al., 2008).

Recruitment of microglia to Aβ plaques followed by clearance of Aβ was also observed in the transgenic mouse model PDAPP in which APP, the pre-aggregate form of Aβ, is over expressed. In 20-month-old mice corteces, an anti-Aβ antibody 10D5 was injected at sites of already existing Aβ plaques in an attempt to see if this would promote the clearance of the plaques. Microglia were recruited to the site of injection in high abundance 3 days post-injection, coinciding with clearance of the anti-Aβ-stained plaques. Though this clearance was mediated by immunotherapy, it is still positive evidence for microglia to surround and clear Aβ and associated bodies, thereby preventing the spread of Aβ plaques (Bacskai et al., 2001). Microglial internalized Aβ was degraded *via* autophagy as confirmed with co-staining of LAMP1, CD68 and Aβ (Bacskai et al., 2001; Feng et al., 2020). These studies support a neuroprotective role for microglia in clearing and halting the propagation of pathogenic senile plaques. Microglia are also recruited to NFTs and internalize insoluble and soluble tau species in AD postmortem tissue, suggesting that microglia continually surveil for aberrant tau (Bolos et al., 2016; Nilson et al., 2017). In an AD mouse model, microglia have been observed to localize around phosphorylated tau within the dentate gyrus and phagocytose p-tau positive neurons, preventing the spread of phosphorylated tau and progression of neurodegeneration (Asai et al., 2015; Loria et al., 2017).

Microglia are also able to phagocytose exosomes containing Aβ, tau, and α-syn (Yuyama et al., 2012; Wang et al., 2017; Xia et al., 2019). However, microglial phagocytosis may not be neuroprotective, as internalized proteins are recycled and released back into the extracellular matrix as microglial exosomes, where they can further propagate neurodegeneration (Asai et al., 2015; Guo et al., 2020). Depletion of microglia in a mouse AD model resulted in decreased EV-associated oligomeric tau and reduced tau pathology in the brain. The same findings were observed when exosome production was inhibited in microglia *via* the nSMase ceramide synthesis pathway (Asai et al., 2015). Similar proof of microglia-derived exosome mediating propagation has been observed with α-syn, where microglial exosomes containing α-syn were internalized by co-cultured neurons and led to increased α-syn oligomerization . In the same co-culture experiment, when microglial nSMase was inhibited to impair exosome biogenesis, oligomerization of α-syn in neurons decreased (Guo et al., 2020) Thus, evidence supports both a neuroprotective role for microglia in internalizing pathogenic proteins neurons, as well as promoting the propagation of protein aggregation in neurodegeneration.

Membrane lipid composition of EVs also influences microglial exosome internalization. Yuyama et al. observed that GSLs on the outer membrane of exosomes were essential for Aβ binding and oligomerization. However, there was no effect on microglial internalization of glycosphingolipid-depleted exosomes. Previously, this group showed that microglia require phosphatidylserine (PS), a lipid implicated in the autophagy of cellular debris and expressed in the outer membrane of apoptotic cells, was required for microglia to uptake neuronal exosomes (Neumann et al., 2009; Yuyama et al., 2012, 2014). It should be noted that another glial cell type, oligodendrocytes which provide the myelin sheath surrounding neuronal axons in the CNS, have been observed to accumulate tau aggregates (Narasimhan et al., 2020) and α-syn filaments (Tu et al., 1998). Accumulation of these pathogenic aggregates led to oligodendrocyte cell death and neurotoxicity (Tu et al., 1998; Narasimhan et al., 2020), adding further complexity to the non-cell autonomous interactions contributing to the spread of pathogenic protein aggregation and progression of neurodegeneration.

In addition to proteins and lipids, EVs contain nucleic acids including microRNAs (miRNAs) that may mediate interactions with neurons. Analysis of EVs from primary rat microglia cultured with inflammatory Th1 cytokines revealed a microglial-specific miRNA that reduces dendritic spine density when internalized by hippocampal neurons (Prada et al., 2018). Specific extracellular and EV-associated miRNAs in CSF have been found to be associated with neurodegenerative diseases such as AD and PD (Lusardi et al., 2017; Grossi et al., 2021). Characterization of miRNAs trafficked to EVs and whether they mediate

neuroprotective or neurotoxic effects in a neurodegenerative context is an emerging area of study.

CONCLUSION

The spread of pathologic protein aggregates throughout regions of the brain in neurodegenerative diseases is associated with the progression of clinical disease. A significant amount of evidence now supports EVs as important vehicles mediating the propagation of pathogenic protein aggregates in multiple neurodegenerative diseases. Alterations in lipid metabolism also alter disease progression by promoting oligomerization of aggregate-prone pathogenic proteins and impairing EV biogenesis. Although this review focused on the neuropathology related to AD and PD, EVs and lipid metabolism alterations are implicated in several other neurodegenerative diseases characterized by pathogenic protein aggregates, including DLB, Huntington's, ALS, prion disease, and other tauopathies and α-synucleinopathies. While the specific proteins involved in aggregates for each of these diseases are unique, there may be a common underlying pathogenic mechanism regulating the spread of these aggregates that could be exploited therapeutically to slow or halt neurodegeneration. Elucidating the alterations in lipid metabolism may reveal possible druggable targets in lipid pathways to ameliorate neurodegenerative processes, as well as identify potential biomarkers of disease progression.

Studies of tissues from *GBA* carriers and GBA deficient model organisms and cultured cells have provided some of the most compelling evidence for the role of lipid metabolism in the progression of neurodegeneration. Lipid composition has widespread cellular influences affecting disease progression, including cell autonomous vesicular trafficking and autophagy, exosome biogenesis, cell signaling, and cell survival. In this review, we examined the evidence for the lipid composition of membranes directly interacting with neurodegenerative proteins to promote aggregation and pathology. Ceramide metabolism is of particular interest, given its multiple avenues of influences that are all implicated in neurodegeneration, including exosome biogenesis, membrane fluidity, and curvature, cell signaling, regulation of apoptosis, autophagy, and inflammation. These studies suggest that alterations in lipid metabolism can have a profound effect in accelerating disease progression. As the lipid abnormalities found in AD, PD and other aggregate-prone neurodegenerative diseases have similarities in common in modifying protein aggregation, elucidation of lipid-mediated mechanisms to slow neurodegeneration holds significant promise as a therapeutic target. Improvements in detection and annotation of lipids from tissues of patients, and animal or cell culture models are resulting in a complex lipid landscape that is increasingly difficult to interpret. Continued lipidomic analysis of patient tissues, isogenically controlled model organisms, and cell culture models will be crucial to identify the key lipid alterations involved in pathogenesis and disease progression.

Understanding the role of glia in the propagation of protein aggregation and disease progression is becoming a major focus in neurodegeneration. However, it remains unclear which glial cells may have neuroprotective vs. neurotoxic effects. Several studies suggest that glia may initially have neuroprotective effects in clearing extracellular pathogenic free-floating and EV-associated proteins that would otherwise seed and propagate pathology in recipient neurons. However, uptake of pathogenic EVs may overwhelm glial endolysosomal trafficking, leading to toxicity of glia as further propagation of pathologic proteins within glial EVs. Recent studies using co-cultures of iPSC-derived neurons and glia are starting to elucidate the complex interactions between these cell types in health and disease. Investigations using iPSC-derived organoid models may be necessary to further understand complex interactions between these cell types that are perturbed in neurodegenerative diseases. Further studies are necessary to elucidate the role of glia in reducing or promoting the propagation of neurodegenerative pathology. These studies could reveal alternative therapeutic targets in harnessing the increased uptake of pathogenic aggregate-prone proteins by glia to reduce the spread of neurodegenerative pathology and slow disease progression.

Lipid metabolism alterations contribute to the progression of neurodegeneration through numerous pathways influencing the spread of pathology. While much focus has been on the study of these aggregate-prone proteins, the lipids surrounding these proteins in EVs, endocytic vesicles, lipid rafts, and cell membranes are altered in the context of neurodegeneration, promoting aggregation and influencing the spread of pathogenic protein aggregates from cell to cell, suggesting that lipid alterations may be as important as the pathogenic proteins themselves.

AUTHOR CONTRIBUTIONS

REE, BL, AK, and MYD performed the literature review and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Crosstalk Between the NLRP3 Inflammasome/ASC Speck and Amyloid Protein Aggregates Drives Disease Progression in Alzheimer's and Parkinson's Disease

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Two key pathological hallmarks of neurodegenerative diseases, including Alzheimer's disease (AD) and Parkinson's disease (PD), are the accumulation of misfolded protein aggregates and the chronic progressive neuroinflammation that they trigger. Numerous original research and reviews have provided a comprehensive understanding of how aggregated proteins (amyloid β, pathological tau, and α-synuclein) contribute to the disease, including driving sterile inflammation, in part, through the aggregation of multi-protein inflammasome complexes and the ASC speck [composed of NOD-, LRR-, and pyrin domain-containing protein 3 (NLRP3), Apoptosis-associated speck-like protein containing a C-terminal caspase activation and recruitment domain (ASC), and inflammatory caspase-1] involved in innate immunity. Here, we provide a unique perspective on the crosstalk between the aggregation-prone proteins involved in AD/PD and the multi-protein inflammasome complex/ASC speck that fuels feed-forward exacerbation of each other, driving neurodegeneration. Failed turnover of protein aggregates (both AD/PD related aggregates and the ASC speck) by protein degradation pathways, prionoid propagation of inflammation by the ASC speck, cross-seeding of protein aggregation by the ASC speck, and pro-aggregatory cleavage of proteins by caspase-1 are some of the mechanisms that exacerbate disease progression. We also review studies that provide this causal framework and highlight how the ASC speck serves as a platform for the propagation and spreading of inflammation and protein aggregation that drives AD and PD.

Keywords: inflammasomes, NLRP3, ASC, Alzheimer's disease, Parkinson's disease, protein aggregation, neuroinflammation, autophagy-lysosomal degradation

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INTRODUCTION

Alzheimer's disease (AD) is the leading cause of dementia and is in the top 10 causes of death worldwide (Lane et al., 2018; Breijyeh and Karaman, 2020). It is characterized clinically by progressive memory loss, functional, and cognitive impairment (Lane et al., 2018; Breijyeh and Karaman, 2020). Three key components of the pathophysiology of AD include the accumulation

of extracellular Amyloid-β (Aβ) plaques, intracellular hyperphosphorylated tau neurofibrillary tangles, and chronic neuroinflammation (Lane et al., 2018; Breijyeh and Karaman, 2020). Early stages of the disease classically affect the hippocampus and then progress to diffuse cerebral cortex involvement (Lane et al., 2018; Breijyeh and Karaman, 2020).

Parkinson's disease (PD) is the second leading cause of neurodegeneration. PD is primarily a movement disorder that classically presents with bradykinesia, cogwheel rigidity, resting tremor, and postural imbalance (Cacabelos, 2017). The pathophysiology of PD is characterized by the accumulation of intracellular α-synuclein (α-syn) aggregates known as Lewy bodies and chronic inflammation with degeneration of dopaminergic neurons in the basal ganglia (Cacabelos, 2017).

There are numerous other neurodegenerative diseases that share commonalties in their pathogenesis including amyotrophic lateral sclerosis (ALS) (Liu and Wang, 2017), Huntingtin's disease (Lois et al., 2018), and the range of tauopathies including supranuclear palsy, frontotemporal dementia (FTD), corticobasal degeneration, FTD and parkinsonism linked to chromosome 17, and others (Arendt et al., 2016). All of these are characterized by misfolded protein aggregation and chronic neuroinflammation. Though each condition is characterized by unique disease-relevant proteins that affect specific brain regions, each condition shares several commonalities including: protein misfolding and aggregation into β-sheet rich fibrillar aggregates, maladaptive innate immune responses with chronic progressive neuroinflammation, defective protein-quality control and degradation, substantial neuronal cell loss, and some form of cognitive or functional impairment (Gan et al., 2018; Ciccocioppo et al., 2020). This review will primarily focus on literature relevant to AD and PD though it is likely that much of the discussion can be extended to these other neurodegenerative proteinopathies.

Having shown by many studies to be primary drivers of the disease, pathologic protein aggregates have been targeted as potential therapy for AD/PD. For example, the AN1792 vaccine, and the monoclonal antibodies Bapineuzumab, Solanezumab, Gantenerumab, Crenezumab, and BAN2401 are clinical candidates for AD (Schneider et al., 2014). Recently, the FDA has conditionally approved Aduhelm®, an immunotherapy against amyloid pathology, requiring post-approval monitoring due to controversy among the scientific community about its effectiveness and potential side effects, especially amyloid-related imaging abnormalities (ARIA) linked to cerebral edema and intracerebral hemorrhage (Tampi et al., 2021). In the past two decades, many therapeutics have only targeted protein build-up in AD and PD, failing to consider other co-etiologies. There is consensus that this might have contributed to significant failure rate (>99%) in translating pre-clinical studies to clinical utility. Among many co-occurring pathological signatures, neuroinflammation may play a more critical role in disease progression than was previously emphasized by the amyloid cascade hypothesis, which viewed neuroinflammation as a byproduct of amyloid toxicity. In the last decade, an inflammation-based hypothesis has gained favor to explain the mechanism of neurodegenerative disease progression whereby inflammation may serve as an inciting event early in the disease that contributes to protein misfolding and aggregation which reinforces neuroinflammation in a positive-feedback manner, ultimately leading to neurodegeneration (McGeer and McGeer, 2013). Finally, recent genome-wide association studies (GWAS) have identified several genes in the immune pathways showing strong association to sporadic AD (e.g., *CD33, TREM2* etc.) and PD (e.g., *BST1, HLA*) (Billingsley et al., 2018; Neuner et al., 2020).

Many studies have shown a link between these misfolded protein aggregates and inflammation. The nod-like receptor family pyrin domain containing 3 (NLRP3) inflammasome is one of the most well characterized sensors of danger signals in cells, most associated with neurodegenerative diseases, and serves as a connection point between neuronally-derived misfolded protein aggregates and the damaging neuroinflammation associated with neurodegeneration (de Alba, 2019; Swanson et al., 2019). The NLRP3 inflammasome is a component of the innate immune system that responds to pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) in a pattern recognition receptor (PRR)-dependent manner (de Alba, 2019; Kelley et al., 2019; Swanson et al., 2019). It is a multi-protein complex composed of NLRP3, which serves as an intracellular sensor for various danger signals, the apoptosis-associated speck-like protein containing a caspase activation and recruitment domain or CARD domain (ASC) which serves as an adaptor protein, and caspase-1 which carries out the enzymatic cleavage of interleukin (IL)-1β, IL-18, and gasdermin-D among others (de Alba, 2019; Swanson et al., 2019). In settings of acute inflammation, the NLRP3 inflammasome components and its byproducts undergo clearance to avoid excessive or sustained activation of inflammatory signaling upon resolution of the inflammatory stimuli.

Aβ aggregates have been shown to activate the transcription of inflammatory cytokines and proteins required for the assembly of the NLRP3 inflammasome through toll-like and scavenger receptor signaling pathways (Udan et al., 2008; Liu et al., 2020). Activation of the receptor recruits myeloid-differentiation primary response gene 88 (MyD88) to the cytoplasmic domain of the receptor. MyD88 then recruits IL-1 receptor-associated kinase (IRAK) proteins, namely IRAK4 and IRAK1, which are sequentially phosphorylated and can activate TNF receptor-associated factor 6 (TRAF6). TRAF6 is an E3-ligase that K63-pulyubiquitinates itself and NF-κB essential modulator (NEMO), recruiting transforming growth factor-β-activated kinase 1 (TAK1) and TAK1 binding proteins (TABs) to phosphorylate the inhibitor of NF-κB kinase (IKK) complex, which then phosphorylates the inhibitor of NF-κB (IκB) protein. This allows for the release of NF-κB from its inhibitory IκB complex, which is subsequently degraded, so that NF-κB can translocate to the nucleus and initiate the transcription of NLRP3, ASC, pro-caspase-1, pro-IL-1β, and pro-IL-18 (Kawai and Akira, 2007). This is referred to as the priming step, or signal 1, for inflammasome activation (**Figure 1**). Aβ aggregates have also been shown to directly trigger the activation step, or signal 2, for NLRP3 inflammasome assembly (**Figure 1**) in microglia (Halle et al., 2008). The mechanism by which this occurs is not entirely clear but has been demonstrated to involve the phagocytosis of the Aβ aggregates by microglia which then escape

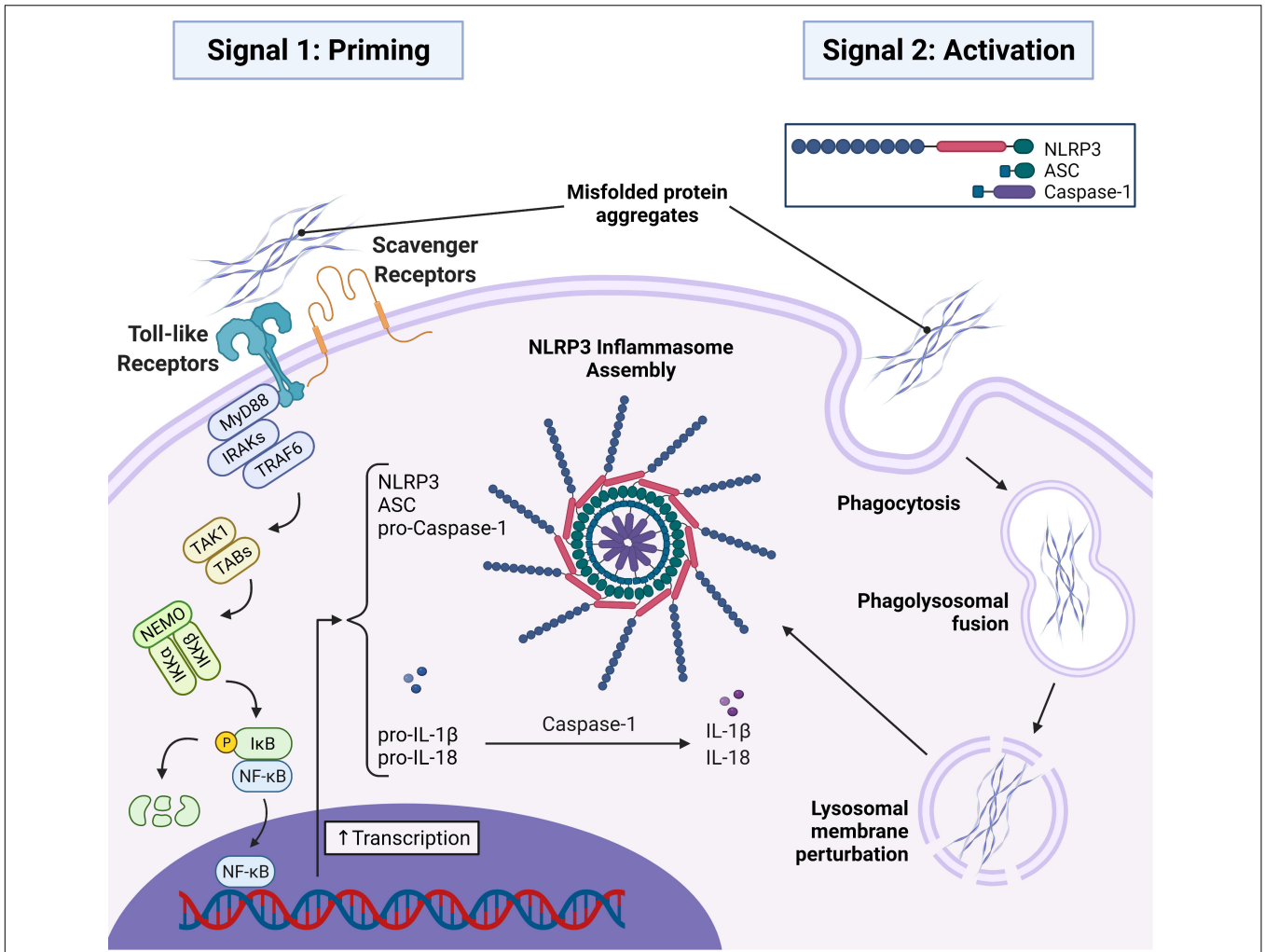

FIGURE 1 | AD and PD related misfolded protein aggregates (Aβ, tau, and α-syn) initiate signal 1 and signal 2 required for assembly of the NLRP3 inflammasome and subsequent ASC speck. In signal 1, misfolded protein aggregates bind to toll-like and scavenger receptors on the microglial cell surface initiating a MyD88-dependent signaling transduction event resulting in the translocation of NF-κB into the nucleus to initiate the transcription of important inflammasome components and the immature forms of the inflammatory cytokines pro-IL-1β and pro-IL-18. In signal 2, misfolded protein aggregates are taken into the cell via phagocytosis and trafficked to the lysosome where they disrupt the lysosomal membrane and trigger assembly of the inflammasome. Oligomerization of NLRP3 then recruits ASC which oligomerizes with NLRP3 via homotypic interactions between PYD regions of both proteins. Pro-caspase-1 is then recruited to the oligomerized ASC via homotypic interactions between CARD regions. Dimerization of pro-caspase-1 triggers an autolytic cleavage event producing two active caspase-1 enzymes that can mature pro-IL-1β and pro-IL-18 into the mature IL-1β and IL-18. MyD88, myeloid differentiation primary response gene 88; IRAK, IL-1 receptor-associated kinase; TRAF6, TNF receptor associated factor 6; TAK1, transforming growth factor-β-activated kinase-1; TAB, TAK1 binding proteins; NEMO, NF-κB essential modulator; IKKα/β, Inhibitor of NF-κB Kinase; IκB, Inhibitor of NF-κB; NF-κB, nuclear factor-kappaB; IL-1β/18, interleukin-1β/18.

lysosomal degradation through damage and destabilization of the lysosome (Halle et al., 2008), upon which they can trigger NLRP3 inflammasome and ASC speck assembly in a manner similar to large crystals (Martinon et al., 2006). Whether or not NLRP3 inflammasome activation by protein aggregates can occur independent of lysosomal damage is unclear, though dysregulation of mitochondrial homeostasis by α-syn has been shown to be another contributor to inflammasome activation (Xie and Chung, 2012; Zhong et al., 2018). Similar findings have demonstrated identical triggering of both the priming (signal 1) and activation (signal 2) steps of the NLRP3 inflammasome by pathological phosphorylated tau aggregates by us (Jiang et al., 2021) and others (Stancu et al., 2019). Additionally, α-syn aggregates have also been shown to trigger both signals (Codolo et al., 2013; Gordon et al., 2018).

NLRP3 oligomerizes upon activation by intracellular protein aggregates. This oligomerization then recruits monomeric ASC which oligomerizes with NLRP3 via homotypic interactions between the pyrin domain (PYD) of ASC and the PYD of NLRP3 (de Alba, 2019; Swanson et al., 2019). Upon aggregation of ASC with NLRP3, pro-caspase-1 is recruited and binds to ASC via homotypic CARD-CARD interactions between the two proteins. The assembly of these three proteins forms the basic functional unit of the inflammasome complex. When two

monomers of pro-caspase-1 dimerize on the ASC scaffolding, they are activated through their intrinsic auto-cleavage capacity yielding two functional caspase-1 enzymes that can then activate numerous other proteins involved in the inflammatory cascade (de Alba, 2019; Swanson et al., 2019). In addition to forming the basic inflammasome complex, NLRP3 inflammasome activation can also result in the formation of a dense structure ∼1 μM in diameter referred to as the ASC speck (de Alba, 2019; Swanson et al., 2019).

The ASC speck is a supramolecular aggregate of the inflammasome complex composed primarily of ASC that serves as a signal amplification platform for enhanced cytokine maturation by caspase-1 (Dick et al., 2016). Upon inflammasome activation and recruitment of ASC to oligomerized NLRP3, ASC continues to aggregate via homotypic interactions between its PYD region into large helical fibrils. Numerous ASC fibrils are then cross-linked via homotypic interactions on the ASC-CARD region into a dense ASC speck. The ASC speck continues to recruit pro-caspase-1 to its surface and functions via the same mechanism as the basic inflammasome complex at a larger scale (de Alba, 2019).

Recent studies have implicated an important role for the NLRP3 inflammasome and the ASC speck in the propagation and spreading of neuroinflammation and misfolded protein aggregation in neurodegenerative diseases and are reviewed here. The goal of this review is not to expand on the inflammasome in neurodegenerative disease, but to highlight our current understanding of this emerging paradigm of the inflammasome/ASC speck as an active contributor to AD/PD pathology through three key topics: (1) How failed protein aggregate clearance and regulation mechanisms contribute to the accumulation of microglial inflammasome protein aggregates and neuronally derived (AD/PD related) misfolded protein aggregates in parallel; (2) How prionoid activity, cross-talk, and cross-seeding of these aggregates contributes to a viscous feed-forward cycle resulting in AD/PD disease progression; and (3) How targeting the microglial inflammasome protein aggregates for therapeutics shows potential in breaking this cycle for the treatment of AD and PD. We encourage readers to refer to other reviews (Pereira et al., 2019; Venegas and Heneka, 2019; Hanslik and Ulland, 2020; Tan et al., 2020) which describe inflammasomes and the possible role that they play in the development and progression of neurodegenerative diseases such as AD and PD. Because microglia are the primary immune cells of the brain, this review will focus on the role of microglia in neuroinflammatory processes relevant to AD and PD.

REGULATION OF INFLAMMASOME AND ASC SPECK ASSEMBLY BY POST-TRANSLATIONAL MODIFICATIONS

Phosphorylation and Dephosphorylation

Phosphorylation and dephosphorylation are the most common post-translational modifications (PTM) and are regulated by the interplay of hundreds of kinases and phosphatases. The regulation of NLRP3 inflammasomes have been extensively reviewed in Gong et al. (2018). Phosphorylation or dephosphorylation of various amino acids on NLRP3 are important for directly regulating the oligomerization of NLRP3 as well as the NLRP3-ASC interaction. One study showed that phosphorylation of NLRP3 at serine (S) 5 prevents the accidental oligomerization of NLRP3 and that phosphatase (PP)2A activity is important for NLRP3 activation (Stutz et al., 2017). Another study showed that dephosphorylation at tyrosine (Y) 861 by Protein Tyrosine Phosphatase Non-receptor Type 22 (PTPN22) allows for enhanced NLRP3 oligomerization indicating that tyrosine phosphorylation is an important regulator of aberrant inflammasome activation (Spalinger et al., 2016).

Additionally, phosphorylation can indirectly regulate NLRP3 inflammasome priming, inhibition, and degradation through the delicate balance between ubiquitination and deubiquitination of NLRP3. C-Jun N-terminal kinases (JNK)-mediated phosphorylation of NLRP3 at S194 is a critical priming step for inflammasome activation by promoting NLRP3 deubiquitination and oligomerization (Song et al., 2017). Conversely, Protein Kinase A (PKA)-mediated phosphorylation of NLRP3 at S291 (Guo et al., 2016) and S295 (Mortimer et al., 2016) can act as a critical brake to stop NLRP3 activation and promotes its ubiquitin-mediated degradation. Similarly, the Src family kinase LYN-mediated phosphorylation of NLRP3 at Y918 also promotes its ubiquitin-mediated degradation by the proteasome to negatively regulate inflammasome activatability (Tang et al., 2021).

Phosphorylation and constitutive interaction of ASC with IKKα prior to inflammasome activation is important for its negative regulation and sequestration in the nuclear compartment (Martin et al., 2014). Phosphorylation by IKKi is important for its translocation to the cytosol upon inflammasome priming, which then recruits the phosphatase PP2A to inhibit the negative regulatory kinase activity of IKKα (Martin et al., 2014). ASC phosphorylation by the TAK1-JNK pathway and Spleen Associated Tyrosine Kinase (SYK)-Protein-Tyrosine Kinase 2-Beta (PYK2) pathway in numerous models have been identified as contributing to ASC oligomerization during inflammasome activation and phosphorylation at Y144 in mouse ASC (human Y146) is critical for ASC Speck formation (Hara et al., 2013; Okada et al., 2014; Chung et al., 2016). A recent study has also demonstrated that Y60 and Y137 phosphorylation is also critical for ASC oligomerization and ASC speck formation and that ASC dephosphorylation may also play a critical role in promoting ASC speck assembly (Mambwe et al., 2019).

Ubiquitination and Deubiquitination

Protein ubiquitination is a complex PTM mediated by a large variety of enzymes. In general, the addition of ubiquitin to a protein is regulated by an E3 ubiquitin ligase and its removal is regulated by deubiquitinases (Komander and Rape, 2012; Zheng and Shabek, 2017; Grumati and Dikic, 2018). The modification of proteins by the ubiquitin system is a tightly regulated balance between these classes of enzymes (Clague et al., 2019). Regulation of the NLRP3 inflammasome by the ubiquitin system occurs

primarily through the function of numerous E3 ubiquitin ligases on each sub-component of the inflammasome and are reviewed extensively in Lopez-Castejon (2020).

Multiple E3 negative regulators that affect NLRP3 levels or activation have been identified including *FBXL2* (Han et al., 2015), *PARKIN* (Kang et al., 2016), and *TRIM31* (Song et al., 2016), which provide a signal for proteasomal degradation, *MARCH7* (Yan et al., 2015), which provides a signal for degradation by autophagy, and *ARIH2* (Kawashima et al., 2017) and *CUL1* (Wan et al., 2019), which bind and ubiquitinate NLRP3 to maintain its inactive state. Positive regulation of NLRP3 by the ubiquitin system has been observed with the interplay of numerous E3 enzymes including *PELI2* (Humphries et al., 2018), *TRAF6* (Xing et al., 2017), and *TRIM33* (Weng et al., 2014), the E2 enzyme *UBC13* (Ni et al., 2021), as well as the deubiquitinating enzymes *BRCC3/ABRO1* (Py et al., 2013; Ren et al., 2019), *USP7* and *USP47* (Lopez-Castejon et al., 2013), and *UCHL5* (Kummari et al., 2015). Negative regulation of the NLRP3 and AIM2 inflammasomes by ubiquitination of ASC promoting proteasomal degradation has also been shown by the *TRAF6* E3 enzyme (Chiu et al., 2016) which, interestingly, has been shown to be a positive regulator of NLRP3. Taken together, these studies indicate competing roles of the enzyme depending on the activating stimuli and cellular environment. Positive regulators that are required for the activation of ASC include the *TRAF3* (Siu et al., 2019) and *LUBAC* E3 enzymes (Elliott et al., 2014; Rodgers et al., 2014; Gurung et al., 2015) as well as the *USP50* deubiquitinating enzyme (Lee et al., 2017). Regulation of caspase-1 by the ubiquitin system has been implicated in numerous E3 IAPs (inhibitor of apoptosis proteins) as well as the *LUBAC* member *SHARPIN*, however, these mechanisms appear to be complex and are not yet well understood (Lopez-Castejon, 2020). While mutations in PARKIN have been directly implicated in the pathogenesis of familial PD (Dawson and Dawson, 2010), it is not well understood how integral each of these enzymes are in orchestrating the inflammasome response that contributes to AD/PD.

Much of our understanding of the role of the ubiquitin system on regulating the inflammasome focuses on regulating the activation and formation of the inflammasome complex through the regulation of its subcomponents. Some research has indicated crosstalk between the inflammasome and ubiquitin system. For instance, Eldritch et al. showed that caspase-1 can inactivate the E2 enzyme *UBE2L3* which decreases the proteasomal-tagging ubiquitination of pro-IL-1β, thus allowing for an enhanced IL-1β inflammatory response (Eldridge et al., 2017).

Other Post-translational Modifications

Other PTMs that have been shown to regulate the inflammasome but are less commonly described include *S*-nitrosylation, SUMOylation, ADP-ribosylation, and proteolytic processing of the inflammasome components, many of which are reviewed in Yang et al. (2017). *S*-nitrosylation by nitric oxide (NO) of NLRP3 can inhibit ASC oligomerization and inflammasome assembly and *S*-nitrosylation of caspases, including caspase-1, can inhibit its catalytic function to suppress IL-1β and IL-18 maturation and release (Kim et al., 1998; Hernandez-Cuellar et al., 2012; Mao et al., 2013). Interestingly, inflammasome activation tends to upregulate NO generation suggesting an autoregulatory mechanism (Yang et al., 2017). Recent studies have demonstrated that SUMOylation of NLRP3 by *UBC9*-directed *SUMO1* and *TRIM28*-directed *SUMO1–3* are important for inflammasome activation and that de-SUMOylation by the SUMO-specific protease *SENP3* can reduce inflammasome activation (Shao et al., 2020; Qin et al., 2021). ADP-ribosylation of NLRP3 by the *Mycoplasma pneumoniae* CARDS-toxin can trigger inflammasome activation for a robust inflammatory response (Bose et al., 2014). Enterovirus 71 protease 2A and 3C (Wang et al., 2015) as well as *Mycobacterium tuberculosis* ZMP1 protease (Master et al., 2008) inhibit NLRP3 inflammasome activity through proteolytic cleavage of NLRP3.

There Are Numerous Strategies to Regulate ASC Speck Assembly

PTM of the NLRP3 inflammasome components is highly important for regulating the activation of the inflammasome. In fact, many PTMs are required for inflammasome assembly to occur (Yang et al., 2017). This highly complex and versatile process contributes to the distinct inflammatory states in response to a variety of damaging stimuli, as well as the cellular decision to either stop the inflammatory cascade or to progress to pyroptosis (Yang et al., 2017; Gong et al., 2018; Lopez-Castejon, 2020; Guo et al., 2021). The various PTM systems may thus be an attractive target for controlling the activation of the inflammasome for the management of many inflammatory conditions, including AD and PD. Regulation of inflammasome assembly can also occur more upstream of inflammasome proteins, such as through regulation of the NF-κB pathway (Afonina et al., 2017) or transcriptional repression by methylation (Yi, 2021). There are clearly numerous cellular strategies to regulate the formation of the NLRP3 inflammasome and ASC speck. Later, we will discuss how failure to regulate and degrade the NLRP3 inflammasome/ASC speck, once it has been assembled, may contribute to the progression of AD/PD.

THE ROLE OF THE ASC SPECK IN THE CHRONIC PROGRESSIVE NEUROINFLAMMATORY STATE IN ALZHEIMER'S DISEASE AND PARKINSON'S DISEASE

Propagation of Inflammation via the ASC Speck

Franklin et al. (2014) published a groundbreaking study on the extracellular activities of the ASC speck, identifying a mechanism for a prion-like propagation of inflammation from macrophage to macrophage in a paracrine fashion, that opened the door for a new paradigm in inflammasome-related pathology. They found that ASC speck formation upon activation of the NLRP3, NLRP1, NLRC4, and AIM2 inflammasomes preceded cell death via pyroptosis, upon which the speck is released into the

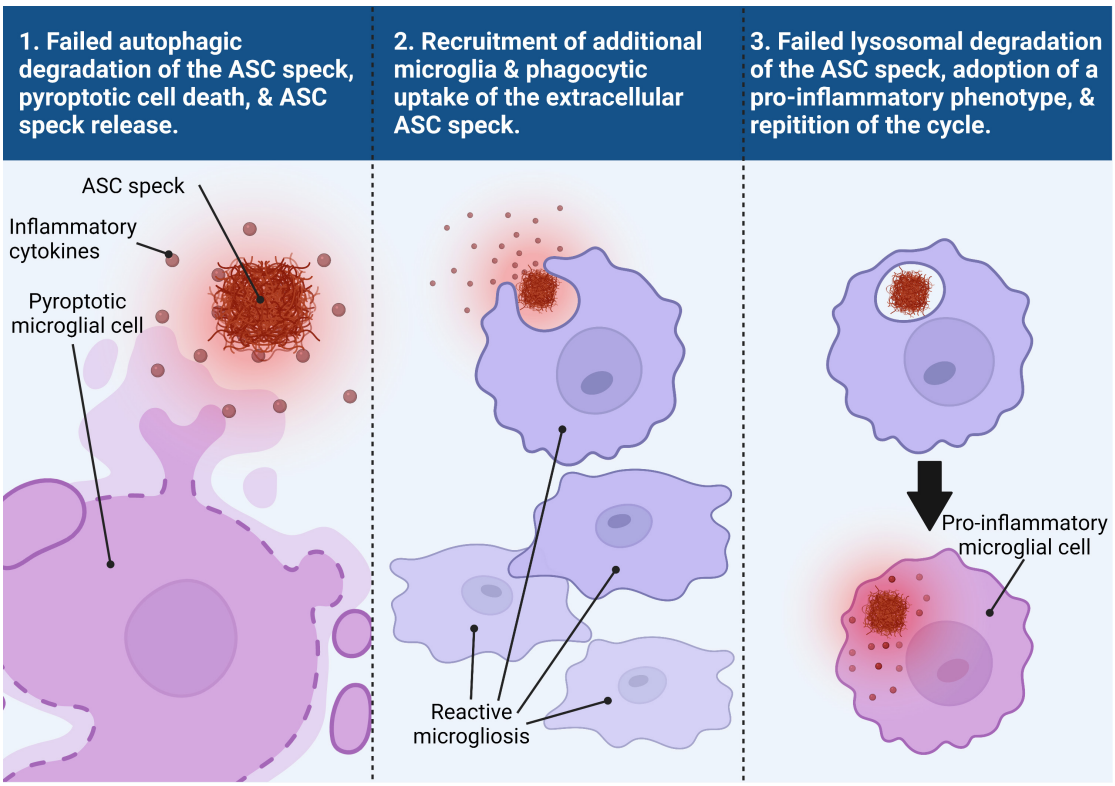

FIGURE 2 | Trans-microglial propagation of inflammation via ASC speck. Upon activation and assembly of the ASC speck from the NLRP3 inflammasome in response to misfolded protein aggregates, the microglial cell fails to degrade the ASC speck via autophagic clearance mechanisms (panel 1). Persistence of the ASC speck can result in either a non-pyroptotic exocytosis of the ASC speck or pyroptotic cell death, resulting in the release of inflammatory cytokines and the ASC speck into the extracellular space (panel 1). The extracellular ASC speck remains functionally active and the presence of inflammatory cytokines helps to recruit additional reactive microglia to the site of inflammation (panel 2). The ASC speck can then be taken up by another microglial cell where it can be degraded by the lysosomes (panel 3). Failed degradation of the ASC speck and possible lysosomal damage induces the microglial cell to adopt a pro-inflammatory phenotype (panel 3). This microglial cell can then undergo the same process as the previous microglial cell, perpetuating a viscous inflammatory cycle.

extracellular space where it is still functionally active for the maturation of caspase-1 and IL-1β (Franklin et al., 2014). The extracellular ASC speck is also taken up by other phagocytic cells where it can either be degraded by the phagolysosomal pathway or behave like an exogenous danger signal by inducing lysosomal damage and further promote inflammasome activation in a similar manner to other fibrillar protein aggregates like Aβ and tau (Franklin et al., 2014). This work confirmed and expanded upon similar work that showed that NLRP3 inflammasome particles are secreted extracellularly where they can continue to mature caspase-1 in the extracellular space as well as within other macrophage cells upon phagocytosis of the particle (Baroja-Mazo et al., 2014). While extracellular ASC specks have been commonly seen in models of neurodegenerative disorders, not all microglia that form ASC specks undergo pyroptosis. It is possible that ASC specks may be "actively" released into the extracellular space in a manner that does not involve cell death though this has not been shown. Our recent studies have even suggested the presence of ASC specks in the cerebrospinal fluid of patients with AD and related tauopathies (Jiang et al., 2021). A model summarizing this process is shown in **Figure 2**.

In the context of neurodegenerative disease, this prion-like propagation of inflammation by the ASC speck is problematic because it contributes to the toxic, chronic progressive inflammatory state that plays a central role in neurodegeneration (Lee et al., 2019). Thus, prion-like pro-inflammatory activity of the ASC speck appears to be a maladaptive innate immune response. However, prion-like polymerization has been shown to be an evolutionarily conserved mechanism of signal transduction in innate immune defense (Cai et al., 2014) indicating that it must confer evolutionary benefits. Understanding how to better regulate this activity may be essential for development of therapeutics that limit the damaging effects of dysregulated inflammation in various diseases.

Failed Degradation of the Inflammasome and ASC Speck

PTMs of the assembled inflammasome have been shown to be necessary for degradation of the inflammasome, including the ASC speck, via the autophagosome. Autophagy can negatively modulate inflammasome activity and pyroptosis through the degradation of PAMPs/DAMPS, degradation of the active

inflammasome or ASC speck after PTM via the ubiquitin system, or degradation of the downstream products of inflammasome activity such as IL-1β and IL-18 (Guo et al., 2021).

It has been known for some time now that inflammasome activation triggers autophagy pathways in an inflammasome sensor-dependent (NLRP3-dependent) manner indicating an autoregulatory mechanism for resolution of inflammatory signaling. Inflammasomes co-localize with autophagosomes and lysosomes that contain inflammasome components (Shi et al., 2012). Inflammasomes are K63 polyubiquitinated and recruit the autophagic adaptor protein, p62, for delivery of the inflammasome to the autophagosome (Shi et al., 2012). It is currently not completely understood why these regulatory mechanisms fail in the setting of pyroptosis and chronic inflammatory states (Guo et al., 2021) where the ASC-speck can escape the clearance mechanism and become prionoid. The ASC speck's prion-like structure is resistant to proteolytic degradation much like the misfolded protein aggregates in AD and PD (Franklin et al., 2014). Thus, failure of autophagic clearance mechanisms of the ASC speck results in a substantial lost opportunity for degradation that allows it to persist to the point that the inflammatory response becomes toxic.

Neurodegenerative diseases such as AD and PD are characterized by failure of neuronal protein degradation systems leading to the accumulation of misfolded protein aggregates (Ciechanover and Kwon, 2015; Jiang and Bhaskar, 2020). It is unclear if this is primarily due to down-regulation of these pathways due to environmental stressors, decreased activity due to cellular aging, or some other mechanism. Heneka et al. (2018) proposed that old age contributes to immuno-senescence of microglia resulting in an altered hyper-responsive inflammatory response that may predispose the brain to chronic inflammatory states. This alone may possibly contribute to the failure of these clearance mechanisms. Whether the failure of the autophagic clearance mechanism for the ASC-speck in microglia is part of a global failure in autophagy that results in the accumulation of AD/PD related proteins in neurons in these neurodegenerative diseases needs to be further explored. Moreover, if there is a common node for failure of the autophagy-lysosomal clearance pathway in both neurons and microglia, identifying this will be important for development of targeted therapeutics.

Microglial Polarization Can Determine Proteinopathy Disease Progression or Clearance

Microglia generally exist in two major states: a "resting" state during which the microglia primarily perform an active surveillance role, and an "activated" state where microglia can perform a number of immune related tasks (Yao and Zu, 2020). The function performed by the "activated" microglia is dependent upon a process called polarization where the microglial cell is induced into a number of phenotypes with specific roles. The classical division are the M1 and M2 phenotypes, with a number of M2 subtypes playing various maintenance roles. In general, the M1 phenotype carries out pro-inflammatory behaviors while the M2 phenotype has an anti-inflammatory/pro-phagocytic role (Yao and Zu, 2020). In neurodegenerative disease, the role of M2 microglia in early stages of the disease has a neuroprotective role. However, as the disease progresses, prolonged and excessive activation of M1 microglia generates the neuroinflammatory state that exacerbates pathological damage and neuronal cell death (Floden and Combs, 2011). Our current understanding of microglial polarization with regards to AD has been reviewed by Yao and Zu (2020).

With the emergence of powerful single cell and single nucleus RNA sequencing allowing for profiling of microglia, the concept of M1, M2 and acquired deactivation phenotype of microglia is fading away. Based on single cell RNA sequencing, previous studies have identified a subset of microglia called "disease-associated microglia" (DAM), which show downregulation of *CX3CR1, P2RY12, CD33,* and *TMEM119*; and upregulation of *TYROBP, APOE, B2M,* and *TREM2* and appear to contribute to the disease process (Butovsky and Weiner, 2018; Deczkowska et al., 2018). There is an interesting subset of DAMs driven by Triggering Receptor Expressed on Myeloid Cells 2 (TREM2)-signaling that specifically sense damage within the CNS called "neurodegeneration-associated molecular patterns" (NAMPs), tailored to contain/remove CNS damage and provide homeostasis (Deczkowska et al., 2018). NAMP-responsive DAMs appear to play a protective role in neuroinflammatory diseases under some conditions and an exacerbating role in other conditions (Butovsky and Weiner, 2018; Deczkowska et al., 2018). It is unclear what may determine this conflicting function, but timing and disease progression may contribute to this duality (Mathys et al., 2017). It is unclear if the specific signaling that trigger NAMP-responsive DAMs are also responsible for preventing microglial ASC speck/inflammasomes from undergoing clearance via ubiquitin-proteosome system or autophagy-lysosomal pathways, or how the ASC speck/inflammasomes may play a role in dysregulation of the DAM phenotype contributing to loss of protective function.

ASC Speck Release May Be an Attempt to Recruit Help to Clear Large Aggregates

The release of ASC specks into the extracellular environment, including during pyroptosis, in response to misfolded protein aggregates may be an attempt to recruit more phagocytic cells to clear these protein aggregates. Broderick and Hoffman (2014) speculated that ASC speck release into the extracellular environment in response to urate crystals that are too large to be phagocytosed by macrophages may serve as a signal amplification mechanism to recruit the assistance of neighboring cells in the immune response. Indeed, microglia that undergo inflammasome activation in response to Aβ aggregates release chemotactic factors for the recruitment of neighboring cells (Halle et al., 2008). Brain sections of AD patients commonly show activated microglia clustered around Aβ plaques (Nichols et al., 2019) as well as in apposition to pathological tau structures (Bolós et al., 2015). Similarly, in PD, a reactive microgliosis can typically be seen in brain regions with substantial α-syn

pathology (Sanchez-Guajardo et al., 2015). Yet, it remains a mystery why innate immune cells may hinge upon ASC speck-mediated amplification despite the prevailing use of the cytokine/chemokine system for trafficking and recruitment of leukocytes/myeloid cells. Perhaps, such a scenario may occur primarily to only amplify IL-1β/IL-18 signaling in the context of a specific disease stage.

THE ROLE OF THE ASC SPECK IN THE ACCUMULATION AND SPREADING OF ALZHEIMER'S DISEASE/PARKINSON'S DISEASE RELATED MISFOLDED PROTEIN AGGREGATES

The ASC Speck Induces Pro-aggregatory Post-translational Modifications of Tau and α-Synuclein

NLRP3 inflammasome activity has been shown to be directly attributable to the pathogenesis of some of the misfolded protein aggregates in neurodegenerative diseases. Caspase-1 activity has been shown to cleave α-syn (at Asp121) to a highly aggregation prone form (Wang et al., 2016). Studies have also shown that caspase-cleaved tau (at Asp421) can also become prionoid and seed tau aggregation (Cotman et al., 2005) and can activate microglia (Cotman et al., 2005; Zilka et al., 2012) and thus drive the disease. We have previously shown that loss of tau can prevent neurodegeneration in a lipopolysaccharide (LPS) model of systemic inflammation in *Cx3cr1*$^{-/-}$ mice (Maphis et al., 2015a). Recently, we showed that doxycycline- or vaccine-mediated suppression of pathological tau blocks NLRP3, ASC, and IL-1β expression in microglia (Jiang et al., 2021).

NLRP3 inflammasome activity is also indirectly attributable to the pathogenesis of AD and PD related protein aggregates through inflammatory signaling-mediated upregulation of pro-aggregatory PTMs of tau and α-syn. Inflammatory signaling, such as through caspase cleaved IL-1β, upregulates neuronal kinase activity resulting in tau hyperphosphorylation and has been reviewed in Barron et al. (2017). Upregulation and activation of p38 mitogen-activated protein kinase (MAPK) (Li et al., 2003; Kitazawa et al., 2005), cyclin-dependent kinase 5 (CDK5) (Kitazawa et al., 2005; Roe et al., 2011), Glycogen Synthase Kinase 3 Beta (GSK-3β) (Kitazawa et al., 2005; Roe et al., 2011; Sy et al., 2011), *JNK* (Kitazawa et al., 2005; Roe et al., 2011), and extracellular signal-regulated kinase (ERK2) (Roe et al., 2011) have all been implicated in the pro-aggregatory hyperphosphorylation of various tau residues in response to inflammatory signaling.

The link between inflammatory signaling and pro-aggregatory PTMs of α-syn is not as well established, though one study demonstrated that LPS induced inflammation triggers α-syn phosphorylation at S129 and accumulation of α-syn aggregates that spread from the olfactory bulb to the substantia nigra and striatum resulting in PD-like pathology (Niu et al., 2020). The level of systemically circulating phosphorylated α-syn also has a positive correlation with the levels of NLRP3 and IL-1β in human PD patients (Chatterjee et al., 2020). There are numerous PTMs that promote the aggregation of α-syn (reviewed in Zhang et al., 2019) such as Casein kinase 2 (CK2)-mediated (Fujiwara et al., 2002; Smith et al., 2005) and G Protein-Coupled Receptor Kinase 2 (GRK2)-mediated (Feany and Bender, 2000) phosphorylation at S129, Seven *in absentia* homolog (SIAH)-mediated ubiquitination (Lee et al., 2008; Rott et al., 2008), Protein Inhibitor of Activated STAT 2 (PIAS2)-mediated SUMOylation (Rott et al., 2017), and nitration (Hodara et al., 2004). Whether IL-1β signaling directly contributes to these PTMs needs further investigation. Together these studies suggest a crosstalk between the NLRP3 inflammasome/ASC speck and neuron-derived protein aggregates in AD and PD.

The ASC Speck as a Scaffold for Protein Aggregation

Based on such crosstalk, it is conceivable that the inflammasome proteins and the AD/PD protein aggregates may cross-seed. Sahillioğlu and Özören (2015) reported that ASC specks have an intrinsic property to co-aggregate cytosolic proteins on their surface through non-specific hydrophobic interactions. They speculated that this may indicate a role for ASC specks as supramolecular platforms for antigen presentation in innate immune cells during intracellular infection. They used HEK293T cells expressing m-Cherry-ASC to detect ASC speck formation and co-localization of the ASC speck with a panel of enhanced green fluorescent protein (EGFP)-fused short peptides, including the complement protein C3 as well as the model antigen ovalbumin. They found a diversity of cytosolic proteins that could co-aggregate on the ASC speck that primarily indicated non-specific hydrophobic interactions. They further speculated that antigen presentation on the ASC speck may allow for cross-presentation of antigens on MHC-II. Cytoplasmic proteins are mostly displayed by MHC-I, however, cytoplasmic proteins that enter the autophagic pathway are presented on MHC-II. Using phorbol-12-myristate-13-acetate (PMA)-differentiated THP-1 macrophages, they observed that purified extracellular ASC specks are engulfed by the phagocytic cell and contained within an acidified organelle, likely the phagolysosome, where it is then degraded over time and trafficked away in small tubular vesicles consistent with the morphology of tubular vesicles responsible for MHC-II dependent presentation of extracellular antigens (Sahillioğlu and Özören, 2015). ASC speck-dependent cross presentation of antigens on MHC-II has not been confirmed. Ciccocioppo et al. (2020) commented that misfolded protein aggregates look like PAMPs and thus activate a range of PRRs to activate the innate immune response in an attempt to clear the inciting stimulus, and when this response fails to clear the aggregates, the immune response remains locked in a toxic pro-inflammatory cascade.

Aggregation of Aβ by the ASC Speck

Heneka et al. (2013) demonstrated a clear link between NLRP3 inflammasome activity and Aβ-related pathology and its clearance in AD. They demonstrated that NLRP3 deficient APP/PS1 mice show no caspase-1 cleavage of pro-IL-1β and

had IL-1β levels similar to non-transgenic control mice (Heneka et al., 2013). NLRP3$^{-/-}$ and Caspase-1$^{-/-}$ APP/PS1 mice show improved delay-dependent and spatial memory relative to APP/PS1 with intact NLRP3 inflammasome pathways. Hippocampal synaptic plasticity as measured by long term potentiation (LTP) was also protected in NLRP3$^{-/-}$ and Caspase-1$^{-/-}$ APP/PS1 mice relative to the LTP suppression observed in APP/PS1 mice (Heneka et al., 2013). NLRP3 deficient mice were also protected from neurobehavioral disturbances as demonstrated by open field testing. NLRP3$^{-/-}$ and Caspase-1$^{-/-}$ APP/PS1 mice had reduced Aβ plaque load in both hippocampus and cortex even though levels of APP expression and processing by β-secretase-1 were unaffected suggesting that the decrease in aggregated Aβ was due to enhanced clearance mechanisms. Furthermore, they clearly demonstrated that NLRP3$^{-/-}$ and Caspase-1$^{-/-}$ APP/PS1 had enhanced phagocytosis of amyloid-β plaques with evidence of degradation by lysosomes. There was also evidence of enhanced insulin-degrading enzyme release by microglia in inflammasome deficient APP/PS1 mice. They also characterized microglial phenotype and found that NLRP3 and caspase-1 deficiency resulted in a skewed microglial phenotype toward the pro-phagocytic M2 phenotype. The upregulation of nitric oxide synthase-2 in M1 microglia in APP/PS1 mice results in nitration of tyrosine on Aβ accelerating its aggregation and seeding of new plaques. NLRP3$^{-/-}$ and Caspase-1$^{-/-}$ APP/PS1 had significantly less nitrated Aβ as well as smaller plaque sizes (Heneka et al., 2013). This study demonstrated that there is a clear link between NLRP3 inflammasome/ASC speck activation and AD progression, especially regarding the accumulation of Aβ plaques. Furthermore, NLRP3 inflammasome/ASC speck activation contributes to a pro-inflammatory microglial phenotype that reduces protein clearance mechanisms.

Venegas et al. (2017) demonstrated that extracellular ASC specks directly cross-seed Aβ aggregation *in vitro* and *in vivo*. Exposure of cultured mouse primary microglia to Aβ_{1-42} caused the formation and release of ASC specks which were shown to associate with TAMRA-labeled Aβ_{1-42} very rapidly in the extracellular environment (Venegas et al., 2017). Incubation of the supernatant derived from non-transgenic mouse primary microglia stimulated with LPS and ATP to produce inflammasomes with Aβ_{1-42} resulted in detectable Aβ_{1-42} aggregation while ASC-deficient microglia did not (Venegas et al., 2017). Purified ASC specks incubated with Aβ_{1-42} accelerated aggregation speed in a concentration-dependent manner. The decreased lag phase of Aβ_{1-42} aggregation in the presence of the ASC speck indicates a cross-seeding ability of the ASC speck through its interactions with the Aβ_{1-42} peptide. Interestingly, this cross-seeding ability was specific to the Aβ_{1-42} peptide and did not occur with the reverse sequence of Aβ_{1-42} or with the model protein bovine serum albumin (BSA). Co-sedimentation assays in which the insoluble pellet fraction is separated from the cell supernatant, and both are analyzed to determine the presence of Aβ and ASC, showed that ASC-specks alone remain in the supernatant but when incubated with Aβ_{1-42} or Aβ_{1-40} will co-sediment into the pellet fraction together (Venegas et al., 2017). The *in vitro* co-sedimentation findings were corroborated by immunoprecipitation studies *ex vivo* in brain samples from APP/PS1 mice (Venegas et al., 2017). Immunohistochemical studies revealed that ASC specks were found in the core of extracellular Aβ plaques in APP/PS1 mouse models as early as 4-months as well as in human AD patients including the early mild cognitive impairment stages of AD (Venegas et al., 2017). APP/PS1-ASC$^{-/-}$ mice showed reduced Aβ plaque deposition and had reduced spatial memory deficits as assessed by Morris water maze. Purified ASC specks that were injected into the hippocampus of 3-month-old APP/PS1 mice showed increased size and number of Aβ plaque deposition relative to the contralateral non-injected side without changes in phagocytosis, amyloid-precursor protein (APP) production, or a-/b-C-terminal fragments of APP (Venegas et al., 2017). APP/PS1-ASC$^{-/-}$ mice injected with brain homogenates from aged APP/PS1 mice in the hippocampus showed significantly reduced size, amount, and spreading of Aβ plaques relative to APP/PS1 mice that were not deficient in ASC (Venegas et al., 2017). This could not be explained by changes in Aβ degradation. Similarly, APP/PS1 injected with brain homogenates from ASC$^{-/-}$ APP/PS1 mice showed reduced Aβ plaque deposition. These results demonstrate that the ASC speck can serve as a scaffold for cross-seeding of Aβ aggregation even in very early stages of AD and thus plays a role in the progression and spreading of Aβ pathology in the brain.

Aggregation of α-Synuclein by the ASC Speck

Similar to AD, human PD patients have elevated NLRP3, ASC, and caspase-1 localized exclusively within Iba1^{+} microglia in the substantia nigra or detectable as extracellular ASC and systemically circulating NLRP3, caspase-1, and IL-1β (Gordon et al., 2018; Chatterjee et al., 2020). Wang et al. (2016) demonstrated that caspase-1 can be found at the core of Lewy bodies extracted from human PD patients' brains. In this study, they stained Lewy bodies for caspase-1 and α-syn and showed a caspase-1 positive core (∼10 microns in diameter) surrounded by α-syn (Wang et al., 2016). While they did not stain for ASC to determine if the caspase-1 positive core was an ASC speck, based on the size of the core and what is known about the ASC speck's ability to cross-seed protein aggregation, we speculate that ASC specks can likely be found in the core of α-syn Lewy bodies similarly to Aβ plaques.

Additionally, Gordon et al. (2018) used three animal models of PD to study inflammasome related dopaminergic neurodegeneration that is driven by mitochondrial dysfunction, oxidative stress, or α-syn pathology. These animal models used 6-hydroxydopamine (6-OHDA) administration, MitoPark transgenic mice, and pre-formed α-syn fibril (PFF) injected mice to induce PD (Gordon et al., 2018). In each animal model, NLRP3, ASC, and caspase-1 were elevated at early stages, even prior to α-syn fibril formation, confirming that NLRP3 inflammasome activity can be stimulated by α-synuclein oligomers or protofibrils and can contribute to α-syn fibril deposition. Primary mouse microglia incubated with α-syn PFFs exhibited robust NLRP3 inflammasome activation that was delayed relative to ATP stimulation (24 h vs. 1 h) likely because

inflammasome activation by fibrils cannot occur until the fibrils are phagocytosed and escape lysosomal degradation (Gordon et al., 2018). They also observed significant levels of extracellular ASC from primary microglia exposed to α-syn PFFs despite not detecting any pyroptosis, suggesting that ASC specks may be released in a non-cell death-dependent manner, possibly via exosomal excretion. α-syn PFFs also activated primary microglia without any priming, suggesting that α-syn fibrils can act at signal 1 and signal 2, though the IL-1β release in unprimed cells were only ∼35% of the levels released in cells that were primed with LPS (Gordon et al., 2018). Injection of fibrillar α-syn in the striatum of WT mice results in deposition of pathological hyperphosphorylated α-syn aggregates in neurons and microglia of the nigrostriatal system as well as in the cortex (Gordon et al., 2018).

Another group used a chronic neurotoxicant, 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), model of PD and found that NLRP3 deficiency protected against nigral dopaminergic neurodegeneration, prevented microgliosis and astrogliosis in the substantia nigra and prevented α-syn aggregate deposition in the substantia nigra (Ou et al., 2021). NLRP3 deficiency also protected against MPTP induced impairments in nigral autophagy pathways in the midbrains of mice as measured by the expression of the autophagy related proteins, LC-II and p62 (Ou et al., 2021). It is unclear if this failure in autophagy is cell-specific (neurons vs. microglia) or global. These studies suggest a similar crosstalk between a-syn aggregates and inflammasomes in the cross-seeding process in PD to what was demonstrated with Aβ in AD.

Aggregation of Tau by the ASC Speck

Previous studies have also demonstrated a link between inflammasome/microglial inflammatory activity and tau neurofibrillary tangle (NFT) seeding/accumulation in rodent models of tauopathy. Ising et al. (2019) showed that NLRP3 activation is elevated in FTD patients as evidenced by elevated ASC, caspase-1, and mature IL-1β levels. Additionally, Tau22 mouse models of FTD that were deficient in ASC ($ASC^{-/-}$) or NLRP3 ($NLRP3^{-/-}$) had reduced levels of phosphorylated tau in the hippocampus, CA1 cell body region, and granular cell layer of the dentate gyrus as assessed by AT8 staining; they also had decreased aggregated tau levels as assessed by Thioflavin T fluorescence assays; and they exhibited rescue in spatial memory relative to Tau22 mice that had intact NLRP3 inflammasome pathways (Ising et al., 2019). In Stancu et al. (2019), TauP301S (PS19) transgenic mice deficient in ASC ($ASC^{-/-}$) were injected with pre-aggregated tau seeds in the frontal cortex at 3 months of age. The results showed significantly decreased seeding ability of tau relative to PS19 mice that were not deficient in ASC indicating a significant role for the inflammasome in tau propagation in an ASC-dependent manner (Stancu et al., 2019). Another group previously demonstrated that microgliosis and microglial activation precede tau neurofibrillary tangle burden in PS19 mice and treating PS19 with an anti-inflammatory molecule, FK506, significantly reduced tau pathology and extended the life-span of PS19 mice (Yoshiyama et al., 2007). We have previously demonstrated that adoptive transfer of purified microglia from hTau-*Cx3cr1*$^{-/-}$ mice that exhibit a highly inflammatory phenotype, into the brains of non-transgenic recipient mice was sufficient to induce tau pathology in a manner dependent upon IL-1β-IL-1R axis (Maphis et al., 2015b). In our recent study, we have demonstrated that myeloid-cell restricted deletion of MyD88 blocked both signal 1 (priming) and signal 2 (inflammasome activation) and myeloid-cell specific deletion of ASC prevented (signal 2) for NLRP3 inflammasome activation and, in both cases, it reduced maturation of IL-1β, reduced tau pathology, and improved memory in the hTau mouse model of tauopathy (Jiang et al., 2021).

A previous study by Asai et al. (2015) showed that depletion of microglia and inhibition of exosome synthesis mitigated the propagation of tau and tau spreading across brain regions. Much of their definitive work was performed in a model using an adeno-associated virus vector expressing human P301L mutant tau in a neuron-specific manner, by injecting it into the entorhinal cortex of C57BL/6j mice. Intracortical inject of P301L FTDP-17 tau exhibited rapid propagation of tau from the entorhinal cortex to the dentate gyrus in 4 weeks. They also corroborated their findings using TauP301S mice. This study focused on the role of tau-containing exosome release from microglia as a method for tau spreading, which is another important way that microglia contribute to AD/PD disease progression, though not the focus of this review. The depletion of microglia using PLX3397, a colony stimulating factor 1 receptor inhibitor, in P301S mice demonstrated reduced AT8 (pS202/pT205 positive) tau burden at 3.5 months of age which supported their claims that microglia play an important role in the spreading of protein aggregates and disease progression (Asai et al., 2015). We speculate that depletion of microglia using PLX3397 may have also reduced ASC speck-mediated aggregation of tau in addition to exosomal spreading, though this was not investigated. Though we will not address them in this review, recent studies have also indicated that the NLRP3 inflammasome may play an important role in promoting exosome release from microglia (Si et al., 2021).

Taken together, these results suggest that microglia can promote seeding/spreading of numerous AD/PD related misfolded protein aggregates as well as pathological modifications of tau and a-syn with a multitude of different mechanisms (**Figure 3**). Importantly, inflammasome activity and/or assembly of the ASC speck underscores many of these mechanisms.

Inhibitors of NLRP3 or Antibody-Neutralization of Inflammasomes Reduce Disease Burden

Given its apparent role in AD and PD pathogenesis and disease progression, the NLRP3 inflammasome pathway has become a target for intervention. Several studies have used small molecule inhibitors of NLRP3 to prevent inflammasome assembly and seen significant reductions in inflammation as well as rescue of cognitive function in animal models of AD and PD. Use of targeted antibody therapies against inflammatory signaling molecules and complexes, such as the ASC speck, is also a growing area of therapeutic development. In this section, we

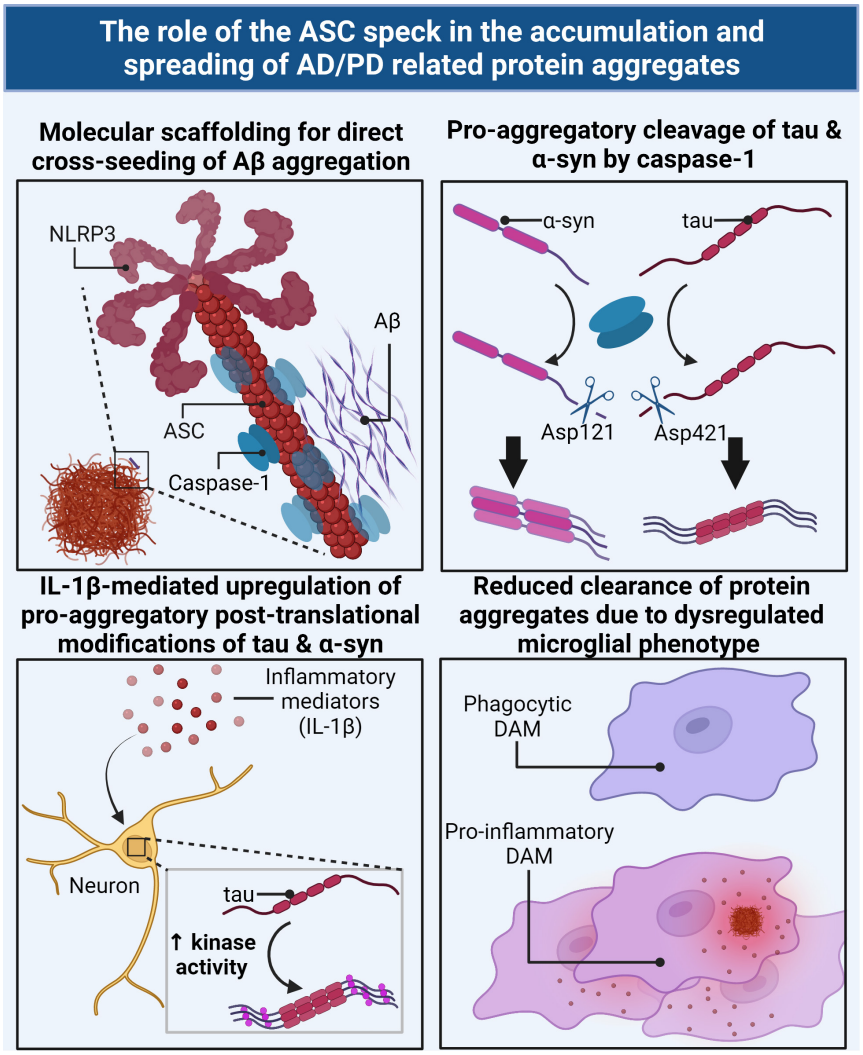

FIGURE 3 | The ASC speck directly and indirectly contributes to the accumulation and spreading of AD/PD related misfolded protein aggregates via several mechanisms. The ASC speck can directly cross-seed the aggregation of Aβ by serving as a molecular scaffold for aggregation **(top left)**. Caspase-1, the effector component of the inflammasome/ASC speck, can directly cleave tau and α-syn monomers into a pro-aggregatory form for seeding nucleation **(top right)**. IL-1β, the downstream product of the ASC speck, can act as a cellular signal to upregulate key enzymes for the pro-aggregatory PTMs of tau and α-syn **(bottom left)**. The ASC speck contributes to the dysregulation of protective disease-associated microglia (DAMs) and subsequent skewed pro-inflammatory phenotype resulting in a decrease in phagocytic clearance of AD/PD related misfolded protein aggregates **(bottom right)**.

will highlight recent studies that have targeted the NLRP3 inflammasome pathway using these therapeutic strategies.

Dempsey et al. (2017) showed reduced Aβ plaque burden, reduced inflammation, and rescue of cognitive function in the APP/PS1 animal model of AD using the NLRP3 inhibitor, MCC950. Cultured primary microglia from C57B/L6 mice were incubated with LPS and ATP or LPS and Aβ for NLRP3 activation. Pre-incubation with MCC950 attenuated the production of IL-1β in both groups as well as caspase-1 immunoreactivity (Dempsey et al., 2017). Only the LPS + ATP group exhibited lactate dehydrogenase release indicating loss of cell viability, but this was attenuated by MCC950. As expected, MCC950 had no effect on TNFα or IL-6 levels, which are expressed independent of inflammasome activation. MCC950 administration in primary microglia incubated with LPS and Aβ showed enhanced phagocytosis of Aβ relative to untreated microglia *in vitro*. These findings were confirmed *in vivo* using the APP/PS1 mouse model (Dempsey et al., 2017). Treatment of 12-month-old APP/PS1 mice with MCC950 every second day for 3 months showed a decrease in plaque number and soluble $A\beta_{1-40,1-42}$ suggesting increased phagocytic activity, though various indicators of microglial phenotype did not reach significance to indicate promotion of an M2 phenotype. The anti-inflammatory and pro-phagocytic effects of MCC950 administration in APP/PS1 mice correlated with improvement in cognitive function as assessed by spontaneous alternation in the T-maze and the novel object recognition test (Dempsey et al., 2017).

Another study using CRND8 APP transgenic mice aged 9 months injected with a novel NLRP3 small molecule inhibitor, JC-124, reduced levels of caspase-1 relative to untreated transgenic mice, reduced the size and number of Aβ oligomers and plaques, and reduced β-secretase cleavage of APP (Yin et al., 2018). Microgliosis was reduced by JC-124 in TgCRND8 mice but a concomitant astrocytosis was observed. Reduction in oxidative stress was also observed following treatment with JC-124 as assessed by the oxidative stress markers heme oxygenase-1 (HO-1) and 4-hydroxynonenal (HNE). There were also improvements in synaptophysin levels suggesting a synaptoprotective effect of JC-124 in TgCRND8 mouse models (Yin et al., 2018).

To confirm the role of the ASC speck in the cross-seeding of Aβ protein aggregates, Venegas et al. (2017) used antibody neutralization studies against the ASC speck. Co-incubation of an anti-ASC antibody with purified ASC-specks and Aβ prevented ASC speck-induced aggregation of Aβ in an antibody concentration dependent manner (Venegas et al., 2017). This was confirmed *in vivo* by co-injecting APP/PS1 hippocampi with brain homogenates from aged APP/PS1 mice with either anti-ASC antibodies or an isotype-specific IgG. The results showed that anti-ASC antibodies reduced the rostral-caudal spreading of Aβ plaque deposition as well as reduced Aβ monomers and oligomers without affecting the APP production or degradation pathways (Venegas et al., 2017). Thus, antibodies against the ASC speck may be a useful strategy for inhibiting its cross-seeding potential and for mitigating the inflammatory response in AD, which may also likely be effective in PD.

In the context of tauopathy, MCC950 administration in LPS-primed primary microglia exposed to pre-aggregated tau seeds prevented inflammasome assembly and IL-1β maturation (Stancu et al., 2019). MCC950 administration in PS19 transgenic mice injected with pre-aggregated tau seeds reduced exacerbation of tau pathology as assessed by AT8 staining in a dose dependent manner as well as microgliosis as assessed by Iba1 staining (Stancu et al., 2019). It was noted that reduction in microgliosis could either be due to reduction in inflammasome assembly (or ASC speck formation) and thus reduced inflammatory cytokine signaling, or reduction in tau pathology, or a combination of the two (Stancu et al., 2019).

In the context of PD, MCC950 blocked NLRP3 inflammasome activity including ASC fibril formation in response to α-syn PFF aggregates in primary mouse microglia (Gordon et al., 2018). MCC950 also blocked inflammasome activation in the 6-OHDA, MitoPark, and PFF models of PD (Gordon et al., 2018). Daily oral dosing of MCC950 in the acute toxicant 6-OHDA model of PD also protected against nigrostriatal dopaminergic degeneration and behavioral deficits *in vivo* (Gordon et al., 2018). The same findings were observed in the chronic progressive PFF mouse model of PD in which chronic dosing of MCC950 prevented progressive motor and behavioral deficits as well as dopaminergic degeneration in response to pathological α-syn aggregates *in vivo* (Gordon et al., 2018). Chronic dosing of MCC950 also prevented the pathological spreading of α-syn aggregates from the PFF injection site in the striatum throughout the nigrostriatal system as well as into cortical brain regions (Gordon et al., 2018). This suggests that NLRP3 inflammasome activity is central to the propagation and spreading of α-syn throughout the brain in PD and can thus be targeted with therapeutic strategies.

Several therapeutics that act upstream of inflammasome activation to inhibit NLRP3 have all shown therapeutic effects in animal models of PD. Han et al. (2019) used kaempferol, a small molecule that inhibits NLRP3 inflammasome activity by promoting the ubiquitin-mediated autophagic degradation of NLRP3, and demonstrated reduced behavioral deficits, reduced nigral dopaminergic neurodegeneration, and enhanced tyrosine hydroxylase activity in an LPS-injected mouse model of PD as well as reduced dopaminergic neurodegeneration and microglial activation in an $A53T^{tg/tg}$ α-syn over-expressing mouse model of PD. Yao et al. (2019) used fingolimod (FTY720), an upstream inhibitor of NLRP3 activation by reducing reactive oxygen species and p65-mediated activation of NLRP3, in the MPTP mouse model of PD and showed reduced behavioral deficits, reduced dopaminergic neurodegeneration, and increased dopamine release in addition to reduced levels of IL-1β, IL-6, and TNFα. Xu et al. (2019) had similar findings using DDO-7263, a novel Nrf2-ARE activator that inhibits NLRP3 inflammasome activation, in the MPTP mouse model of PD.

Even inhibiting the downstream components of the inflammasome pathway have shown some therapeutic effect in animal models of PD. Injections of the caspase-1 inhibitor, Ac-YVAD-CMK, into the brains of two rat models of Parkinson's disease (LPS-induced and 6-OHDA induced) reduced neurodegeneration of dopaminergic neurons in the substantia nigra and reduced the expression of inflammasome components (Mao et al., 2017).

Taken together, these studies indicate that inhibition of the NLRP3 inflammasome/ASC speck at various stages of its pathway (inhibiting inflammasome assembly, its downstream inflammatory signaling components, or the ASC speck's interaction with other proteins) has potential to reduce or prevent disease progression in AD and PD.

DISCUSSION

We have known for some time now that inflammation is a core characteristic of neurodegenerative diseases. It was previously believed to be a byproduct of the toxicity of the misfolded protein aggregates but over the last decade there has been significant progress in understanding how inflammation plays a major role in driving the disease. Within the last 5 years, several groups have begun to elucidate the active role that the NLRP3 inflammasome and ASC speck play in exacerbating misfolded protein aggregation and damaging neuroinflammation that drives disease progression and spreading throughout the brain (**Figure 4**). Thus, paving the way to a new paradigm in inflammasome biology that has broadened our understanding of the pathogenesis of neurodegenerative diseases like AD and PD. There are still many unanswered questions surrounding this new paradigm and many new opportunities for therapeutic development to be explored.

It seems that the ASC speck can cross-seed Aβ aggregation directly by acting as a molecular scaffold for aggregation

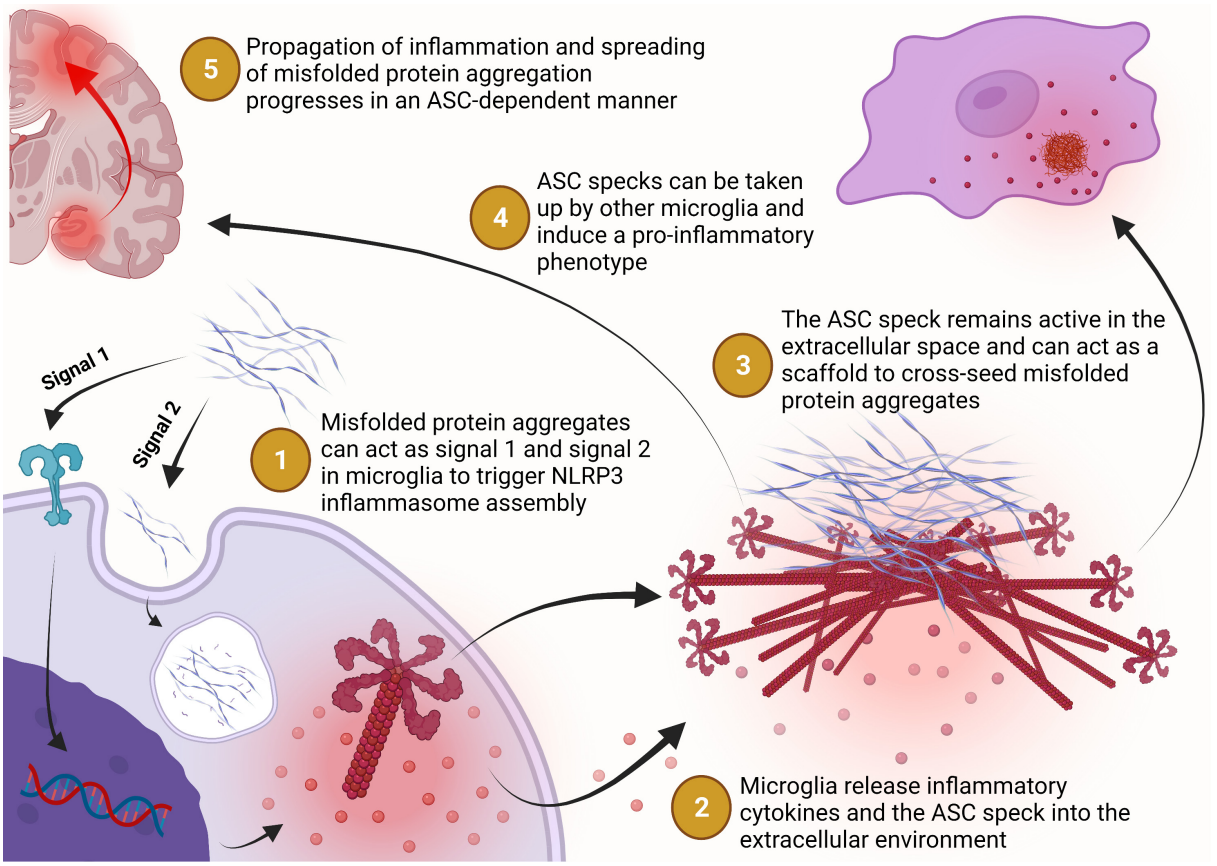

FIGURE 4 | Misfolded protein aggregates bind to toll-like or scavenger receptors which triggers the expression of NLRP3 and caspase-1 to prime the cell for inflammasome assembly in a MyD88-NFκB signaling dependent manner. Extracellular misfolded protein aggregates may be internalized by the phagolysosomal pathway but can escape lysosomal degradation and trigger assembly of the NLRP3 inflammasome (step 1). The NLRP3 inflammasome catalyzes the maturation of interleukin-1β and interleukin-18 which can be transported extracellularly or released into the extracellular environment during pyroptosis for inflammatory signaling (step 2). Assembly of the NLRP3 inflammasome may progress to the formation of the ASC speck, a supramolecular complex with enhanced inflammatory signaling capacity, which may then be released into the extracellular environment following pyroptosis or some other non-pyroptic mechanism (step 2). Once in the extracellular space, the ASC speck remains functionally active for inflammatory cytokine maturation, pro-aggregatory modifications of other proteins, and cross-seeding of neuronally derived misfolded protein aggregation (step 3). The ASC speck can also propagate inflammation from one microglial cell to another by inducing a pro- inflammatory phenotype in cells that phagocytose the ASC speck but fail to degrade it (step 4). Together, these activities of the ASC speck implicate its role in the propagation of inflammation and spreading of protein aggregation in a vicious cycle that contributes to the chronic progressive nature of Alzheimer's disease and Parkinson's disease (step 5).

and plays an important role in triggering the aggregation of tau and α-syn through a variety of mechanisms including upregulation of pro-aggregatory PTMs. This prion-like activity is particularly interesting because the ASC speck does not have the same β-pleated sheet fibrillar structure that is required for aggregation of the AD/PD related protein aggregates. Conversely, these misfolded protein aggregates can prime and activate the aggregation of NLRP3 inflammasome components into the prion-like ASC speck. It is unclear mechanistically how these protein aggregates trigger the activation of the NLRP3 inflammasome complex, but it is fascinating that there seems to be bi-directional cross seeding that triggers prionoid protein aggregation.

PTMs of the proteins involved in the inflammasome complex is important for regulating inflammasome assembly in response to various cellular stress signals as well as the decision to assemble the supramolecular ASC speck complex. The advances in single cell and single nucleus RNA sequencing that have allowed for the profiling of NAMP-responsive DAMs in AD and PD will likely permit further understanding of how misfolded protein aggregates contribute to the cellular decision to commit to the formation of the ASC speck.

Failed degradation of the ASC speck by microglial autophagy pathways results in the persistence of the active ASC speck contributing to an unregulated inflammatory response. This seems to coincide with a failure in the degradation of AD/PD related misfolded protein aggregates in neurons. Further research is necessary to understand why these degradation pathways fail in neurodegenerative diseases and to understand if there is a common link in the failure of both microglial and neuronal protein aggregate degradation pathways. Understanding how this pathological process occurs will be

useful for identifying ways to generate therapeutics for disease prevention and intervention.

Therapeutics targeting the NLRP3 inflammasome and ASC speck have shown promising results in animal models of AD and PD. A multi-pronged approach targeting inflammasome/ASC speck assembly and activity, misfolded protein aggregates, and protein clearance mechanisms will likely be the future direction for therapeutic design in the treatment of neurodegenerative proteinopathies like AD and PD. With this new understanding of mechanisms contributing to disease progression, the future for generating effective treatments in the clinical management of AD and PD seems hopeful.

AUTHOR CONTRIBUTIONS

JH performed the literature search and drafted the manuscript. KB edited the manuscript. Both authors contributed to the article and approved the submitted version.

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REVIEW
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Contribution of Autophagy-Lysosomal Pathway in the Exosomal Secretion of Alpha-Synuclein and Its Impact in the Progression of Parkinson's Disease

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Parkinson's disease (PD) is caused by the degeneration of dopaminergic neurons due to an accumulation of intraneuronal abnormal alpha-synuclein (α-syn) protein aggregates. It has been reported that the levels of exosomal α-syn of neuronal origin in plasma correlate significantly with motor dysfunction, highlighting the exosomes containing α-syn as a potential biomarker of PD. In addition, it has been found that the selective autophagy-lysosomal pathway (ALP) contributes to the secretion of misfolded proteins involved in neurodegenerative diseases. In this review, we describe the evidence that supports the relationship between the ALP and α-syn exosomal secretion on the PD progression and its implications in the diagnosis and progression of this pathology.

Keywords: autophagy-lysosomal pathway, α-syn exosomal secretion, Parkinson's disease progression, biomarker, degradation

INTRODUCTION

Parkinson's disease (PD) is the second more common neurodegenerative disease globally, and it is associated with age (Dorsey et al., 2018). The PD incidence is estimated from 5 to more than 35 new cases per 100,000 individuals, depending on demographic differences (Poewe et al., 2017). The pathology prevalence ranges from 41/100,000 individuals in the fourth decade of life to more than 1,900/100,000 among those 80 and older (Pringsheim et al., 2014). Have been described that PD is more prevalent in men (1,729/100,000, >65 years) than in women (1,644/100,000) (Pringsheim et al., 2014; Riedel et al., 2016). Although the risk of developing PD is higher in men, women have a higher mortality rate and faster clinical progression (Cerri et al., 2019).

Parkinson's disease is a complex neurodegenerative disorder clinically characterized by bradykinesia, tremor, rigidity, later postural reflexes instability, and progressive paralysis (Jankovic, 2008). Some non-motor symptoms such as dementia, depression, anxiety, and sleep disorder may precede motor symptoms for more than a decade, affecting several neurotransmitter pathways (Langston, 2006). PD coexists with dementia in over 25% of the cases and depression in over 30% of the cases in some countries (Riedel et al., 2016). As a motor disorder, PD affects patients' quality of life, making social interaction more difficult and worsening their financial condition due to the high medical expenses associated with the pathology.

Part of the PD symptomatology is caused by the degeneration of dopaminergic (DA) neurons of Substantia Nigra pars compacta (SNpc) and loss of the dopaminergic fibers that innervate the striatum (Dauer and Przedborski, 2003). The appearance of the first symptoms correlates with a 30% loss of dopaminergic neurons (Fearnley and Lees, 1991), indicating degeneration of the neurotransmission integrity in the basal ganglia circuits. Currently, PD has no cure, and the potential treatment to prevent or revert this pathology arises as a substantial challenge (Troncoso-Escudero et al., 2020). Therefore, it is essential to obtain a better understanding of the correlation between associated cellular mechanisms and the clinical features of PD to develop strategies to optimize the prevention, diagnosis, and treatment.

About 10% of PD cases are associated with genetic mutations (Selvaraj and Piramanayagam, 2019), which includes: (i) mutation in the *SCNA* gene that encodes for the alpha-synuclein protein (α-syn) (Bras et al., 2020), (ii) mutations in the *LRRK2* gene, which encodes leucine-rich repeat kinase 2, are a cause of autosomal dominant forms of PD (Zimprich et al., 2004; Tolosa et al., 2020), (iii) heterozygous mutations of the *GBA1*, encoding for lysosomal enzyme glucocerebrosidase (GCase), are a strong risk factor for PD and can lead to α-syn accumulation (Avenali et al., 2020). While 90% of PD cases are classified as idiopathic, the evidence indicates that the histopathology characteristic of PD is the accumulation of intraneuronal abnormal protein aggregates, including the α-syn protein. These aggregates of the amyloid type are called Lewy bodies and are constituted mainly by oligomers of α-syn and ubiquitin (Spillantini et al., 1998; Schulz-Schaeffer, 2010). Idiopathic cases show an increase of endogenous wild-type α-syn (Golbe et al., 1990; Michell et al., 2005; Shprecher et al., 2018), forming aggregates of this protein that have a toxic effect on dopaminergic neurons, triggering neurodegenerative processes (Petrucelli et al., 2002; Volpicelli-Daley et al., 2016).

Mitochondrial toxins have been identified in epidemiological studies as contributors to "sporadic" PD in humans. In this context, animal and *in vitro* models used to study PD are based on the administration of neurotoxins, generating oxidative stress and mitochondrial dysfunction. 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), paraquat (PQ; 1, 10-dimethyl-4,40-bipyridinium), and rotenone are conventionally used in PD modeling (Chia et al., 2020), as they can be uptaken by DA neurons through dopamine transporters, inhibiting complex I of the mitochondrial electron transport chain, leading to ATP depletion, increasing reactive oxygen species, and ultimately resulting in neuronal death (Betarbet et al., 2002; Devi et al., 2008; Potashkin et al., 2010). PQ, a commonly used herbicide, shares structural similarities with MPP+, the active metabolite of MPTP. PQ crosses the blood-brain barrier, generates reactive oxygen and nitrogen species (ROS/RNS), and causes the loss of SNpc DA neurons in animal models (Castello et al., 2007). In rats, chronic rotenone exposure leads to α-syn aggregation, DA neurodegeneration, and behavioral defects (Hoglinger et al., 2003). This toxin induced the cytosolic accumulation of α-syn through the *de novo* synthesis, rather than a reduction of degradation by chaperone-mediated autophagy (CMA), suggesting a mechanism independent from lysosomal degradation (Sala et al., 2013). Indeed, rotenone regulates α-syn phosphorylation, reducing protein phosphatase 2A (PP2A) activity (Wang et al., 2016).

Classical pharmacological therapies for PD patients are dopamine precursors as levodopa, L-dopa, and L-3,4-dihydroxyphenylalanine. Other treatments include dopamine agonists such as amantadine, apomorphine, pramipexole, and monoamine oxidase inhibitors (MAO) or catechol-*O*-methyltransferase (COMT). The sustained administration of these drugs induces a "wearing-off phenomenon" and additional psychomotor, cardio-cerebrovascular, and hormones regulation problems (Cacabelos, 2017). Novel biotherapies, as natural products, should achieve dopaminergic protection to avoid neurodegeneration, enhancing dopaminergic neurotransmission. However, the cellular and molecular events involved in PD must be broadly explored to design and develop efficient treatments (Solayman et al., 2017; Wang et al., 2017; Troncoso-Escudero et al., 2020).

ALPHA-SYNUCLEIN (α-SYN): A HALLMARK IN PARKINSON'S DISEASE

α-syn protein is expressed at high levels in the central nervous system (CNS), specifically neurons. It is found in presynaptic terminals as a monomeric, unfolded, and soluble protein (Maroteaux et al., 1988), bound with high affinity to the membranes of synaptic vesicles (Burre et al., 2010). α-syn was described in neuromuscular junctions (Askanas et al., 2000), suggesting other cellular functions in addition to its activity in the CNS. Although α-syn is enriched in synaptic boutons, which sprout from axons of different neurochemical phenotypes, α-syn is not present in all synaptic terminals. In agreement, not all terminals accumulate the protein in neurodegenerative disorders (Totterdell et al., 2004). Furthermore, the expression of α-syn is not limited to the nervous system. This protein is present in the cerebrospinal fluid (CSF), in plasma (El-Agnaf et al., 2003; Forland et al., 2018), as well as is expressed in the erythropoietic lineage cells (Nakai et al., 2007) and peripheral lymphocytes (Kim et al., 2004).

To clarify the α-syn function, knockout mice for the *SCNA* gene were generated. Although knockout mice were viable and fertile, with a lack of spontaneous neurodegeneration signs, this model displays alterations in activity-dependent dopamine release from axons in the striatum (Abeliovich et al., 2000). In addition, the triple knockout mice lacking the three variants of syn (α, β, and γ) were generated, showing no neurodegeneration (Greten-Harrison et al., 2010). However, it was possible to observe synapse-structure modifications and a decrease in the synaptic terminal size in an age-dependent manner (Greten-Harrison et al., 2010). Lack of α-syn in the transgenic mice model showed less mobilization of glutamatergic vesicles (Gureviciene et al., 2007) and increased the expression levels of proteins involved in vesicle traffic, such as SNAREs, synapsins, and complexines (Greten-Harrison et al., 2010). In addition, it has been described that the participation of α-syn in vesicle homeostasis is Ca^{2+}-dependent (Lautenschlager et al., 2018). Overall, these data suggest a direct physiologic role of

α-syn in synaptic transmission in the CNS, especially in the dopamine system.

Different factors can trigger α-syn aggregation, including point mutations (Narhi et al., 1999; Rutherford et al., 2014), truncations (Li et al., 2005), posttranslational modifications of α-syn (Duda et al., 2000; Fujiwara et al., 2002; Lazaro et al., 2014), and wild-type SCNA gene duplication or triplication (Singleton et al., 2003; Fuchs et al., 2007). An increase in somatic copy number of the *SNCA* gene in CNS neurons, especially from the SN region, was reported in a cohort of PD patients, contributing to the sporadic α-syn accumulation (Mokretar et al., 2018). However, it is still unknown what prompts the accumulation of wild-type α-syn into toxic aggregates. Evidence indicates that toxic α-syn conformers can act as seeds for the misfolding and aggregation of the native protein. For instance, α-syn preformed fibrils (PFFs), synthetically produced, were added in neuronal cultures and taken up inside cells, recruiting α-syn endogenous into protein aggregates (Luk et al., 2012a,b). The inoculation of PFFs into the brain of young adult A53T *SNCA* mice (overexpressing mutated human α-syn) generated *in vivo* aggregates and PD-like symptoms in mice (Luk et al., 2012b). Moreover, species of α-syn isolated from A53T transgenic mice induce aggregation of α-syn in primary neuronal cultures (Colla et al., 2018), indicating a potential trigger role of α-syn aggregates on wild-type soluble α-syn.

Nevertheless, not only protein interactions determine the status of α-syn aggregation. α-syn contains a lipid-binding domain that allows its binding to vesicles at the presynaptic terminal (Lashuel et al., 2013). However, in pathological conditions or modified lipids composition, this interaction can potentiate conformational changes in α-syn protein, prompting it to aggregation (Marschallinger et al., 2020). Recently, it was reported that the lipid alteration in membrane compartments, as instability of lipid raft microdomains, promoted by aging, and neurotoxins, as the MPTP, could affect α-syn aggregation (Galvagnion, 2017; Canerina-Amaro et al., 2019). Caveolins, a subgroup of lipid rafts, act as scaffolding proteins that recruit other proteins and lipids, leading to colocalization and interaction of proteins involved in vesicular transport, signal transduction, and receptor trafficking (Hanzal-Bayer and Hancock, 2007). The central protein controlling caveolae formation is caveolin-1 (Cav-1). Cav-1 is widely expressed in the central and peripheral nervous systems (Boulware et al., 2007), regulating neurotrophin signaling pathways and synaptic remodeling (Bilderback et al., 1999; Suzuki et al., 2004). In addition, Cav-1 modulates neurotransmitter receptor signaling (Bhatnagar et al., 2004; Francesconi et al., 2009).

aveolin-1 is also involved in the aging process. Since Cav-1 expression is upregulated in old rat brain and aged human cortex (Park et al., 2000; Kang et al., 2006), suggesting that overexpression of Cav-1 may induce aging phenotypes (Wheaton et al., 2001; Lee et al., 2015). Some evidence suggests that scaffold proteins such as Cav-1 may be involved in the pathogenesis of several neurodegenerative disorders, including PD (Hashimoto et al., 2003; Benarroch, 2007). Age-related expression of Cav-1 may affect the cell-to-cell transmission of α-syn, contributing to the pathogenesis of PD (Ha et al., 2021). Cav-1 overexpression facilitated the uptake of α-syn into neurons and the formation of additional Lewy body-like inclusion bodies (Ha et al., 2021). Immunoprecipitation experiments demonstrated that the double mutant alpha-synuclein protein (A30P/A53T) interacts with Cav-1 present in both cytoplasmic and inner membrane extracts of the mouse brain, suggesting that the double mutation of α-syn increases the affinity for Cav-1 in the cytosol. These results suggest a direct interaction between Cav-1 and α-syn under non-physiological conditions (α-syn overexpressed or α-syn mutated) (Madeira et al., 2011). Furthermore, colocalization experiments using SH-SY5Y cells demonstrate that α-syn and caveolin interact directly and mediate endocytosis and colocalize to a lesser extent along the endocytosis pathway with early endosome antigen 1 (EEA1) and Rab7-positive late endosomes (Fakhree et al., 2021). EEA1 is an early endosomal Rab5 effector protein that has been implicated in the docking of incoming endocytic vesicles before fusion with early endosomes (Fakhree et al., 2021), and Rab7, a member of the Rab family of small GTPases, is a ubiquitously expressed protein that plays a vital role in the regulation of the trafficking, maturation, and fusion of endocytic and autophagic vesicles (McCray et al., 2010).

A recent work using human iPSC-derived cerebral organoids found that 3D-cultures from donors carrying homozygous *APOE4* allele presented aggregation of α-syn, loss of synaptic integrity, and impairment on lipids metabolism, resulting in accumulation of lipid droplets (Zhao et al., 2021). APOE4 isoform is a known risk factor for late-onset Alzheimer's disease (AD) development. In this study, researchers also reported a boosted interaction of APOE4 itself with α-syn in postmortem brain samples from Lewy bodies disease patients (Zhao et al., 2021), confirming a link between lipid metabolism and α-syn aggregation. As previously mentioned, heterozygosis variants in the gene encoding GCase (*GBA1*) represent a significant PD genetic risk factor (Avenali et al., 2020). Indeed, about 10% of PD patients present mutations in the gene that codifies to GCase (Sidransky et al., 2009). GCase is a lysosomal enzyme that catalyzes the hydrolysis of the glycolipid glucosylceramide. Homozygous mutations in *GBA1* cause Gaucher's disease, the most prevalent recessively inherited lysosomal lipid storage disease, characterized by neurodegeneration and peripheral symptoms (Han et al., 2020). *GBA1* variants associated with PD present a decreased enzymatic activity, resulting in the accumulation of the substrate glucosylceramide, as shown in CSF samples from PD patients (Huh et al., 2021). Accumulated glucosylceramides were reported to promote wild-type α-syn aggregation in *in vitro* studies (Taguchi et al., 2017). Of note, a small-molecule modulator (activator) of GCase reduces pathological α-syn aggregates and restores lysosomal function in PD patient midbrain neurons (Mazzulli et al., 2016b). In a bilateral correlation, α-syn aggregation also causes impairment on GCase activity and lysosomal dysfunction (see below).

The overexpression of α-syn selectively induced apoptotic programmed cell death in primary dopamine neurons (Zhou et al., 2000), neuroblastoma cell lines, and hippocampal primary neurons (Mahul-Mellier et al., 2015). The causal relationship between α-syn aggregation and cellular toxicity was investigated

by assessing the effect of inhibiting fibrillization on α-syn-induced cell death. It was reported that exogenous α-syn fibrils bind to the plasma membrane and act as nucleation sites for the formation of endogenous α-syn fibrils, promoting the accumulation and internalization of the aggregates that finally turn on the activation of both the extrinsic and intrinsic apoptotic cell death pathways in cellular models (Mahul-Mellier et al., 2015).

It has been described that secreted α-syn can be internalized by neighboring cells *via* endocytosis (Desplats et al., 2009), demonstrating the cell-to-cell transmission of α-syn accumulation and providing evidence of the pathological mechanism to explain PD progression and other synucleinopathies (Desplats et al., 2009; Hansen et al., 2011). Recent works also showed α-syn transference cell-to-cell through the formation of tunneling nanotubes (TNTs; Abounit et al., 2016; Dieriks et al., 2017). The α-syn uptake by cells depends on the fibrillization (Luk et al., 2009) and oligomeric (Lee et al., 2008) state of α-syn. Oligomers of α-syn have more significant cytotoxicity in recipient cells than soluble monomers of α-syn (Desplats et al., 2009; Emmanouilidou et al., 2010). It was reported that a single intrastriatal injection of synthetic α-syn fibrils initiates a pathological α-syn transmission sufficient to cause PD-like neurodegeneration in non-transgenic mice (Luk et al., 2012b). Furthermore, extracellular α-syn has been shown to activate microglia and astroglia, enhancing neurodegeneration, indicating a cell non-autonomous mechanism (Zhang et al., 2005; Klegeris et al., 2006).

An abundance of synaptic vesicle-related proteins like CD9 (exosomes), Clathrin, AP-2 complex, and dynamin (clathrin-mediated endocytosis), dynein, dynactin, and spectrin (retrograde transport), synaptosomal-associated protein 25, vesicle-associated membrane protein 2, and syntaxin-1 (synaptic vesicle fusion) are present in α-syn-containing protein inclusions purified from *post mortem* brain tissues from dementia with Lewy bodies (DLB) patients (McCormack et al., 2019). Different models of intercellular transmission of α-syn, not mutually exclusive, have been proposed, such as the α-syn cellular release, movement, and uptake, by different mechanisms, including exocytosis, exosomes, TNTs, glymphatic flow, and endocytosis (Valdinocci et al., 2017). In this regard, different types of vesicles are released from the cells depending on the metabolic and homeostatic cellular status into the extracellular space (extracellular vesicles, EVs), such as exosomes. EVs act as a shuttle for cargo delivery between cells, participate in cell-to-cell communication, and have a potential pathogenic role in the cell-to-cell transmission of toxic aggregated proteins in a neurodegenerative disease context.

EXOSOMAL α-SYN SECRETION IN PARKINSON'S DISEASE AND ITS IMPACT ON DISEASE PROGRESSION

Exosomes are small vesicles (40–100 nm in diameter) released into the extracellular space by various cell types, including neurons, astrocytes, microglia, and lymphocytes. Exosomes are generated from multivesicular bodies (MVB) that, after fusion with the plasma membrane, releases intraluminal vesicles – exosomes – containing membrane components, proteins, lipids, and microRNAs (Kowal et al., 2014; Hessvik and Llorente, 2018). Moreover, this EV population can be detected in body fluids such as blood, urine, and CSF (Thery et al., 2006; Keller et al., 2011).

Increasing evidence suggests that the secretion of α-syn, and oligomeric species, is associated with membrane vesicles, as exosomes (Alvarez-Erviti et al., 2011; Danzer et al., 2012; Emmanouilidou and Vekrellis, 2016). The secretion of exosomal α-syn is a calcium-dependent mechanism (Emmanouilidou et al., 2010). The mechanism of exosomes internalization is not entirely decoded, and it seems to depend on the type of recipient cells (Fruhbeis et al., 2013; Nanbo et al., 2013; Svensson et al., 2013; Tian et al., 2014). The pathways caveolin-dependent, clathrin-dependent, and macropinocytosis are not involved in the internalization of exosome-associated oligomeric α-syn (Delenclos et al., 2017). Furthermore, heparin sulfate proteoglycans (HSPGs), transmembrane, and lipid-anchored cell surface receptors modulate the internalization exosomes containing Aβ monomer (Kanekiyo et al., 2011) and α-syn recombinant fibrils (Holmes et al., 2013). However, in contradictory results, another group reported that the deficiency of HSPG did not attenuate the up-taking of α-syn exosomes (Delenclos et al., 2017), suggesting that this pathway is not critical for the α-syn oligomers internalization.

Neurons secrete α-syn by non-canonical cellular pathways that may involve the participation of chaperones UPS19 and DNAJ/HSC70 complex (Lee et al., 2016; Bieri et al., 2018). Although the mechanism of exosomal α-syn secretion and uptake has not been elucidated, it is more apparent that the secreted vesicular α-syn is readily internalized compared to free α-syn oligomers (Delenclos et al., 2017; Gustafsson et al., 2018), conferring toxicity on the neighboring cells (Emmanouilidou et al., 2010; Danzer et al., 2012).

Exosomes may provide a catalytic environment for nucleation of α-syn aggregation (Grey et al., 2015). Vesicles containing-α-syn have been shown to increase the oligomerization status of α-syn (Lee et al., 2005; Grey et al., 2015), and oligomers negatively impact cellular health more than monomers. α-syn species with presumably lost physiological functions or altered aggregation properties may shift the cellular processing toward vesicular secretion. Fluorescent protein tags on the N-terminus of α-syn alter intracellular dynamics (Goncalves and Outeiro, 2013) and induce vesicular secretion (Jang et al., 2010). N-terminal protein tags on α-syn lead to altered membrane-binding properties and may form particularly pathogenic and stable forms of aggregated α-syn that could increase cell-to-cell spreading (Gustafsson et al., 2018).

A minor fraction (0.1–2%) of secreted α-syn are associated with EVs, whereas most of the protein can be found free in the extracellular space (Danzer et al., 2012; Shi et al., 2014). Even though the EV-associated fraction of extracellular α-syn is slight, such vesicles are considered biologically active (van Niel et al., 2006) and molecules in this environment could be more efficiently delivered to other cells

(Subra et al., 2010). Interestingly, the exosomal α-syn levels of neuronal origin in plasma correlate significantly with motor dysfunction, a parameter of the severity of the disease (Shi et al., 2014), highlighting the exosomes containing α-syn as a potential biomarker of PD (**Figure 1A**). α-syn and DJ-1, also known as Parkinson's disease protein 7 (PARK7), an antioxidant, transcriptional co-activator, and molecular chaperone, presence in plasma neural-derived exosomes were significantly higher in PD patients (Zhao et al., 2018). Recently, it was described that exosomes derivated from saliva also contain α-syn and may be used as a potential biomarker in PD (Cao et al., 2019).

Is the secretion of exosomal α-syn an intercellular transmission mechanism that increases toxicity in the brain?, or could it be a cellular protective response against the intracellular accumulation of α-syn? This response has not been elucidated, and there is controversial literature about it. In this context, it has been described that exosomes isolated from brain tissue of patients with DLB injected into the brain of wild-type mice generate the misfolding of the endogenous α-syn protein (Ngolab et al., 2017). Notable, CSF exosomes from PD patients induce oligomerization of α-syn in a reporter cell line in a dose-dependent manner (Stuendl et al., 2016). About the secretion and uptake of α-syn *via* EVs in cultured cells, it has been reported that disease-causing mutants, as A53T α-syn, displayed increased association with EVs (Gustafsson et al., 2018). It has been described that γ-syn, another protein family member of synucleins, can be oxidized and initiate α-syn aggregation. γ-syn secreted in exosomes from neuronal cells can be transmitted to glial cells and cause the aggregation of intracellular proteins (Surgucheva et al., 2012).

Interestingly, pramipexole, an agonist of the dopamine receptor family, is used as a treatment for PD patients. After 12 weeks of pharmacological therapy, the patient's motor performance was statistically improved, and the α-syn content in serum exosomes was lower after the treatment (Luo et al., 2016). Although these results propose a correlation between the α-syn content in serum exosomes and motor symptoms, the mechanism to explain it is still unknown.

Not only neurons would participate in the transmission of α-syn exosomal since microglia also can capture exosomes from the plasma of patients with PD. α-syn induces an increase of exosomal secretion by microglia, and these exosomes showed a high level of MHC class II molecules and TNF-α (Chang et al., 2013). More recently, it was described that the secretion of exosomal human α-syn from the microglia could facilitate its aggregation (Guo et al., 2020) and propagation, possibly through dysregulation of autophagy (Xia et al., 2019).

On the other side, on physiological conditions, it is possible to detect monomers of α-syn, which are degraded by the ubiquitin-proteasome system (UPS; Bennett et al., 1999) and the chaperone-mediated autophagy (CMA; Cuervo et al., 2004; Vogiatzi et al., 2008; Mak et al., 2010). Oligomers, however, are efficiently degraded by the autophagy-lysosomal pathway (ALP; Ebrahimi-Fakhari et al., 2011). Both processes, exosomes secretion of α-syn and degradation by ALP, occur in a coordinate balance (Fussi et al., 2018; **Figure 1B**). Nevertheless, the mechanism involved in the balance of autophagy and α-syn exosomal secretion in neurons has not been elucidated.

IMPACT OF AUTOPHAGY-LYSOSOMAL PATHWAY IN PARKINSON'S DISEASE PROGRESSION

Autophagy (derived from the Greek words for "self" and "eating") is an evolutionarily conserved lysosomal pathway that digests long-lived proteins, protein aggregates, stress RNA granules, and abnormal cytoplasmic organelles. Based on the type of substrate, mode of cargo recognition, transport, and delivery to the lysosome, three types of autophagy have been described: microautophagy, CMA, and macroautophagy in PD (Martinez-Vicente and Cuervo, 2007; Kenney and Benarroch, 2015). During microautophagy, the cargo is taken into the lysosome or late endosome through its membrane invagination, being quickly degraded in the lysosomal lumen (Frake et al., 2015; Bento et al., 2016). In the CMA, the cargo containing an aminoacidic sequence binds to cytosolic chaperones, is recognized and imported into the lysosomal lumen by a receptor on the lysosomal membrane (Kaushik and Cuervo, 2008). Macroautophagy (hereafter referred to as autophagy or autophagy-lysosomal pathway) is a highly regulated mechanism that forms a double-membrane vesicle called the autophagosome to isolate the cargo that will be degraded (Bento et al., 2016). After maturation, autophagosomes fuse with lysosomes to degrade their content by the activity of lysosomal acid hydrolases (**Figure 1B**). Lysosomes' proper function is central to concluding several convergent pathways, including autophagy and endocytosis. Under basal conditions, autophagy is an active quality control process that prevents metabolic and oxidative stress in the cell by degrading aggregated proteins and damaged or dysfunctional organelles. Starvation-induced autophagy is a cellular response to nutrient deprivation that recycles macromolecules to offer substrates for metabolism (Mizushima et al., 2008).

In a pathological context, it has been found that selective autophagy contributes to the clearance of misfolded proteins involved in neurodegenerative diseases such as tau, SOD1, and α-syn (Vidal et al., 2014; Frake et al., 2015). Moreover, evidence has demonstrated a link between PD and mitophagy (selective mitochondrial autophagy). Mitophagy is mediated by binding selective autophagy receptors simultaneously to ubiquitinated proteins in the mitochondria surface and proteins from the autophagy machinery, such as the LC3-II family proteins (Pickles et al., 2018; Conway et al., 2020). The most studied mitophagy pathway is dependent on two proteins, PTEN-induced kinase 1 (PINK1) and Parkin (Narendra et al., 2010). In this pathway, PINK1 accumulates on depolarized mitochondria, triggering the translocation of Parkin from the cytosol, eliciting the ubiquitination of several mitochondrial proteins, including mitofusin 1 and 2 (MFN1 and MFN2), translocase of outer membrane 20 (TOM20), and voltage-dependent anion-selective channel 1 (VDAC1; Bayrhuber et al., 2008; Wang et al., 2011; Sarraf et al., 2013). Notably, mutations in *PINK1* and *Parkin*

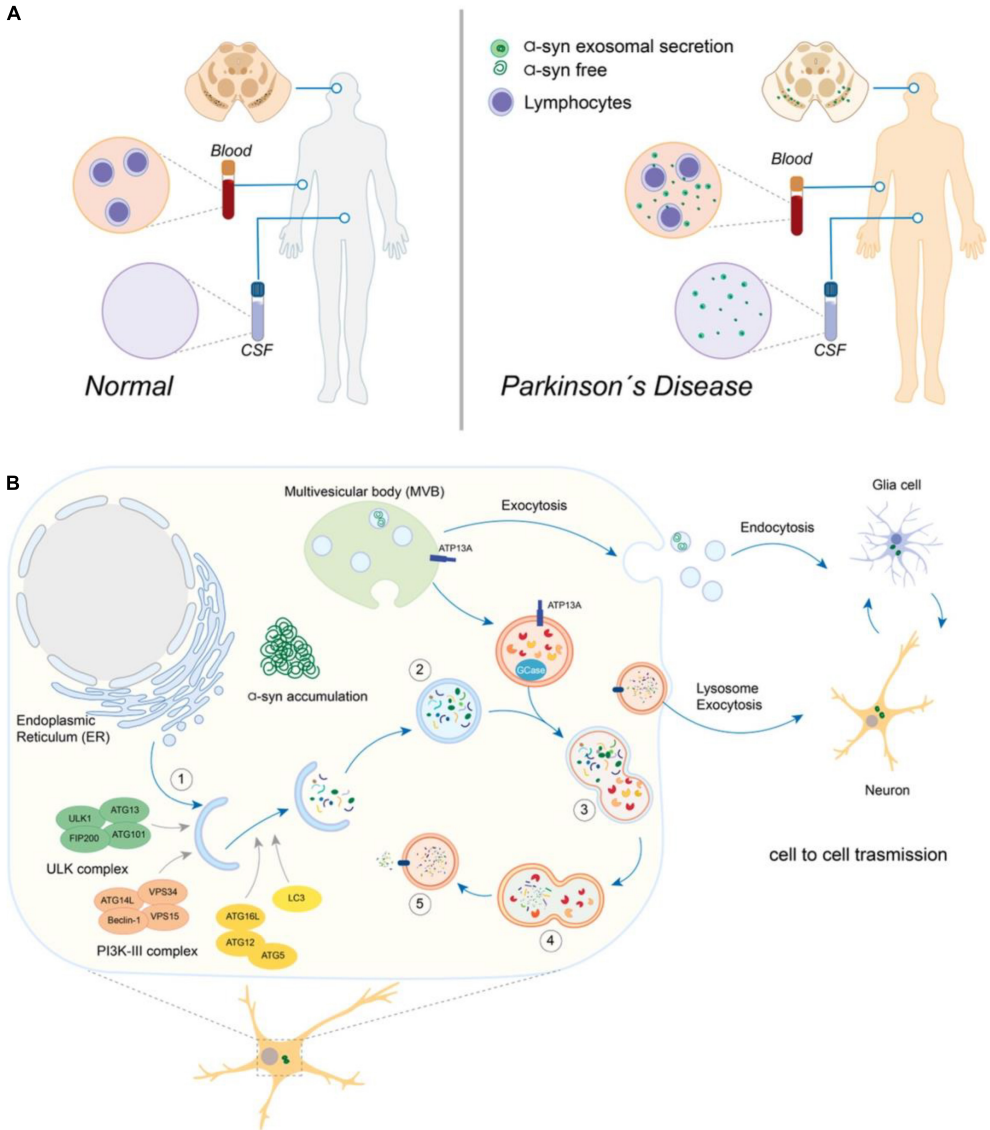

FIGURE 1 | Crosstalk between the autophagy-lysosomal pathway (ALP) and the exosomes secretion in Parkinson's disease. **(A)** The presence of soluble free or exosomes-containing α-synuclein (α-syn) derived from Substantia Nigra can be detected in cerebrospinal fluid (CSF) and blood samples from Parkinson's disease patients, potentially contributing to the early disease's diagnosis and progression monitoring. **(B)** Overview of the autophagy-lysosomal pathway (ALP) and exosomes secretion. In (1), the formation of the initial membrane that will originate the double-vesicle autophagosome (2) depends on several complex proteins' actions (shown in different colors). (3) The fusion of autophagosomes with lysosomes is a final step of the pathway, originating the autolysosome (4), where the substrates are finally degraded into their monomeric components that can be recycled back to the cytosol (5). Multivesicular bodies (MVB) originate the exosomes vesicles, which are secreted by exocytosis and participate in the cell-to-cell transmission of α-syn (neurons and glia cells).

are associated with familial parkinsonism, while the loss of PINK1 function induces oxidative stress and mitophagy (Valente et al., 2004; Dagda et al., 2009). Moreover, the parkinsonian neurotoxin MPP+ (the active metabolite of MPTP) induces autophagy and mitophagy depending on autophagy proteins ATG5, ATG7, and ATG8, but independently of the protein Beclin 1 (Chu et al., 2007).

Autosomal dominant mutations in the gene *LRRK2* encoding the protein leucine-rich repeat kinase 2 are among the most common causing familial PD (Zimprich et al., 2004). Mutations in *LRRK2* have been shown to reduce mitochondria trafficking in rat neurons (Godena et al., 2014; Hsieh et al., 2016), impair the mitophagy activity in PD-derived cells (Bonello et al., 2019; Wauters et al., 2020), and increase aggregation of α-syn in mice models and human iPSC-derived dopaminergic neurons (Bieri et al., 2019). In a recent work, researchers studied α-syn spreading levels in CSF from patients carrying different DLB and PD mutations using an α-syn real-time amplification assay (Brockmann et al., 2021). Interestingly, CSF samples from patients harboring mutations in *PINK1* or *Parkin* did not show

positive α-syn seeding activity. However, CFS samples from *LRRK2* PD patients (78%) showed an elevated α-syn positivity, only exceeded by CSF samples from DLB (100%) or PD (93%) patients carrying *GBA1* mutations (Brockmann et al., 2021). Notably, this higher α-syn seeding activity in CSF from *GBA1* patients was associated with lower levels of proteins related to α-syn clearance, including autophagy, lysosomal function, and endocytosis pathways in the same samples, suggesting *GBA1* mutations promote a negative correlation between α-syn accumulation and degradation pathways, mainly associated with lysosomal dysfunction (Brockmann et al., 2021). This work also found decreased levels of proteins from the UPS and neurosecretion processes in CSF from *GBA1*-carrying patients, which increase the urge to elucidate the mechanisms involved in this broad reduction in degradation pathways proteome (Brockmann et al., 2021) and to check if this data is reflected in CNS samples.

The failure of the protein quality control systems, especially lysosomal-dependent degradation, promotes the accumulation of α-syn (Desplats et al., 2009). Heterozygous mutations in the *GBA1* gene encoding lysosomal enzyme GCase are strong risk factors for PD (Avenali et al., 2020). GCase is an N-glycosylated protein synthesized and transported in vesicles from the ER-to-Golgi apparatus, where it is correctly folded through a maturation process before reaching the lysosomes. α-syn aggregation generated by wild-type *SNCA* triplication in PD patients iPSC-derived dopaminergic neurons was reported to disrupt the ER-GA trafficking by inhibiting the SNARE protein ykt6 (Cuddy et al., 2019), depleting lysosomes from acid hydrolases, and increasing the accumulation of insoluble immature GCase in ER (Stojkovska et al., 2021). Interestingly, using a pharmacological enhancer of ER proteostasis plus a farnesyltransferase inhibitor (FTI), which restores ykt6 activity, was reestablished GCase maturation and lysosomal activity, becoming a promising therapeutic strategy for future studies in synucleinopathies (Stojkovska et al., 2021).

Recent works have suggested that the secretion of exosomes containing α-syn could result as a protection mechanism against the blockage of autophagy-dependent α-syn clearance (Fussi et al., 2018). In particular, the silencing of ATG5, a key protein involved in the extension of the phagophore membrane in autophagic vesicles (Pyo et al., 2005), increases the secretion of α-syn *via* exosomes, which are associated with a decrease in cell death α-syn induced (Fussi et al., 2018). In accordance, the inhibition of lysosomal function in α-syn overexpressing neural cell lines generated an increase of exosomal secretion of α-syn, promoting a cell-to-cell transfer of α-syn (Alvarez-Erviti et al., 2011). Other evidence also shows that the ALP inhibition reduces intracellular α-syn while increasing the secretion of smaller oligomers, exacerbating the uptake, inflammation, and cellular damage (Poehler et al., 2014). Moreover, has been reported a secretion of aggregated α-syn by exosomes and Rab11a-associated pathways and by membrane shedding (Poehler et al., 2014). It was confirmed that the ALP inhibition promotes the release and transmission of α-syn *via* EVs with a hybrid autophagosome-exosome phenotype, increasing the ratio of extracellular α-syn/intracellular α-syn and its association with EV in neuronal cells (Minakaki et al., 2018). GCase loss-of-function was also associated with α-syn secretion. Studies in transgenic mice harboring the human mutation A53T in *SNCA* found that inhibition of GCase increases the exosome-associated α-syn oligomers release (Papadopoulos et al., 2018). The plasma exosomal/total α-syn ratio is associated with GCase activity, and it correlates with severity (motor deficiency) in PD patients (Cerri et al., 2018; Johnson et al., 2020), proposing the link between lysosomal dysfunction with increased exosome secretion. Similar results were reported in fibroblasts derived from PD patients with or without *GBA1,* in which defective GCase activity increased the release of exosomes (Cerri et al., 2021). Isolated exosomes from these cells caused increased levels of phospho-α-syn in SH-SY5Y recipient cells, overexpressing wild-type α-syn (Cerri et al., 2021). Interesting, this effect was not due to a seeding effect since fibroblasts are α-syn-free. The researchers hypothesize that fibroblast-derived from patients harboring *GBA1* mutations promote changes in the lipid composition of recipient cells, which may account for the increased phospho-α-syn, posttraductional modification that increases the formation of insolubleα-syn forms (Canerina-Amaro et al., 2019). Besides releasing exosomes, MVBs can be eliminated through the ALP by a direct fusion with lysosomes or autophagosomes (Fader et al., 2008; Vanlandingham and Ceresa, 2009; Szatmari et al., 2014; Teixeira et al., 2021). α-syn itself can disturb the ALP activity, promoting potential positive feedback to its secretion. Notably, α-syn fibrils have been shown to impair lysosomes' morphology from inside the organelle lumen, reducing the ALP-dependent clearance of aggregates and defective organelles. Moreover, lysosomes filled with α-syn fibrils can be transferred to neighboring cells through TNTs or secretion vesicles, contributing to the disease's spread (Dilsizoglu Senol et al., 2021). There is evidence for a loop between the lysosome and α-syn proteoforms (Wildburger et al., 2020).

Some genes encoding proteins involved in intracellular vesicle trafficking and lysosome transport are risk genes associated with PD (Abeliovich and Gitler, 2016; Mazzulli et al., 2016a). For example, Kufor-Rakeb syndrome (KRS) is caused by an autosomal recessive mutation in the *PARK9* gene encoding ATP13A2 (transmembrane lysosomal type 5 P-type ATPase protein) characterized by juvenile-onset parkinsonism. Interestingly, a mutation in this gene was described in a Chilean family patient for the first time (Ramirez et al., 2006). *PARK9* encodes a lysosomal ATPase involved in cation homeostasis, and its loss of function leads to lysosomal dysfunction (Gitler et al., 2009; Kong et al., 2014; Tsunemi et al., 2014). Interestingly, in *Caenorhabditis elegans* and dopamine cell culture models, it was described that ATP13A2 would have a protective role against the accumulation of misfolded α-syn and cellular toxicity (Gitler et al., 2009). However, the overexpression of ATP13A2 increases the release of exosomes, promoting the secretion of α-syn in primary cortical neurons (Tsunemi et al., 2014). The evidence suggests that the ATP13A2 protein regulates the release of α-syn *via* EVs through the modification of the biogenesis of exosomes by a functional interaction with the lysosomal sorting complex required for transport (ESCRT; Tsunemi et al., 2014). Additionally, the enhanced secretion of

exosome-associated α-syn may explain the increased viability in neurons of the SNpc in sporadic PD patients by overexpressing ATP13A2 (Kong et al., 2014). A recent work has shown that PD mutations in *ATP13A2* increase α-syn intracellular accumulation by impairing lysosome exocytosis using iPSC-derived neurons from PD patients (Tsunemi et al., 2019). The mechanism by ATP13A2 modulates lysosomal exocytosis is by mediating Ca^{2+} homeostasis in these organelles. Interestingly, a pharmacological agonist of the lysosomal Ca^{2+} channel, TRPML1, recovers lysosomal exocytosis, correcting α-syn secretion defects and decreasing intracellular accumulation in ATP13A2 patient neurons (Tsunemi et al., 2019).

What is the contribution of neighboring cells of neurons in PD? Microglia isolated from adult mice, in contrast to microglia from young mice, display phagocytosis deficits of free and exosome-associated α-syn oligomers (Bliederhaeuser et al., 2016). The neuronal α-syn secreted by exosomes or lysosomal exocytosis is partially endocytosed by astrocytes, which contribute to reducing the α-syn spread between neurons. Indeed, degradation of α-syn is more efficient in astrocytes than neurons (Tsunemi et al., 2020). However, the iPSC-derived astrocytic protection against the α-syn accumulation and propagation is partially lost by *ATP13A2* mutations, resulting in the increased accumulation and propagation of α-syn between neurons (Tsunemi et al., 2020), suggesting that astrocytic lysosomal dysfunction indirectly contributes to the α-syn neuronal pathology.

Moreover, impaired biogenesis of MVBs by a dominant-negative mutant of vacuolar protein sorting 4 (VPS4) interferes with the lysosomal targeting of α-syn and facilitates α-syn secretion (Hasegawa et al., 2011). The hypersecretion of α-syn in VPS4-defective cells was restored by the functional disruption of recycling endosome regulator Rab11a. VPS4, a master regulator of MVBs sorting, may serve as a determinant of lysosomal targeting or extracellular secretion of α-syn (Hasegawa et al., 2011). Another member of the endosomal protein sorting, VPS35, is also associated with PD (Vilarino-Guell et al., 2011). VPS35 is part of the retromer complex, which mediates the endosome-to-Golgi recovery of membrane proteins. The VPS35 D620N mutation causes an autosomal-dominant form of PD, and the cells expressing the mutant form have impaired autophagy. The defects in autophagy can be explained in part by the abnormal traffic of the transmembrane autophagy protein ATG9A (Zavodszky et al., 2014).

Another traffic protein associated with PD is Secretory Carrier Membrane Protein 5 (SCAMP5), a regulator of membrane trafficking enriched in the brain, identified as an autophagy inhibitor that promotes exosomal secretion of α-syn (Yang et al., 2017). SCAMP5 is a novel coordinator of autophagy and exosome secretion, induced under protein stress by Bafilomycin A1 to clear toxic proteins *via* the exosomes rather than ALP (Yang et al., 2017).

All these results support the idea that exists a connection between autophagy, lysosomal homeostasis, and α-syn exosomes secretion on the PD progression (Xu et al., 2018). Moreover, autophagy modification (gain and loss function) impacts exosomes released into the extracellular space *in vitro* (Hu et al., 2020). Although, the full mechanism underlying these processes *in vivo* and the effect on the progression of the PD remains poorly understood (**Figure 2**). Overall, several lines of evidence propose that a correction in lysosomal function can boost dopaminergic neurons' survival in PD, avoiding α-syn aggregation. However, it is open to whether the increase in the α-syn secretion, by exosomes or other vesicles kinds, can result in a progression spread of the disease in an *in vivo* long-term study.

CLINICAL ASPECTS OF AUTOPHAGY-LYSOSOMAL PATHWAY AND PARKINSON'S DISEASE PROGRESSION

As mentioned, oligomers and fibrils α-syn degradation is mediated by autophagy, connecting the role of lysosomes to the etiology/progression of PD (Webb et al., 2003; Lee et al., 2004). Concerning the above, the expression of genes from the autophagy pathway [UNC- 51- like kinase (ULK) 3, autophagy-related (Atg) 2A, Atg4B, Atg5, Atg16L1, and histone deacetylase 6] were evaluated in peripheral blood mononuclear cells (PBMCs) of patients with PD. Researchers observed a decrease in the expression of autophagy regulatory components in patients with PD, while they reported an increase of α-syn protein levels in PBMCs compared to controls (Miki et al., 2018). However, the comprehensive mechanisms of the dynamic interaction of ALP-MVBs for the secretion of α-syn *via* EVs have not been fully elucidated. It has been recently described that a significant percentage of the proteins detected in tissue-purified Lewy bodies from DLB patients and cytoplasmic glial inclusions (CGI) of oligodendrocytes from multiple systemic atrophy (MSA) patients are synaptic vesicle proteins, including CD9 associated with exosomes (McCormack et al., 2019). This fact suggests that the misfolding or accumulation of α-syn, characteristic of synucleinopathies, contributes to the vesicle-mediated transport of these protein inclusions (McCormack et al., 2019). A recent study demonstrated that the inhibition of dynamin-related protein 1 (Drp1) improved both mitochondrial function and autophagic flux in experimental models of α-syn (Fan et al., 2019). The following key step is to determine if the Drp1 inhibition confers neuroprotection through the abolished autophagic impairment induced by α-syn in *in vivo* models of PD.

Several pharmacological agents targeting ALP components, especially lysosomal function, are active research topics in preclinical and clinical phases to PD and other synucleinopathies. They include the previously mentioned FTI, which restores the SNARE ykt6 activity, reestablishing lysosomal hydrolases maturation, and finally, the lysosomal activity (Stojkovska et al., 2021) pharmacological agonists of lysosomal Ca^{2+} channel, TRPML1, which has been shown to restore lysosomal exocytosis, enhancing α-syn secretion and decreasing accumulation in *ATP13A2* patient iPSC-derived neurons (Tsunemi et al., 2019); or the ambroxol, a cough syrup approved by the FDA since 1971, which has been reported to reduce α-synuclein levels *in vitro* and *in vivo* (Migdalska-Richards et al., 2016), and to increase

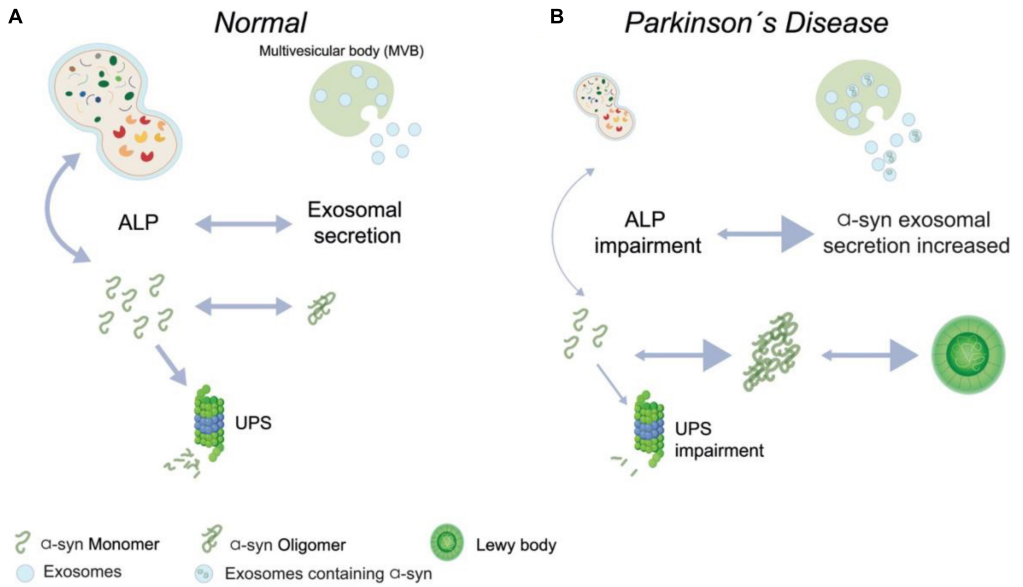

FIGURE 2 | Contribution of autophagy-lysosomal pathway and α-syn secreted in Parkinson's disease. **(A)** In normal conditions, it is possible to detect monomers of alpha-synuclein (α-syn), which are degraded by Ubiquitin Proteasome System (UPS), and oligomers of α-syn are efficiently degraded by Autophagy-Lysosomal (ALP) pathway or can be secreted by exosomes. Both degradation and secretion processes occur in a coordinate balance. **(B)** On Parkinson's Disease condition, abundant oligomers and fibrils of α-syn are formed, and it less degraded by autophagy due to an impairment of this pathway, and it is possible to observe an increased secretion of α-syn by exosomes.

GCase expression and activity (Migdalska-Richards et al., 2017). Ambroxol was shown to restore lysosomal exocytosis (Magalhaes et al., 2018) and promote ER folding. Recently, ambroxol treatment in PD patients harboring or not *GBA1* mutations has shown promising results regarding α-syn secretion in CSF (Mullin et al., 2020). However, although these drugs are promising hopes, they need to be tested in different mutations associated with PD, considering the broad clinical and physiological variability between them (Cerri et al., 2018; Johnson et al., 2020).

CONCLUSION AND PERSPECTIVES

This review summarized the antecedents that demonstrate altered autophagy in PD and some evidence that proposes a link between ALP components and the exosomal secretion of α-syn. To elucidate the mechanisms that explain the relation between ALP and the secretion of EVs in PD is still a field in study. However, some clues of the crosstalk between exosomes and autophagy have been proposed [review in Xu et al. (2018), Gudbergsson and Johnsen (2019), Buratta et al. (2020), Xing et al. (2021)].

The origin of extracellular vesicles can offer additional information. For example, mitochondrial-derived vesicles are a candidate as biomarkers in body fluids of PD patients may provide clues to understand the association between mitochondrial dysfunction and systemic inflammation in PD (Picca et al., 2019). Interesting proposals are in the therapy field based on autophagic degradation and exosomal secretion. The development of an α-syn nano-scavenger for PD capable of stimulating nuclear translocation of TFEB (master regulator of autophagy), promoting autophagy and calcium-dependent exosome secretion for the clearance of α-syn (Liu et al., 2020). While α-syn expression can be reduced by antisense oligonucleotides (ASOs), the big challenge is delivering ASOs efficiently and safely into the neurons. Exosomes can be a safe and highly effective ASO delivery method (Yang et al., 2021). Promising ALP and exosomal secretion research are developed on *in vitro* models. However, further validations in animal models and physiology-related conditions are required.

Recently, *in vitro* study reported that proteins from SARS-CoV-2, which causes COVID-19, interact with α-syn, speeding up the formation of amyloid plaques (Semerdzhiev et al., 2021) open an interesting research field associating virus infections with PD or other neurodegenerative diseases development.

AUTHOR CONTRIBUTIONS

DS, MC-O, JA, MN, and RV wrote and edited the manuscript. DS and RV prepared the figures. DS, MN, and RV planned the manuscript. All authors contributed equally to the critical reading of the final manuscript, including figures.

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REVIEW
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Insights Into the Role of Heat Shock Protein 27 in the Development of Neurodegeneration

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Small heat shock protein 27 is a critically important chaperone, that plays a key role in several essential and varied physiological processes. These include thermotolerance, apoptosis, cytoskeletal dynamics, cell differentiation, protein folding, among others. Despite its relatively small size and intrinsically disordered termini, it forms large and polydisperse oligomers that are in equilibrium with dimers. This equilibrium is driven by transient interactions between the N-terminal region, the α-crystallin domain, and the C-terminal region. The continuous redistribution of binding partners results in a conformationally dynamic protein that allows it to adapt to different functions where substrate capture is required. However, the intrinsic disorder of the amino and carboxy terminal regions and subsequent conformational variability has made structural investigations challenging. Because heat shock protein 27 is critical for so many key cellular functions, it is not surprising that it also has been linked to human disease. Charcot-Marie-Tooth and distal hereditary motor neuropathy are examples of neurodegenerative disorders that arise from single point mutations in heat shock protein 27. The development of possible treatments, however, depends on our understanding of its normal function at the molecular level so we might be able to understand how mutations manifest as disease. This review will summarize recent reports describing investigations into the structurally elusive regions of Hsp27. Recent insights begin to provide the required context to explain the relationship between a mutation and the resulting loss or gain of function that leads to Charcot-Marie Tooth disease and distal hereditary motor neuropathy.

Keywords: small heat shock protein 27 (Hsp27), Charcot-Marie-Tooth disease (CMT), distal hereditary motor neuropathy (dHMN), α-crystallin domain (ACD), heat shock protein

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INTRODUCTION

The proteome is a vast collection of proteins each of which contributes to the maintenance of cellular homeostasis (Kim et al., 2013; Bakthisaran et al., 2015; Webster et al., 2019). Diverse environmental and physiological stressors can lead to various deleterious effects including structural damage of proteins (Sun and MacRae, 2005; Kim et al., 2013; Webster et al., 2019). With so many threats overwhelming the integrity of the proteome it is imperative that the cell ensures a robust defense mechanism to keep its proteins structurally viable and functional

(Eyles and Gierasch, 2010; Kim et al., 2013). Cellular stressors such as heat shock can often result in partial protein misfolding that can then lead to various disorders including neurodegeneration (Eyles and Gierasch, 2010; Webster et al., 2019). The integrity of protein structure and function is maintained by a class of proteins known as molecular chaperones (Sun and MacRae, 2005; Bhatt et al., 2018; Rodriguez et al., 2020). These molecular chaperones sustain protein homeostasis through assistance with protein folding or the stabilization of partially misfolded proteins (Sun and MacRae, 2005; Kim et al., 2013). A subclass of molecular chaperones, known as heat shock proteins, can assist in the folding of nascent polypeptides or aid in the stabilization and the refolding of non-native proteins (van Montfort et al., 2001; Fu, 2014; Enriquez et al., 2017; Bhatt et al., 2018). They are also involved in other pathways such as degradation of irreversibly damaged proteins and unfolding of other proteins for translocation across a membrane (Haslbeck et al., 1999; Shashidharamurthy et al., 2005; Richter et al., 2010).

Heat shock proteins are broadly conserved amongst six major classes. These include Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, and the small heat shock proteins (Sun and MacRae, 2005; Richter et al., 2010; Fu, 2014; Bakthisaran et al., 2015; Enriquez et al., 2017; Rodriguez et al., 2020). Small heat shock proteins (sHsps) are molecular chaperones that do not require the hydrolysis of ATP (Bova et al., 2002; Sobott et al., 2002). Their primary role is to maintain a partially denatured protein in a folding-competent state to protect it from irreversible and detrimental aggregation (Borrelli et al., 2002; Bakthisaran et al., 2015). Small heat shock protein 27 is a ubiquitously expressed chaperone involved in many physiological and metabolic pathways (Parcellier et al., 2005). In addition to protein folding, it has been implicated in the regulation of redox states, stabilization of the cytoskeleton, anti-apoptotic activity, and in important interactions with transcription factors and mRNA processing proteins (D'Ydewalle et al., 2011; Chalova et al., 2014; Fu, 2014). Hsp27 (HspB1) belongs to the subgroup of small heat shock proteins that encompass 10 human sHsps. These small heat shock proteins are encoded by the genes HspB1–10 and have been categorized into two main classes (Bakthisaran et al., 2015; Muranova et al., 2015; Webster et al., 2019). Class I sHsp include HspB1 (Hsp27), HspB5 (α-B-crystallin), HspB6 (Hsp20), and HspB8 (Hsp22; Kappe et al., 2003; Bakthisaran et al., 2015). These sHsps are widely distributed with expression in nearly all cells and tissues where they perform various roles (Kappe et al., 2003; Mainz et al., 2015; Mymrikov et al., 2017). The second class of small heat shock proteins includes HspB2, HspB3, HspB4 (αA-crystallin), HspB7 (cvHsp), HspB9, and HspB10 (ODF1) and are restricted to certain types of tissues such as the eyes, heart, and testes (Kappe et al., 2003; Bakthisaran et al., 2015).

Small heat shock proteins are characterized by a conserved sequence of approximately 80–100 residues known as the α-crystallin domain (ACD; Fu, 2014; Bakthisaran et al., 2015; Muranova et al., 2015; Clouser et al., 2019). The ACD is flanked by a variable hydrophobic amino terminal region (NTR) and a short flexible c-terminal region (CTR; Garrido et al., 2012; Hochberg et al., 2014; Rajagopal et al., 2015; **Figure 1**). Small heat shock proteins form dimers *via* β-sheets formed by the ACD. These dimers serve as building blocks of class 1 sHsp that organize into large oligomeric complexes that are stabilized by their N-terminal and C-terminal regions (Lelj-Garolla and Mauk, 2012; McDonald et al., 2012; Aquilina et al., 2013; Rajagopal et al., 2015). The dimers are thought to be the smallest functional form of small heat shock proteins (Lambert et al., 1999; Hayes et al., 2009). These complexes engage in rapid dimer exchange creating a dynamic equilibrium between large multimers of various sizes and dimers (Bova et al., 2000, 2002; Sobott et al., 2002; Rajagopal et al., 2015; **Figure 2**). The stress pathway activates p38 mitogen-activated protein kinase, which in turn phosphorylates mitogen-activated protein kinase activated protein kinase (MAPKAPK2; Kostenko and Moens, 2009; Bakthisaran et al., 2015; Lang et al., 2021). Phosphorylation by MAPKAPK2 shifts the equilibrium to small oligomers and dimers whereas protein phosphatase 2A activity reverses this process and favors the formation of large multimers (Cairns et al., 1994; Kostenko and Moens, 2009; Katsogiannou et al., 2014; Bakthisaran et al., 2015; **Figure 2**). This system might be a way to store large amounts of Hsp27 to quickly react to stress without the need for *de novo* protein synthesis (Peschek et al., 2013).

Small heat shock proteins can be perturbed by a myriad of inherited and sporadic mutations that result in the formation of neurological disorders. For example, various missense mutations in the small heat shock protein 27 (Hsp27) cause the neuropathies Charcot-Marie-Tooth Disease Axonal Type 2F (CMT2F) and Distal Hereditary Motor Neuropathy 2B (dHMN2B; Houlden et al., 2008; Tanabe et al., 2018). These neuropathies are commonly inherited disorders that exclusively affect the peripheral nervous system (Holmgren et al., 2013). They are closely related in their key features of disease progression and only differ by the point mutation that leads to the respective disease. CMT2F is a subset of the more broadly categorized forms of CMT, specifically characterized by axonal degeneration of the peripheral nerves (Ismailov et al., 2001; Schwartz, 2019). The characteristic symptom of CMT2F includes the slow progressive loss of motor function manifested as weakness and muscle atrophy of the distal limbs (Evgrafov et al., 2004; Gentile et al., 2020). Additionally, there can be decreased tendon reflexes of the ankle and legs, as well as physical deformities of the feet (Webster et al., 2019; Gentile et al., 2020). In cases of sensory neuron involvement, afflicted individuals lose pain and temperature sensation (Szigeti and Lupski, 2009). The disease, although progressive over many years, does not affect the life span (Schwartz, 2019). Individuals with CMT2F typically experience the onset of symptoms during adulthood and may require treatment through physical therapy, mobility aids, orthopedic shoes, and/or surgery (Evgrafov et al., 2004). More severe forms of CMT2F can result in hearing loss and vocal cord impairment, in addition to acute distal limb pain (Bakthisaran et al., 2015).

While CMT2F manifests as a motor and sensory neuropathy, Distal Hereditary Motor Neuropathy Type II (dHMNIIB) exclusively affects motor neurons (Geuens et al., 2017; Schwartz, 2019). dHMNIIB clinically presents similarly to CMT2F as they are related diseases caused by some of the same mutations (Tanabe et al., 2018). Individuals experience lower limb weakness

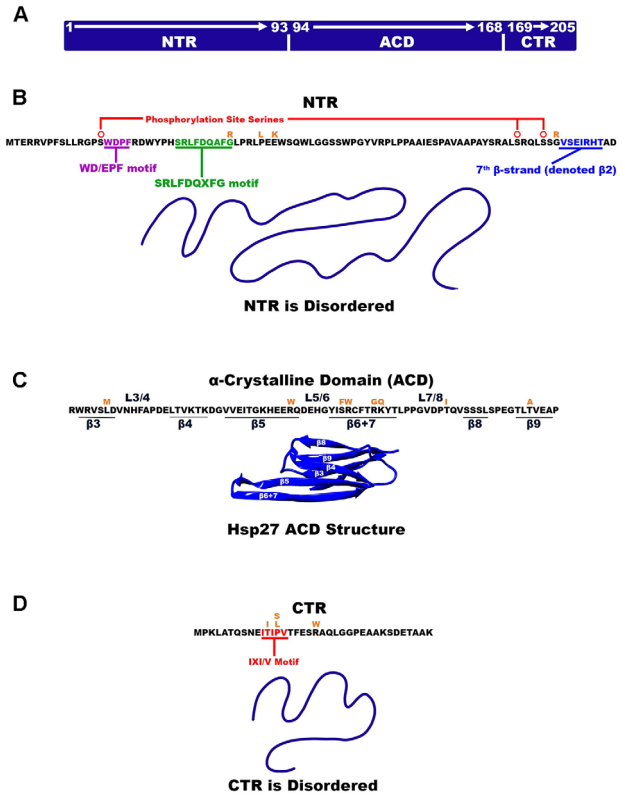


FIGURE 1 | Hsp27 Structural organization. **(A)** Hsp27 is made up of three regions where each region is identified as NTR, ACD, and CTR. The residue numbers that make up each region are also denoted. **(B)** The amino acid sequence of the disordered NTR is listed and the two motifs WD/EPF and SRLFDQxFG are highlighted with red and green, respectively. β-strand β-2 is highlighted in cyan. **(C)** The ACD amino acid sequence is listed as well as the location of β-strands and their connecting loops. The ACD is the only part of Hsp27 that is ordered (PDB 2N3J). **(D)** Hsp27 disordered CTR highlighting the IXI/V motif in red. Amino acid mutations are illustrated in orange above the amino acid being mutated.

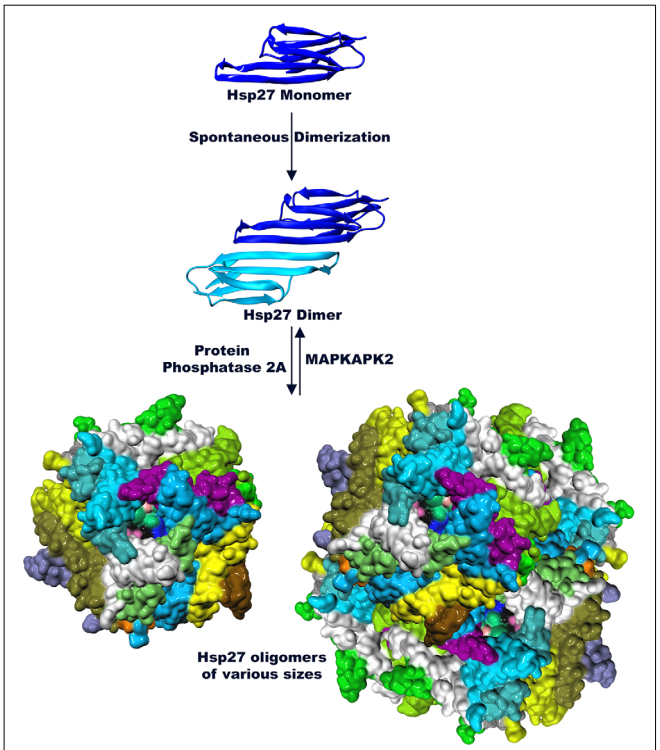


FIGURE 2 | Hsp27 is organized into higher order structures (PDB 6DV5 and 2N3J). The monomer of Hsp27 assembles into dimers *via* the ACD. Dimers in turn interact with each other to form large highly dynamic multimers. Phosphorylation by MAPKAPK2 dissociates oligomers of Hsp27 and favors dimer formation while Protein Phosphatase 2A shifts the equilibrium towards multimer formation.

due to muscle loss and deteriorating leg mobility (Dierick et al., 2007; Chung et al., 2008; Tanabe et al., 2018). The typical onset of the disease occurs in individuals between the ages of 15 and 45 and is a progressive disease that severely compromises the quality of life (Dierick et al., 2007; Chung et al., 2008; Schwartz, 2019). Both CMT2F and dHMNIIB have autosomal dominant patterns of inheritance indicating that only one copy of a mutated gene is sufficient to cause disease (Evgrafov et al., 2004; Schwartz, 2019). These neuropathies are described as being clinically and genetically heterogenous (Ackerley et al., 2006; Dierick et al., 2007; Echaniz-Laguna, 2015; Tanabe et al., 2018). This means that the same mutation can result in different clinical presentations and different mutations can manifest as the same disease, further complicating the understanding of the pathomechanisms. There are many mutations in Hsp27 that have been implicated in hereditary neuropathies (Muranova et al., 2019; Vendredy et al., 2020). A number of these mutations result in either CMT2F, dHMN2B, or both. There have been several reports linking mutated Hsp27 to disturbances in the cytoskeletal components of axons (Almeida-Souza et al., 2011; D'Ydewalle et al., 2011; Nefedova et al., 2017). Several substrates of Hsp27, such as Tau and α-synuclein, are involved in stabilizing cytoskeletal components and vesicle trafficking (Abisambra et al., 2010; Barbier et al., 2019; Baughman et al., 2020). These are processes that are required for normal motor neuron functioning (Abisambra et al., 2010; Barbier et al., 2019). These relationships have been implicated as indirect causes for many neurodegenerative diseases including CMT2F and dHMN2B (Webster et al., 2019; Muranova et al., 2020; Vendredy et al., 2020).

Hsp27 has been suspected in the progression of other neurodegenerative disorders such as Alzheimer's and Parkinson's disease (Zhang et al., 2014; Hu et al., 2021). They are the top two most common neurological illnesses affecting roughly 12% of people over 60 years of age (Hu et al., 2021). Alzheimer's disease (AD) is also a progressive neurodegenerative disorder localized to the brain, mainly affecting neurons of the cortex (Nussbaum and Ellis, 2003). The classical manifestations of Alzheimer's are an accumulation of amyloid-β plaques and neurofibrillary tangles of hyperphosphorylated tau (Nussbaum and Ellis, 2003). Alzheimer's is the number one cause of dementia and severely diminishes the quality of life of those affected. Parkinson's disease (PD) results in the irreversible degeneration of dopaminergic neurons in the substantia nigra

and includes the aggregation of α-synuclein contained within Lewy bodies and neuritis (Nussbaum and Ellis, 2003; Poewe et al., 2017). Parkinson's disease, like Alzheimer's, can also cause dementia in addition to the classical parkinsonian symptoms including tremors, rigidity, and bradykinesia (Williams and Litvan, 2013). Recently increased attention has targeted the involvement of Hsp27 in the progression of these neurological diseases from a therapeutic standpoint (Venugopal et al., 2019; Baughman et al., 2020; Vicente Miranda et al., 2020; Navarro-Zaragoza et al., 2021). The substrates of Hsp27 such as Tau and α-synuclein are major components in the pathological progression of both AD and PD. Although there is no evidence to suggest that mutations of Hsp27 are linked to AD and PD, the faulty chaperone may contribute to disease by ineffective regulation of these substrates.

Features of the Small Heat Shock Protein 27 Regions

Three major structural regions of Hsp27 can be defined by the structural and functional distinctions between them. The N-terminal region (NTR) of Hsp27 is composed of the first 93 residues and nearly 50% of the Hsp27 primary sequence (Clouser et al., 2019; **Figures 1A,B**). The NTR is inherently disordered and composed of predominantly hydrophobic and aromatic residues. It is thought to be primarily responsible for driving/stabilizing higher order multimers of Hsp27 (Bakthisaran et al., 2015). A less conserved region within the NTR is the WD/EPF motif made of residues 16-WDPF-19 that have been shown to correlate with protein chaperoning activity (Thériault et al., 2004; Lelj-Garolla and Mauk, 2012). Hsp27 is phosphorylated *via* the p38 MAPK pathway by MAPKAPK2, which occurs at serine residues 15, 78, 82 of the NTR (Lambert et al., 1999; Shashidharamurthy et al., 2005; **Figure 1B**). The phosphorylation site at serine-15 precedes the NTR WD/EPF domain while phosphorylation sites 78 and 82 reside near the end of the NTR (Lelj-Garolla and Mauk, 2012). In its unphosphorylated state Hsp27 exists as a large polydisperse oligomeric species with a molecular weight of up to ∼650 kDa (Rogalla et al., 1999). Experimental evidence has demonstrated that tunability of Hsp27 oligomeric size can be achieved through stepwise phosphorylation of the three phosphorylation sites (Hayes et al., 2009; Jovcevski et al., 2015). Phosphorylation of all three sites yields smaller oligomers and dimers whereas phosphorylation of one or two sites results in intermediately sized oligomers (Hayes et al., 2009; Jovcevski et al., 2015). At residue positions, 26–34 and near the center of the NTR is the conserved SRLFDQxFG motif (Pasta et al., 2003). Truncation studies have confirmed that this motif plays a large part in the production and stability of higher order multimers (Pasta et al., 2003). The NTR appears to be a highly flexible region that can adopt several conformations to facilitate oligomerization.

The Hsp27 α crystalline domain (ACD) is comprised of residues 94–168 that are arranged into six β-strands that form two β-sheets (Baranova et al., 2011; Alderson et al., 2019; Nappi et al., 2020; **Figure 1C**). This immunoglobulin-like fold of the ACD is conserved amongst various organisms in both sequence and structure (Weeks et al., 2018; Clouser et al., 2019).

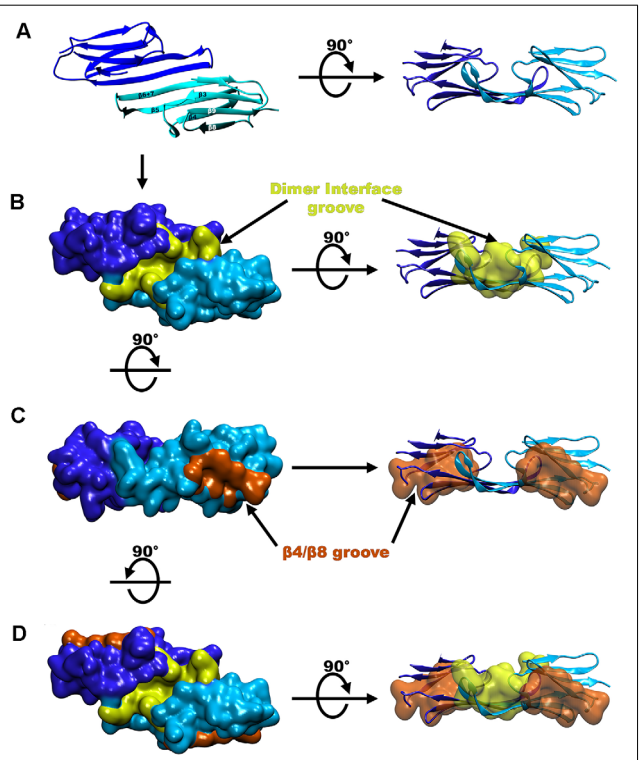


FIGURE 3 | Groove locations in the crystal structure of Hsp27. **(A)** A ribbon diagram of the Hsp27 dimer (PDB 4MJH). **(B)** Hsp27 structure seen in panel **(A)** but in surface representation where the dimer interface groove is shaded yellow. **(C)** Hsp27 β4/β8 groove (colored in orange). **(D)** Representation of the three grooves on a dimeric unit colored the same as in panels **(B,C)**.

The β-strands denoted as 4, 5, and extended strands 6 + 7 make up one β-sheet, whereas β-strands 3, 8, and 9 form another β-sheet (Hochberg et al., 2014; Klevit, 2020). Residues on the carboxy-terminal side of the NTR (84–91) periodically form a seventh β-strand (denoted β2) that integrates into the β3/β8/β9 β-sheet (Collier et al., 2019; Klevit, 2020; **Figure 1B**). The driving force for Hsp27 dimerization is hydrogen bonding and electrostatic interactions between ACDs. This occurs by pairwise alignment of the extended β6 + 7 strands in an antiparallel 2 register (AP2) creating the dimer interface and an extended β-sheet (Aquilina et al., 2013; Rajagopal et al., 2015; Clouser et al., 2019; Boelens, 2020; **Figure 3A**). The extended β-sheet is the sum of two β6 + 7 containing β-sheets forming one longer extended symmetry related β-sheet. The extended β-sheet and two β3/β8/β9 β-sheets from each monomer create a groove that is located right above the dimer interface and between the two β3/β8/β9 β-sheets, therein described as the dimer interface groove (Klevit, 2020; **Figure 3B**). In total, 18 residues with their side chains pointing inward, create the dimer interface groove. Eight of these residues are from each β6 + 7 strand and 10 are from loop regions that connect the β-strands 3 and 4 (L3/4) and β-strands 5 and 6 + 7 (L5/6; Klevit, 2020).

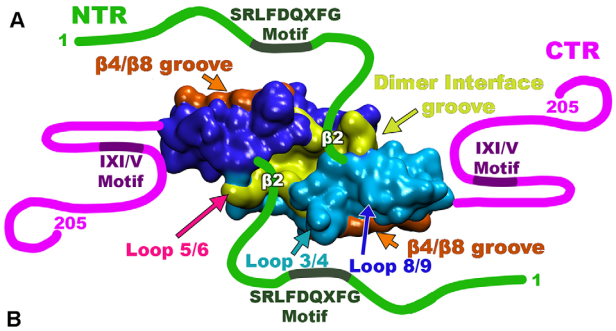
Another structurally notable feature of the ACD is the β4/β8 groove which includes a hydrophobic pocket centered between β-strands 4 and 8 and is located on the outer edge of

the ACD β-sandwich (Ehrnsperger et al., 1997; Klevit, 2020; Reid Alderson et al., 2021; **Figure 3C**). Therefore, within a dimer of Hsp27 there are two hydrophobic grooves located at the edges of each monomer and one shared dimer interface groove created by the linkage of two monomers (Clouser et al., 2019; Boelens, 2020; **Figure 3D**). These grooves provide regions for several client protein and oligomer binding opportunities and will be discussed later in this review.

The C-terminal region (CTR) and extension make up the remaining 37 residues of the Hsp27 primary sequence (**Figure 1A**). The CTR has an abundance of polar and charged residues and is partly responsible for oligomer formation and is thought to aid in the solubility of Hsp27 oligomers and or oligomer/substrate complexes (Pasta et al., 2004; Chalova et al., 2014; Carver et al., 2017; Boelens, 2020). The CTR contains a well-conserved region known as the IXI/V motif at residue positions 179–183 characterized by alternating isoleucine and valine residues with the central residue being a canonical proline (Lelj-Garolla and Mauk, 2012; Carver et al., 2017; **Figure 1D**). This motif contributes to the stabilization of oligomers by interlinking adjacent dimers. The C-terminal extension consists of the last 22 residues and does not contain any secondary structure (Alderson et al., 2017; Carver et al., 2017). The extension including the IXI/V motif is inherently disordered and highly mobile (Alderson et al., 2017). This is partly due to cis-trans isomerization about the 182 and 194 proline residues which causes changes in the adopted disordered states (Alderson et al., 2017). Interestingly, however, the residues near these pralines were shown to convert into a low residual β-strand structure which is thought to have implications in regulating oligomerization stability (Alderson et al., 2017).

Hsp27 NTR ACD and CTR Interact to Form Higher Order Complexes

Recent studies have revealed that the involvement and dynamics of the NTR in oligomer formation is far more complicated than originally thought (Clouser et al., 2019; Collier et al., 2019; Nappi et al., 2020). Various techniques including NMR, HX-MS, and modeling were employed to characterize the NTR of Hsp27 (Clouser et al., 2019). These revealed that various hydrophobic peptide regions of the NTR make multiple contacts with two grooves of the ACD (Clouser et al., 2019). Using six truncated variations of the NTR, a map of the binding combinations of NTR and ACD displayed four separate interactions (Clouser et al., 2019). First, the distal region of the NTR that encompasses the first 13 residues interacts with the β4/β8 groove of the ACD (**Figure 4**). This interaction is thought to be the result of alternating hydrophobic residues on the distal region of the NTR (6-VPFSLL-11) considered to be a motif that can bind the β4/β8 groove (Clouser et al., 2019). The next set of residues of the NTR, that contains serine-15 and the WD/EPF motif (12–27), was shown to bind a negatively charged area made of loops L3/4 and L5/6 (**Figure 4**; Clouser et al., 2019). Interestingly, serine residues 78 and 82, although distant in sequence, were also found near this NTR binding region (Clouser et al., 2019). It is suggested that this phosphorylated serine congregation facilitates the regulation of phosphorylation (Clouser et al., 2019).



The regions displayed in the figure have been determined by peptide mapping and interactions as listed below can occur within the same dimer and between dimers to form oligomers.

ACD Groove/Pocket	Disordered Binding Region		
Dimer Interface Groove	NTR 25-37 (SRLFDQXFG motif)	NTR 74-91	NTR 85-91 (β2)
β4/β8 Groove	NTR 1-13	NTR 85-91 (β2)	CTR 179-183 (IXI/V motif)
Pocket created by Loop 3/4 and Loop 5/6	NTR 12-27		
Pocket created by Loop 3/4 and Loop 8/9	NTR 76-85		

FIGURE 4 | Map of Hsp27 interacting regions. **(A)** Each of the monomers is depicted as either blue or cyan with the dimer interface groove in yellow and the β4/β8 groove for each monomer in orange. Disordered regions are drawn as either green or magenta lines with the binding motifs as labeled. Important interaction loops are indicated by arrows. **(B)** The table at the bottom lists each of the disordered binding regions and the groove/pocket that bind as described by peptide mapping.

Additional negative charges, introduced by phosphorylation, disturb the region, and promote dissociation of the NTR (12–27) from the negatively charged loops (Hayes et al., 2009; Clouser et al., 2019).

The remaining segments of the NTR (residues 25–37 and 74–91) are found to bind the groove created by the dimer interface (**Figure 4**). For example, the NTR (25–37) contains the conserved SRLFDQxFG motif (Pasta et al., 2003; Clouser et al., 2019). This motif binds at the dimer interface groove much like the NTR (74–91) that contains the phosphorylation site serine residues (Klevit, 2020). This suggests that at any given time, several regions of the NTR may be bound to different grooves within the ACD. Another study proposed an additional NTR-ACD binding combination (Collier et al., 2019). Using a previous structure and co-crystallization strategy, an ACD construct was built that included NTR phosphorylation site serines 78 and 82 (Collier et al., 2019). The β2 strand of Hsp27 (residues 84–93) transiently binds the adjacent β3 strand in an antiparallel orientation (Clouser et al., 2019; Collier et al., 2019; Klevit, 2020). The presence of the β2 strand, therefore, alters the space allotted within the dimer interface groove (Collier et al., 2019; Klevit, 2020). Various peptide mimics of the NTR crystallized with an ACD construct (84–171) confirm a hydrogen bonding network between the β2 and β3 strands (Collier et al., 2019). Additionally, a third Hsp27 binding combination has revealed that the β2 strand becomes extended and transiently binds the β4/β8 groove of an adjacent dimer (**Figure 4**; Collier et al., 2019). As mentioned previously, the NTR

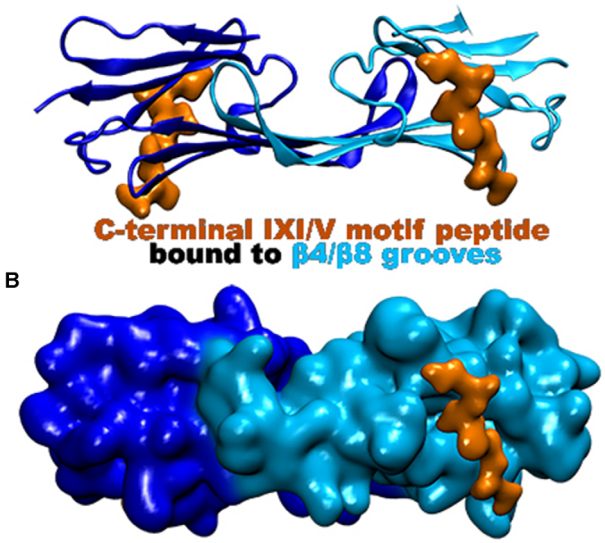


FIGURE 5 | Hsp27 crystal structure (PDB 4MJH) co-crystallized with peptide mimic of the IXI/V motif. The peptide (colored in orange) was found bound to the β4/β8 groove as indicated. **(A)** Ribbon representation of the dimer illustrating the location of the two bound peptides. **(B)** Surface representation of the dimer that better illustrates the β4/β8 groove with the bound peptide.

(12–27) containing serine 15, binds a space between negatively charged loops L3/4 and L5/6. This was found to perturb the phosphorylation site residues S78 and S82 indicating that the three phosphorylation sites are close (Clouser et al., 2019). This is confirmed by the crystal structure (6GJH) revealing that a peptide segment containing the phosphorylation site serines 78 and 82 binds a pocket created by loops L3/4 and L8/9 within the ACD (**Figure 4**; Collier et al., 2019). Therefore, the binding pockets for the NTR regions share the center L3/4 loop indicating that the phosphorylation site serines 15, 78, and 82 are close in space. Recently, an x-ray structure defined a spherical Hsp27 oligomer complex composed of 12 dimers (Nappi et al., 2020). The structure revealed that serine residues 78 and 82 from each monomer share the same area. Collectively, these studies reveal that between adjacent dimers, six phosphorylation sites are arranged near each other. This supports the claim that charge repulsion is the driving force of oligomer dissociation that results in a molecule with increased flexibility (Collier et al., 2019).

The CTR of the small heat shock proteins forms transient interactions with regions on the ACD (Pasta et al., 2004). As seen with many sHsp the IXI/V motif within the CTR fits into the hydrophobic β4/β8 groove in an antiparallel orientation (**Figure 5**; Clouser et al., 2019; Baughman et al., 2020). This interaction is like the NTR distal motif and the NTR β2 strand binding within the groove (**Figure 4**). The outer residues of the IXI/V motif fit into the holes of the hydrophobic groove due to the kink created by the center proline-182 residue (Freilich et al., 2018; Klevit, 2020). This is described as an extended formation of the CTR in the trans-conformation as the canonical proline can undergo cis-trans isomerization about the I-P peptide bond (Alderson et al., 2017). The interaction is found to occur within the same dimeric subunit and between adjacent dimers as well (Reid Alderson et al., 2021). The lowly populated residual β-strand structure may further encourage binding between the ACD and CTR and other binding partners (Alderson et al., 2017). The IXI/V motif is considered a short linear motif (SLiM) that modulates interactions between proteins (Reid Alderson et al., 2021). In the case of Hsp27, those interactions are between the CTR, the ACD, and possibly client proteins (Reid Alderson et al., 2021). A crystal structure of the truncated Hsp27 ACD co-crystallized with a peptide mimicking the C-terminal IXI/V motif has been solveds (**Figure 5**; Hochberg et al., 2014). This assembly shows the C-terminal SLiM in an antiparallel alignment to the β8 strand. The binding of the CTR IXI/V motif within the hydrophobic groove of the ACD acts to further stabilize high molecular weight homo-oligomers of Hsp27. It accomplishes this by transiently linking neighboring dimers indicating that the population of bound and unbound IXI/V motifs is mixed (Boelens, 2020). The binding combinations for these regions occur within a dimer and between adjacent dimers. The transient nature of these interacting regions creates a flexible protein that is unexpectedly stabilized. Hsp27 is a dynamic protein that is continuously exchanging its binding regions allowing for substrate capture.

Hsp27 Regions Involved in Substrate Capture

The three regions of Hsp27 have distinct roles in substrate capture (Jaya et al., 2009; Hochberg et al., 2014; Mainz et al., 2015). The ACD is thought to bind proteins that aggregate *via* the amyloid fibril aggregation pathway (Bakthisaran et al., 2015; Mainz et al., 2015; Freilich et al., 2018; Santhanagopalan et al., 2018; Webster et al., 2019). Client proteins that aggregate *via* this mechanism tend to form β-sheet conformations that resemble β-amyloid fibrils so the attraction between the ACD and the amyloid fibril-like substrate is robust (Mainz et al., 2015; Liu et al., 2020). The neuronal protein α-synuclein is an established substrate of Hsp27 that aggregates *via* the amyloid fibrillar pathway. It was found that full-length Hsp27 and truncated ACD transiently bind monomers of α-synuclein preventing their formation into fibrils (Cox et al., 2016, 2018; Jia et al., 2019). The truncated ACD displayed a lower binding affinity for α-synuclein monomers than the WT indicating that the α-synuclein monomer binding is in large part through the NTR (Jia et al., 2019). Additionally, the isolated ACD of Hsp27 cannot bind to α-synuclein fibrils that are already aggregated (Cox et al., 2018; Selig et al., 2020). Co-sedimentation experiments of Hsp27 lacking the CTR and in the presence of α-synuclein fibrils confirmed that the CTR is required to bind the fibrils (Selig et al., 2020). Therefore, involvement of the NTR and CTR is required when binding fibrillar aggregates of α-synuclein to prevent their elongation (Cox et al., 2018; Selig et al., 2020). These experiments suggest that the ACD alone is not sufficient to protect against amyloidogenic aggregation and that the involvement of all three regions is required.

Further evidence suggests that there is a strict interplay between the three Hsp27 regions to regulate chaperone activity

(Baughman et al., 2018, 2020; Freilich et al., 2018). It has been observed that Hsp27 binds Tau, another protein that aggregates *via* the amyloidogenic pathway (Abisambra et al., 2010; Baughman et al., 2018, 2020; Freilich et al., 2018). This is mediated by an interaction between an IXI/V motif of Tau and the ACD hydrophobic groove suggesting that the groove provides protective benefits against fibril formation (Freilich et al., 2018). However, this study also revealed that using truncated forms of Hsp27 lacking the NTR completely abolished this protective activity, further complicating the mechanism by which Hsp27 binds Tau (Freilich et al., 2018). One study suggested that the chaperone activity of Hsp27 extends beyond the ACD hydrophobic groove and is actually dependent on interactions of NTR to client protein (Baughman et al., 2020). It was subsequently determined that a terminal peptide representing the first 13 residues of the Hsp27 NTR binds the ACD hydrophobic groove that binds Tau 50 residues downstream of the highly disordered NTR region (Clouser et al., 2019). These experiments demonstrate a possibility that a binding competition for the ACD groove exists beyond IXI/V-containing proteins such as Hsp27 CTR, Tau, PCBP1, and others. Mutation of the B4/B8 hydrophobic groove to discourage binding between the groove and any IXI/V motif showed an unexpected increase in chaperone activity towards Tau (Freilich et al., 2018). This suggests that the ACD region is not the actual site for chaperoning activity (Baughman et al., 2020). Instead, there may be a competition between the NTR, CTD, and other IXI/V containing entities for the hydrophobic groove of Hsp27. Therefore, the role of the ACD hydrophobic pocket may be to act as a regulatory limiting factor that controls the extent to which Hsp27 binds and chaperones proteins (Chalova et al., 2014; Freilich et al., 2018; Santhanagopalan et al., 2018). In summary, Hsp27 can protect a large array of structurally diverse client proteins by utilizing its dynamic conformational variability. Mutations of Hsp27 can easily disturb this tightly regulated balance and lead to neurodegenerative disorders.

Missense Mutations Associated With CMT2F and dHMN2B

Mutations within all three regions of Hsp27 are implicated in the neurodegenerative disorders CMT2F and dHMN2B (Arbach et al., 2017; Muranova et al., 2019, 2020; Vendredy et al., 2020). As previously mentioned, there is an abundance of clinical and genetic overlap between the two neuropathies further complicating the elucidation of disease progression. The NTR mutants (**Table 1**) are associated with dHMN and cause overstabilization of oligomers accompanied by a slight increase in size (Muranova et al., 2015). These mutants have decreased chaperoning activity and are resistant to phosphorylation (Nefedova et al., 2013b; Muranova et al., 2015). The NTR is a dynamic region that is constantly undergoing conformational modifications to accommodate oligomerization and substrate binding. Amino acid changes within this region directly affect the interaction of the NTR with the ACD. The effects are either enhanced or diminished binding to the ACD, both of which have negative consequences that can have a direct impact on the oligomerization, phosphorylation, and chaperoning activity since the three are interdependent. Furthermore, mutations of the NTR could potentially result in diminished chaperone activity toward Tau and α-synuclein. Experimental evidence suggests that the NTR is critical in the effective chaperoning of Tau and α-synuclein (Baughman et al., 2020; Selig et al., 2020). Perhaps disease mutations of the NTR could result in diminished chaperone activity of these proteins aggravating the progression of AD and PD. Several models demonstrate that Hsp27 is critical in preventing the formation of α-synuclein inclusions further supporting the notion that ineffective chaperoning will exacerbate disease progression (Cox et al., 2016, 2018; Cox and Ecroyd, 2017).

The ACD is considered a hotspot for mutations that result in CMT2F and dHMN2B (Kalmar et al., 2017). Mutants R127W, S135F, and R136W destabilize the dimer interface groove resulting in increased monomerization that correlates with increased chaperoning activity (Almeida-Souza et al., 2010; Weeks et al., 2018; Alderson et al., 2019). These mutants were shown to be hyperactive and displayed elevated binding to α and β tubulin (Almeida-Souza et al., 2010, 2011; Weeks et al., 2018). This increased binding was correlated with an increased stability of the microtubule network and is an unfavorable consequence since the dynamic polymerizing nature of microtubules is essential to its function (Almeida-Souza et al., 2011; Kevenaar and Hoogenraad, 2015). Hsp27 mutations R140G and K141Q were found to decrease the thermal stability of the oligomers (Nefedova et al., 2013a). This causes changes in the oligomerization and chaperoning ability of Hsp27 as the two are connected. The T151I mutant occurs at the end of the β4/β8 groove within an α-helix and surprisingly did not affect chaperone activity and did not have increased binding to tubulin/microtubules (Almeida-Souza et al., 2010; Klevit, 2020). The T164A mutant was shown to cause dissociation of oligomers accompanied by a decrease in thermal stability (Chalova et al., 2014). The ACD mutations lead to destabilization of the secondary structure at the dimer interface. This may lead to aberrations within the dimer interface groove that disrupt contacts made by the NTR. If the NTR is not regulated properly by the ACD, changes to the quaternary structure and chaperoning ability arise. It was demonstrated that mutations of the ACD to enhance the release of the NTR increased Tau substrate capture (Baughman et al., 2020). Disease mutations of the ACD could strengthen the interaction between the NTR and the ACD. The point mutations of the ACD occur at the dimer interface groove and one near the β4/β8 groove, both regions known to transiently bind stretches of the NTR. A mutation from a charged residue to a hydrophobic residue could amplify the interaction between these ACD binding regions and the hydrophobic NTR resulting in a protein that can no longer effectively chaperone Tau.

Lastly, mutations in the CTR and extension are causative for dHMN. The T180I mutant significantly improves the stability of Hsp27 oligomers and does not alter chaperoning activity (Chalova et al., 2014). However, this mutation could impact the interconversion rate between cis and trans states about the I180-P182 bond (Alderson et al., 2017). Disturbances near this region could explain the increased oligomer stability that is not

TABLE 1 | Hsp27 mutations associated with CMT2F and dHMN2B.

Domain/Motif	Mutation	Structural change	Possible consequences	References
NTR after SRLFDQxFG motif	G34R	Large negatively charged residue reduces flexibility	Reduces interaction of NTR (25–37) with dimer interface groove	Muranova et al. (2015) and Clouser et al. (2019)
NTR	P39L	Substitution to L increases flexibility	Either increase in α-helical structure or NTR-NTR interactions	Muranova et al. (2015), Kalmar et al. (2017), and Clouser et al. (2019)
NTR	E41K	Negative charged residue replaced with positive charge residue	Could disrupt contacts made with ACD	Muranova et al. (2015)
NTR β2	G84R	Large negatively charged residue reduces flexibility	Reduces interaction of NTR (74–91) with dimer interface groove	Nefedova et al. (2013b), Nefedova et al. (2017), and Clouser et al. (2019)
ACD β3	L99M	Replacement of L sidechain could disturb contacts with R140	Possibly disturbs the stability of ACD	Nefedova et al. (2013b) and Nefedova et al. (2017)
ACD β5	R127W	Replacement of R abolishes 3 H-bonds with H103 and E108 in L3/4 loop	Could destabilize structure of ACD	Almeida-Souza et al. (2010), Almeida-Souza et al. (2011), Holmgren et al. (2013), and Weeks et al. (2018)
ACD β6 + 7	S135F	Replacement of polar S with hydrophobic F	Could cause solubility issues	Almeida-Souza et al. (2010), Kalmar et al. (2017), Schwartz et al. (2018), and Weeks et al. (2018)
ACD β6 + 7	R136W	Replacement of R abolishes H-bonds with H124 of β5 of the same monomer	Introduction of another large aromatic residue in the crowded floor of the dimer interface groove could cause destabilization	Almeida-Souza et al. (2010), Almeida-Souza et al. (2011), and Weeks et al. (2018)
ACD β6 + 7	R140G	Substitution to G abolishes intradimer contacts. Additionally, an intradimer salt bridge with N129 is disrupted.	Possibly causes destabilization and collapse of the dimer interface groove	Nefedova et al. (2013a), Nefedova et al. (2017), and Kalmar et al. (2017)
ACD β6 + 7	K141Q	Replacement of K results in loss of positive charge and interaction with E126 in β5 of the adjoining monomer.	Could affect the dynamic contacts that are made with the NTR and the CTR	Nefedova et al. (2013a) and Nefedova et al. (2017)
ACD L7/8	T151I	Replacement of T with a hydrophobic residue at the β4/β8 entrance	The introduction of a hydrophobic residue could disturb binding interactions with the groove	Almeida-Souza et al. (2011)
ACD β9	T164A	Replacement of T with a hydrophobic residue	Alteration of the last ACD strand could affect the orientation of the CTR and cause negative effects on interdimer contacts	Chalova et al. (2014)
CTR IXI/V	T180I	Increases hydrophobicity of CTR	Minimal effects	Chalova et al. (2014)
CTR IXI/V	P182L	Substitution with L abolishes rigidity imposed by the center P	Affects binding of IXI/V to groove	Holmgren et al. (2013), Geuens et al. (2017), and Reid Alderson et al. (2021)
CTR IXI/V	P182S	Substitution with S abolishes rigidity imposed by the center P	Affects binding of IXI/V to groove	Chalova et al. (2014) and Nefedova et al. (2017)
C-terminal Extension	R188W	Substitution of charged R with large and hydrophobic W	This may affect the solubility of Hsp27/substrate complexes lead to co-precipitation	Chalova et al. (2014)

conducive to the mobile nature of Hsp27. The proline at position 182 is the central residue of the IXI/V motif and is susceptible to two different mutations, P182L and P182S. These mutations, as listed in **Table 1**, result in an increase in oligomerization resulting in aggregation and diminished chaperone activity (Chalova et al., 2014). The P182L mutation similarly results in

very large oligomers that have diminished chaperone activity. This was confirmed both *in vitro* and *in vivo* with dHMN patient-derived stem cells (Reid Alderson et al., 2021). It was found that the P182L mutant decreases the affinity of the IXI/V for the β4/β8 groove possibly due to the disruption of the trans configuration of the proline residue which is evidenced to facilitate binding to the ACD (Alderson et al., 2017; Reid Alderson et al., 2021). This liberates the interaction between the CTR and ACD and results in increased binding of the NTR to the ACD groove (Reid Alderson et al., 2021). This explains why the P182L and possibly the P182S result in large oligomers that are unable to chaperone client proteins. The NTR regions are erroneously bound to the ACD limiting their availability to chaperone, resulting in large oligomeric complexes (Freilich et al., 2018; Reid Alderson et al., 2021). This study further revealed that other IXI/V-containing proteins have the potential to bind the Hsp27 hydrophobic groove. One such protein is poly(rC)-binding protein 1 (PCBP1; Geuens et al., 2017; Reid Alderson et al., 2021). The single-stranded nucleic acid binding protein has several neuronal transcript targets suggesting that disruption of PCBP1 may have deleterious effects on downstream proteins that are vital for neuronal activity (Geuens et al., 2017). P182L bound with higher affinity to PCBP1 compared to the WT when using a mutant cell line (Geuens et al., 2017). The increased interaction between the Hsp27, P182L, and PCBP1 caused an overall reduction in translational repression of PCBP1, resulting in elevated protein expression levels (Geuens et al., 2017). The R188W mutation does not alter oligomeric size or stability but does have a significant effect on chaperoning activity (Chalova et al., 2014). The substitution of a charged amino acid with a large hydrophobic residue may affect the solubility of Hsp27/substrate complexes (Chalova et al., 2014). In addition, it is suspected that the R188 mutation could interfere with the formation of the residual β-strand structure formed by residues 193–199 which is hypothesized to regulate oligomer and substrate dynamics (Alderson et al., 2017).

Concluding Remarks

Hsp27 plays a central role in several physiological processes, including maintaining cytoskeletal dynamics, apoptosis, signal transduction, activation of the proteasome, and protein folding (Parcellier et al., 2005; Garrido et al., 2012). It is comprised of several structurally distinct regions, including the NTR, the ACD, and the CTR. The NTR region is primarily responsible for substrate capture with sequences that are equipped to bind substrates. The NTR is generally bound to the ACD, which regulates the NTR, so it is not continuously and arbitrarily fishing for the substrate. The CTR regulates this interaction between the NTR and the ACD by competing with binding sites on the ACD that then render the NTR available for substrate capture. This delicate interplay is disturbed by a myriad of point mutations that cause neurodegenerative diseases. These mutations affect the three key regions of Hsp27 in both divergent and overlapping fashions, thus further complicating our current understanding of disease progression of CMT2F and dHMN2B. Their role in pathological progression may lie in the consequential inability to protect vital neuronal proteins. Similarly, the inability to properly chaperone these client proteins may worsen diseases such as Alzheimer's and Parkinson's disease. Although there have been many breakthroughs during the investigation of these relationships, further work is required to better elucidate the link between disrupted Hsp27 structure and the progression of these debilitating neuropathies. Furthermore, subsequent efforts are required to better understand how the conformational variability of Hsp27 regulates oligomerization, phosphorylation, and substrate capture with respect to Hsp27's encompassing role in human physiology.

AUTHOR CONTRIBUTIONS

BH wrote and prepared the manuscript. RB, ZH, and BH contributed to the editing of the manuscript at all points of the preparation process. All authors contributed to the article and approved the submitted version.

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