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Modeling neuronal intranuclear inclusion disease: A review of animal and human-derived cellular models and mechanistic insights

Xin-Hui Wang[1,2], Ying Jiang[3], Hong Jiang[1,2,4], Lu Shen[1,2,4], Bei-Sha Tang[1,2,4], Yong-Cheng Pan[1,2,*], Qiong Liu[1,2,*]

[1] *Department of Neurology*, *Xiangya Hospital*, *Central South University*, *Changsha*, *Hunan* 410008, *China*

[2] *Key Laboratory of Hunan Province in Neurodegenerative Disorders*, *Xiangya Hospital*, *Central South University*, *Changsha*, *Hunan* 410008, *China*

[3] *Centre for Medical Genetics & Hunan Key Laboratory of Medical Genetics*, *School of Life Sciences*, *Central South University*, *Changsha*, *Hunan* 410078, *China*

[4] *National Clinical Research Center for Geriatric Disorders*, *Xiangya Hospital*, *Central South University*, *Changsha*, *Hunan* 410008, *China*

ABSTRACT

Neuronal intranuclear inclusion disease (NIID) is a rare autosomal dominant neurodegenerative disorder defined by the presence of eosinophilic intranuclear inclusions across both central and peripheral components of the nervous system, as well as multiple visceral organs, resulting in pronounced clinical heterogeneity. Following the discovery of pathogenic GGC repeat expansions in the *NOTCH2NLC* gene as the underlying genetic driver, a diverse array of experimental platforms has been established to probe NIID pathogenesis, including adeno-associated virus-mediated expression systems, transgenic animal models, and patient-derived cellular systems such as brain organoids. Collectively, these models recapitulate key histopathological and behavioral phenotypes observed in NIID and have elucidated multiple molecular and cellular pathways implicated in disease progression. This review systematically examines the current landscape of NIID model systems, highlighting their respective contributions to understanding disease pathogenesis, evaluating their experimental limitations, and identifying avenues for future refinement. Such integrative analysis is critical for advancing the development of more faithful disease models and facilitating the identification of therapeutic targets for NIID.

Keywords: Neuronal intranuclear inclusion disease; *NOTCH2NLC* gene; GGC repeat expansions; Animal models; Human-derived cell models; Pathogenesis

INTRODUCTION

Neuronal intranuclear inclusion disease (NIID) is an autosomal dominant neurodegenerative disorder characterized by widespread accumulation of p62-positive intranuclear inclusions in both the central and peripheral nervous system, as well as in multiple somatic organ systems (Lindenberg et al., 1968; Sone et al., 2016; Takahashi-Fujigasaki, 2003). Clinically, NIID exhibits marked heterogeneity, encompassing a wide range of manifestations such as cognitive decline, muscle weakness, parkinsonism, peripheral neuropathy, tremor, recurrent encephalopathic episodes, and dysautonomia (Sone et al., 2005, 2016). First described in 1968, the condition was originally recognized by the presence of eosinophilic intranuclear inclusions in neuronal and visceral cells (Lindenberg et al., 1968). Prior to 2011, NIID remained exceptionally rare, with most diagnoses made postmortem or through tissue sampling from muscle, rectum, or sural nerve (O'Sullivan et al., 2000; Schuffler et al., 1978; Sone et al., 2005; Zannolli et al., 2002). The introduction of skin biopsy as a diagnostic tool in 2011 has enabled less invasive detection of hallmark inclusions and substantially expanded case identification globally (Sone et al., 2011).

In 2019, independent groups from China and Japan employed long-read sequencing alongside conventional genetic analyses to identify pathogenic GGC repeat expansions (exceeding 60 repeats) in the 5' untranslated region (UTR) of the human-specific *NOTCH2NLC* gene as the causative mutation for NIID (Deng et al., 2019; Ishiura et al., 2019; Sone et al., 2019; Tian et al., 2019). Subsequent investigations revealed that this mutation is not unique to NIID

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*Corresponding authors, E-mail: panyongcheng@csu.edu.cn; lqiong66@csu.edu.cn

but also occurs in a range of clinically distinct neurological disorders, including essential tremor (Ng et al., 2020; Sun et al., 2020; Yan et al., 2021; Zhou et al., 2022), leukoencephalopathy (LEP) (Okubo et al., 2019; Wu et al., 2023), Parkinson's disease (PD) (Ma et al., 2020; Shi et al., 2021; Tian et al., 2019), amyotrophic lateral sclerosis (ALS) (Wan et al., 2023; Yuan et al., 2020; Zhang et al., 2022), Alzheimer's disease (AD) (Jiao et al., 2020; Wu et al., 2022), cerebral small vascular disease (CSVD) (Wang et al., 2023), multiple system atrophy (Fang et al., 2020), inherited peripheral neuropathy (Liao et al., 2022; Wang et al., 2021; Yu et al., 2021b) and oculopharyngodistal myopathy-3 (OPDM3) (Ogasawara et al., 2020; Yu et al., 2021a). This genetic pleiotropy has drawn considerable interest in neurogenetics. Notably, the mutation appears to be largely restricted to East Asian populations—particularly those from China, Japan, and Singapore—likely reflecting a founder effect, with only sporadic cases reported in European and North American cohorts (Yau et al., 2020a, 2020b, 2021a, 2021b).

Recent large-scale cohort studies have stratified NIID into distinct clinical subtypes based on initial and predominant symptomatology, including dementia-dominant (cognitive impairment-dominant), muscle weakness-dominant (neuromuscular disease-dominant), movement disorder-dominant, paroxysmal symptom-dominant (episodic neurogenic event-dominant), and autonomic dysfunction-dominant forms (Tai et al., 2023; Tian et al., 2022). This subclassification highlights the marked phenotypic heterogeneity of NIID. Emerging evidence has further implicated the size and internal structure of GGC repeat expansion in *NOTCH2NLC* as key modulators of clinical expression. For instance, larger repeat expansions appear to be associated with earlier disease onset, with the muscle weakness-dominant subtype also characterized by significantly longer GGC repeat tracts compared to other clinical forms (Tian et al., 2022). Interruptions within the repeat sequence also appear to influence phenotype. Notably, GGA repeat insertion within the GGC repeat sequence (known as GGA interruption) is correlated with neuromuscular presentations (Sone et al., 2019; Sun et al., 2020), while AGC interruptions are associated with parkinsonian features (Ma et al., 2020; Sun et al., 2020).

Since the identification of the pathogenic GGC repeat expansion, there has been a marked increase in reported NIID cases, reflecting both improved diagnostic capabilities and heightened clinical recognition. Despite these advances, the molecular mechanisms driving NIID pathogenesis remain incompletely elucidated, limiting the development of targeted interventions. To address this, a variety of model systems have been established, including murine and *Drosophila* models, as well as human-derived organoid platforms, to investigate disease mechanisms and assess potential therapeutic strategies. This review provides an integrated analysis of currently available NIID models—both *in vivo* and *in vitro*—outlining key insights into disease biology, evaluating experimental limitations, and identifying opportunities for further refinement. This comprehensive overview aims to guide future model development, deepen our understanding of NIID mechanisms, and facilitate the identification of therapeutic targets for NIID.

ADENO-ASSOCIATED VIRUS (AAV)-MEDIATED MOUSE MODELS OF NIID

The establishment of animal models for inherited diseases typically involves introducing pathogenic variants identified in affected individuals into experimental organisms. These mutations are generally classified as either loss-of-function (LOF) or gain-of-function (GOF), with modeling strategies tailored accordingly. LOF mutations are commonly studied through gene knockout, knockdown, or insertion of equivalent pathogenic mutations into the orthologous gene of the model organism. In contrast, GOF mutations are typically modeled by transgenic overexpression or targeted knock-in of the disease-associated variant at the orthologous genomic locus.

In the case of NIID, transcriptomic analyses of postmortem brain tissue, skin fibroblasts, and peripheral blood have demonstrated no significant difference in *NOTCH2NLC* mRNA levels between patients and healthy controls (Ishiura et al., 2019; Sone et al., 2019; Tian et al., 2019). Similarly, in induced pluripotent stem cell (iPSC)-derived forebrain organoids and neurons, protein levels detected using a NOTCH2NL antibody (which does not distinguish between NOTCH2NLA, NOTCH2NLB, and NOTCH2NLC) did not differ significantly between NIID and control samples (Fan et al., 2023). Although GGC repeat expansions in the 5'UTR of certain genes can lead to hypermethylation and transcriptional silencing in other repeat expansion disorders (Santoro et al., 2012), methylation levels in the 5'UTR of *NOTCH2NLC* appear largely unaltered in NIID patient-derived tissues (Ishiura et al., 2019; Sone et al., 2019; Tian et al., 2019), except in select contexts (discussed below). Collectively, these observations suggest that NOTCH2NLC-associated NIID likely arises through a GOF mechanism rather than transcriptional silencing or haploinsufficiency. Accordingly, the predominant strategy for modeling NIID in vivo has involved the ectopic expression of NOTCH2NLC transcripts harboring pathogenic GGC repeat expansions, via AAV-mediated delivery or transgenic mice, to replicate molecular pathology and investigate disease mechanisms.

Ubiquitous *NOTCH2NLC*-100polyG expression mouse model

Although the pathogenic GGC repeat expansions in *NOTCH2NLC* reside within the 5'UTR, they are embedded in a short upstream open reading frame (uORF), suggesting the potential for translation into a polyglycine (polyG)-containing protein via an upstream AUG start codon. Boivin et al. (2021) recently demonstrated that constructs containing 100 GGC repeats generate a protein, termed uN2CpolyG, that undergoes translation and forms p62-positive intranuclear inclusions. These inclusions were also detected in brain and skin tissue biopsies from NIID patients, implicating uN2CpolyG as a likely driver of disease pathology.

To evaluate the pathogenic potential of uN2CpolyG *in vivo*, Boivin et al. (2021) first cloned the full *NOTCH2NLC* uORF containing either 100 GGC repeats (pathogenic) or 12 GGC repeats (control) and assessed cytotoxicity in primary cultured neurons. Overexpression of the expanded construct induced significant neuronal death. To further validate its pathogenicity, the authors generated the first AAV-mediated mouse model expressing uN2CpolyG under the control of a CMV promoter following retro-orbital injection of AAV with a PHP.eB serotype (Boivin et al., 2021). To focus on the toxicity of the polyG protein and exclude potential RNA-mediated GOF effects, the GGC repeats were substituted with alternative glycine-encoding codons (GGA, GGT, and GGG), ensuring polyG protein production while avoiding the formation

of pure GGC RNA hairpins, thereby enabling specific isolation of uN2CpolyG protein toxicity as the primary contributor to phenotypic manifestations.

Mice expressing uN2CpolyG exhibited widespread intranuclear accumulation of p62-positive aggregates across multiple brain regions, mirroring a core neuropathological signature of NIID. As detailed in Table 1, these mice developed progressive neurodegeneration, locomotor deficits, and markedly shortened survival, typically succumbing within 4 to 6 months post-injection. In contrast, control mice showed no overt abnormalities and exhibited a normal lifespan. Although cell-based models revealed that uN2CpolyG interacted with Ku70 and Ku80 proteins, potentially impairing DNA damage repair, neither mislocalization of Ku proteins nor evidence of DNA damage accumulation was observed in cells, brain tissue, or skin samples from NIID patients (Boivin et al., 2021). These findings suggest that impaired DNA repair is unlikely to be the primary mechanism of toxicity. Together, this model establishes a direct pathogenic role for uN2CpolyG and provides a valuable platform for investigating NIID-associated neurodegeneration.

Substantia nigra (SN)-targeted *NOTCH2NLC*-98polyG mouse model

Although pathogenic GGC repeat expansions in *NOTCH2NLC* have been implicated in PD, their direct contribution to dopaminergic neurodegeneration within the SN remains unclear (Liu et al., 2024; Ma et al., 2020; Shi et al., 2021; Tian et al., 2019). To investigate this, Liu et al. (2024) engineered two AAV constructs under the control of the CMV promoter—one expressing the 5 ′UTR of *NOTCH2NLC* harboring 98 GGC repeats and the other encoding green fluorescent protein (GFP) as a control. These AAV constructs were packaged into AAV9 capsids and bilaterally injected into the SN of adult C57BL/6 mice via stereotaxic injection.

By one month post-injection, dopaminergic neurons in the *NOTCH2NLC*-98GGC group exhibited intranuclear or perinuclear polyG inclusions (Table 2). Tyrosine hydroxylase (TH) immunofluorescence revealed a dispersed and weakened signal in axons and dendrites, indicating early pathology. Quantification showed a 26% reduction in TH^+ neurons within the SN compared to the GFP-injected side, which progressed to 69% two months post-injection. In contrast, TH^+ neurons in the adjacent ventral tegmental area (VTA) remained largely unaffected, indicating region-specific vulnerability. Additionally, TH signal intensity in the striatum declined over time, suggesting impaired axonal projections of dopaminergic neurons from the SN to the striatum (Liu et al., 2024). These findings demonstrate that localized expression of *NOTCH2NLC* GGC repeat expansions in the SN elicits progressive, region-specific dopaminergic neuron degeneration, offering new insights into the pathological mechanisms underlying *NOTCH2NLC*-related PD.

Concomitant or separate *NOTCH2NLC*-polyG protein/-GGC repeat RNA expression mouse models

To dissect the relative pathogenic contributions of GGC repeat RNA and the corresponding polyG protein in NIID pathogenesis, Zhong et al. (2024) engineered four AAV constructs under control of the CAG promoter. The first construct (ATG-GGC100) included the 5'UTR and coding sequence of *NOTCH2NLC* harboring 100 GGC repeats, enabling the expression of both uN2CpolyG protein and GGC repeat RNA. The second construct (TAG-GGC100) carried a

Table 1 Comparison of AAV-mediated mouse models for NIID

Mouse model	NOTCH2NLC-100polyG	NOTCH2NLC-98polyG	ATG-GGC100	TAG-GGC100	ATG-GGN100dCT
Publication	Boivin et al., 2021	Liu et al., 2024	Zhong et al., 2024	Zhong et al., 2024	Zhong et al., 2024
Transgene	uORF of *NOTCH2NLC*	uORF of *NOTCH2NLC*	uORF and CDS of *NOTCH2NLC*	uORF and CDS of *NOTCH2NLC*	uORF and CDS of *NOTCH2NLC*
Promotor (AAV serotype)	CAG promotor (PHP.eB)	CMV promotor (AAV9)	CAG promoter (PHP.eB)	CAG promoter (PHP.eB)	CAG promoter (PHP.eB)
GGC repeat size	100	98	100	100	100
Repeat composition	GGN (N represents random A/T/G/C)	GGC with multiple GGA interruptions	GGC with GGA and GGT interruptions	GGC with GGA and GGT interruptions	GGN
Injection age	2 months	2 months	2 months	2 months	2 months
Sex	♂	♂	♂	♂	♂
Injection method	Intravenous injection (retro-orbital sinus)	Stereotaxic injection	Intravenous injection (tail vein)	Intravenous injection (tail vein)	Intravenous injection (tail vein)
Expression distribution	CNS (peripheral tissue: –)	Substantia nigra	CNS (peripheral tissue: –)	CNS (peripheral tissue: –)	CNS (peripheral tissue: –)
Life span	6–8 months	–	5–7 months	Over 9 months	6–9 months
Behavioral deficits					
Cognitive deficits (Y maze)	–	–	Yes (5.5 months)	No	No
Clasping	Yes (5 months)	–	Yes (5.5 months)	No	No
Rotarod deficits	Yes (5 months)	–	Yes (5.5 months)	No	Yes (5.5 months)
Notched bar test deficits	Yes (5 months)	–	Yes (3.5 months)	No	Yes (5.3 months)
Hyperactivity	Yes (5 months)	–	Yes (5.5 months)	No	Yes (5.5 months)
Neuropathology					
Intranuclear inclusions	4 months	3 months	6 months	No	6 months
Neuronal loss	Yes (4 months)	Yes (3 months)	Yes (6 months)	No	Yes (6 months)
Purkinje cell loss	Yes (4 months)	–	Yes (6 months)	No	Yes (6 months)
Demyelination	–	–	Yes (6 months)	No	Yes (6 months)
Reactive astrocytes	Yes (4 months)	–	Yes (6 months)	No	Yes (6 months)

–: Not available; CNS: Central nervous system.

Table 2 Comparison of NIID transgenic mouse models

Mouse model	EIIa-Tg	Myh6-Tg	N2C2-45G	N2C2-32G13S
Publication	Liu et al., 2022	Pan et al., 2023	Tu et al., 2024	Tu et al., 2024
Transgene	*NOTCH2NLC* exon 1 with loxP-stop-loxP		*NOTCH2NLC* exon 1	*NOTCH2NLC* exon 1
Promotor	Cre: EIIa	CreERT2: Myh6	Nestin	Nestin
GGC repeats	98	98	45	45
Repeat composition	GGC with multiple GGA interruptions		GGC	32GGC13AGC
Sex	♂	♂&♀	♂&♀	♂&♀
Expression distribution	Ubiquitous	Cardiomyocyte expression upon tamoxifen	CNS	CNS
Life span	2 months	2 months (post-tamoxifen induction)	Over 18 months	Over 18 months
Cognitive deficits (Y maze)	–	–	–	–
Cognitive deficits (novel object recognition test)	Yes (P31–35)	–	–	–
Ataxia	Yes (P40)	–	–	–
Decreased motivation (open field test)	Yes (P30)	–	No	Yes (12 months)
Anxiety (open field test)	No	–	No	Yes (12 months)
Rotarod deficits	Yes (P40)	–	No	No
Vertical movement deficits (cylinder test)	Yes (P43)	–	No	Yes (12 months)
Intranuclear inclusions	Yes	Yes	Yes	Yes
Purkinje cell degeneration	Yes (P32)	–	–	–
Neuronal loss	Yes (P50)	–	No	Yes (12 months)
Muscle atrophy	Yes (P50)	Yes (3 months)	–	–

–: Not available; P: Postnatal day; CNS: Central nervous system.

TAG stop codon replacing the ATG start site, selectively blocking uN2CpolyG translation while maintaining GGC repeat RNA expression. The third construct (ATG-GGN100dCT) substituted GGC with degenerate GGN, producing a truncated form of uN2CpolyG lacking its native C-terminus while minimizing structured RNA formation. The fourth construct (ATG-GGC11), containing only 11 GGC repeats, served as a negative control. All constructs were packaged in AAV-PHP.eB and systemically delivered via intravenous injection into 2-month-old C57BL/6 mice. By four months post-injection, mice receiving ATG-GGC100 exhibited widespread accumulation of uN2CpolyG-positive inclusions in neurons, astrocytes, and oligodendrocytes, with microglia notably spared. These mice developed progressive neurodegeneration characterized by locomotor and cognitive impairments, shortened lifespan, demyelination of the cerebellum, reactive gliosis, and diffuse white matter pathology. Mice injected with ATG-GGN100dCT displayed similar but attenuated phenotypes, attributed to lower uN2CpolyG expression levels. In contrast, TAG-GGC100 mice lacked inclusions and overt phenotypes, resembling the ATG-GGC11 controls.

Zhong et al. (2024) further reported that uN2CpolyG and uN2CpolyG-dCT inclusions shared similar ultrastructural features under electron microscopy, equivalent condensate dynamics in fluorescence recovery after photobleaching (FRAP) assays, and comparable pro-apoptotic activity *in vitro*. Transcriptomic profiling identified shared molecular perturbations in both ATG-GGC100 and ATG-GGN100dCT mice, with prominent enrichment of microglia-mediated neuroinflammation. These signatures were mirrored in NIID patients, where translocator protein-positron emission tomography (TSPO-PET) revealed a correlation between microglial activation and severity of white matter atrophy. Importantly, microglia ablation in ATG-GGC100 mice ameliorated neurodegenerative phenotypes and transcriptional alterations without altering uN2CpolyG inclusion formation.

Together, these results establish uN2CpolyG as a primary pathogenic driver in NIID and underscore the contribution of microglial activation as a key mediator of neurotoxicity. Targeting the neuroimmune interface may thus represent a promising therapeutic strategy in polyG-associated neurodegenerative diseases.

TRANSGENIC MOUSE MODELS OF NIID

While AAV-mediated approaches have proven effective in recapitulating many aspects of NIID pathology, they also have several limitations, including potential immune activation, declining transgene expression over time, and temporal restriction of gene delivery to postnatal or adult stages. This temporal mismatch is particularly problematic for the natural expression of genes such as *NOTCH2NLC*, which are endogenously expressed from embryonic development (Fiddes et al., 2018; Suzuki et al., 2018). In contrast, transgenic models enable stable, consistent, and heritable gene expression, initiating during embryogenesis and permitting the investigation of early disease processes. These features make transgenic systems especially valuable for investigating the full spectrum of NIID-associated neurodegeneration.

NOTCH2NLC-98GGC conditional transgenic mouse model

Liu et al. (2022) generated the first transgenic mouse model of NIID by inserting exon 1 of *NOTCH2NLC* containing either 98 GGC repeats (pathogenic) or 17 GGC repeats (control) into the Rosa26 locus. Notably, a loxP-stop-loxP cassette placed upstream of the transgene preserved the potential for spatiotemporally controlled expression using the Cre-loxP system. The conditional transgenic mice were crossed with EIIa-Cre mice, which express Cre recombinase from the zygotic stage (Liu et al., 2022), resulting in ubiquitous expression of *NOTCH2NLC*-98GGC or 17GGC throughout the entire organism. Compared to control mice, EIIa;

NOTCH2NLC-98GGC (EIIa-Tg) mice exhibited significant motor and cognitive impairments, widespread p62-positive intranuclear inclusions, and neurodegeneration across multiple brain regions (Table 2), faithfully recapitulating the clinical and pathological features of NIID.

Compared to AAV-mediated models expressing a similar repeat length (Boivin et al., 2021), EIIa-Tg mice exhibited markedly earlier onset and more severe disease features, including intranuclear inclusion formation as early as postnatal day 12 (P12) and premature mortality by two months of age. This accelerated phenotype likely reflects both the broader spatial distribution and earlier initiation of transgene expression. Inclusions were also observed in non-neural tissues, including the gastrocnemius muscle, accompanied by muscle degeneration and abnormal electromyographic profiles, further recapitulating systemic human disease.Mechanistically, Liu et al. (2022) confirmed that the GGC expansion not only produced polyG via canonical ATG-initiated translation, consistent with prior studies, but also generated polyA and polyR peptides, potentially via repeat-associated non-AUG (RAN) translation or ribosomal frameshifting. Notably, the tissue distribution of these proteins varied, with only polyG and polyR detected in the gastrocnemius muscle of EIIa-Tg mice. The authors attributed this selectivity to the presence of GGA interruptions in the repeat sequences derived from patient samples, in contrast to synthetic repeats without interruptions employed in prior studies. Similar interruption-dependent modulation of translation products and toxicity has been reported in other repeat expansion disorders (Perez et al., 2021; Rajan-Babu et al., 2024), although the functional contributions of polyA and polyR in NIID remain to be clarified.

Given the absence of an orthologous *NOTCH2NLC* gene in the murine genome, the authors also established iPSCs from NIID patients and unaffected controls. Differentiation into human neural progenitor cells (hNPCs) recapitulated hallmark pathological features, including ubiquitin- and *NOTCH2NLC*-positive intranuclear inclusions (Sone et al., 2019). Transcriptomic comparisons between the mouse model and patient-derived hNPCs revealed dysregulated alternative splicing as a common molecular signature. Further mechanistic studies identified hnRNPM, an alternative splicing regulator (Cho et al., 2014; Hovhannisyan & Carstens, 2007; Llères et al., 2010; Park et al., 2011; Ramesh et al., 2020), as a direct interactor of both NOTCH2NLC-polyG and -polyA proteins. hnRNPM was found to be sequestered within inclusions, and its overexpression rescued cellular toxicity *in vitro*. These findings suggest that hnRNPM-mediated alternative splicing may play an important role in the molecular pathogenesis of NIID and highlight the potential of targeting this pathway as a therapeutic strategy for the neurodegenerative disease.

Cardiomyocyte-specific *NOTCH2NLC*-98GGC transgenic mouse model

Although NIID primarily affects the central and peripheral nervous systems, accumulating evidence suggests that cardiac involvement may also occur in individuals with NIID or OPDM3, including cardiomyopathy, cardiac insufficiency, valvular regurgitation, postural hypotension, and paroxysmal chest distress (Chen et al., 2020; Gu et al., 2023; Huang et al., 2022; Oyer et al., 1991). However, these observations remain largely correlative, and the mechanistic link between intranuclear inclusions in cardiomyocytes and functional cardiac impairment has yet to be firmly established.

To investigate this connection, Pan et al. (2023) generated a cardiomyocyte-specific transgenic mouse model by crossing conditional *NOTCH2NLC*-98GGC mice with Myh6-CreERT2 (Myh6-Tg) mice, which specifically express tamoxifen-inducible Cre recombinase in cardiomyocytes. Following tamoxifen induction beginning at P60, widespread p62-positive NOTCH2NLC-polyG inclusions were detected in the myocardium of Myh6-Tg mice within one month. These mice developed marked ventricular structural abnormalities, histopathological signs of cardiomyopathy, and progressive decline in cardiac function. Mortality increased steadily, with most mice succumbing by two months post-induction (Pan et al., 2023). These findings indicate that peripheral tissue pathology plays a critical role in the progression of NIID. Subsequent analyses revealed that the mouse model exhibited pronounced mitochondrial dysfunction, characterized by suppression of mitochondria-related genes, impaired electron transport chain activity, and disrupted energy metabolism. Collectively, these results offer mechanistic insight into the systemic nature of NIID and highlight the pathological relevance of peripheral organ involvement in disease pathogenesis.

Transgenic mouse model expressing intermediate GGC repeats in *NOTCH2NLC*

In the general population, *NOTCH2NLC* GGC repeat length typically remains below 40, while expansions exceeding 60 are classified as pathogenic (Ishiura et al., 2019; Sone et al., 2019; Sun et al., 2020; Tian et al., 2019). However, clinical studies have identified individuals with neurodegenerative disorders, including PD, ET, ALS, CSVD, and AD, who carry intermediate-length GGC repeats ranging from 41 to 60 (Ma et al., 2020; Ng et al., 2020; Shi et al., 2021; Wan et al., 2023; Wang et al., 2023; Wu et al., 2022; Yuan et al., 2020). Additionally, the presence of AGC insertions within the GGC repeat tract has been linked to increased risk of parkinsonian phenotypes (Ma et al., 2020; Sun et al., 2020). *NOTCH2NLC* expresses two transcript variants. In transcript variant 1, the GGC repeat lies within the 5'UTR, forming an uORF that is translated independently of the downstream *NOTCH2NLC* coding sequence. In transcript variant 2, the repeat is embedded within the coding sequence, allowing translation as part of the full-length NOTCH2NLC protein itself. Although transcript variant 2 accounts for less than 16% of total transcripts (Wang et al., 2024; Zhong et al., 2021), *in vitro* evidence suggests it may contribute to pathogenicity (Wang et al., 2024). However, the *in vivo* relevance of this variant remains unclear.

To address this, Tu et al. (2024) generated transgenic mouse lines expressing transcript variant 2 of NOTCH2NLC with varying GGC repeat configurations under the control of the human NES promoter. Three lines were established: N2C2-30G (30 GGC repeats, non-pathogenic control), N2C2-45G (45 GGC repeats, intermediate expansion), and N2C2-32G13S (32 GGC repeats with 13 AGC insertions, representing 28.9% AGC content based on patient-derived sequences). Among these, N2C2-32G13S mice exhibited relatively more severe neuropathology, including larger fiber-like NOTCH2NLC-polyG aggregates, reduced TH expression, increased phospho-α-synuclein levels, α-synuclein fiber formation, mitochondrial dysfunction, cortical

hypermyelination, and locomotor deficits emerging between 8 and 12 months of age (Table 2). In contrast, N2C-45G mice developed smaller cortical NOTCH2NLC-polyG aggregates and increased phospho-α-synuclein levels but showed no significant mitochondrial dysfunction or overt behavioral abnormalities (Tu et al., 2024). These findings implicate AGC insertions as a pathogenic modifier that exacerbates repeat-mediated toxicity and accelerates PD-like pathology.

Compared with EIIa-Tg mice (Liu et al., 2022), N2C2-32G13S mice display a milder neurodegenerative phenotype, as evidenced by delayed-onset locomotor deficits and preserved survival beyond 12 months. This divergence likely reflects differences in both repeat size and spatiotemporal expression. EIIa-Tg mice express a substantially longer repeat tract than N2C2-32G13S and N2C2-45G mice (98 GGC vs 45 GGC). Furthermore, *NOTCH2NLC*-98GGC is ubiquitously expressed in EIIa-Tg mice, whereas N2C2-32G13S and 45G mice specifically express the transgene in the central nervous system under the control of the NES promoter.

TRANSGENIC *DROSOPHILA* MODELS OF NIID

In addition to mouse models, transgenic *Drosophila* models offer unique advantages for studying NIID, including rapid generation time, cost-effectiveness, and easier genetic manipulation, facilitating high-throughput phenotypic and mechanistic screening.

Yu et al. (2022) established three *Drosophila* lines expressing the uORF of *NOTCH2NLC* transcript variant 1, carrying either nine GGC repeats (uN2C-GFP) or 100 GGC repeats (uN2CpolyG-GFP), or a GFP-only control, all under UAS control and regulated by Gal4 drivers. Targeted expression of uN2CpolyG-GFP in the eye induced ubiquitin-positive intranuclear inclusions and progressive degeneration of rhabdomeres, a specialized photoreceptor structure of the compound eye. When expressed ubiquitously, uN2CpolyG-GFP caused severe locomotor deficits, reduced lifespan, and mitochondrial swelling, a feature also observed in patient muscle biopsies (Yu et al., 2022). Mechanistic analysis revealed that uN2CpolyG directly interacted with the mitochondrial RNA-binding protein LRPPRC, disrupting mitochondrial oxidative phosphorylation, especially in complex I, and significantly reducing mitochondrial respiratory capacity and ATP synthesis. Notably, administration of IDB, a coenzyme Q analog that enhances electron transfer and mitochondrial complex I activity, effectively restored mitochondrial morphology, rescued locomotor performance, prolonged lifespan, and normalized ATP synthesis in uN2CpolyG-GFP-expressing flies. This Drosophila model, consistent with findings from mouse studies, provides compelling evidence that uN2CpolyG exerts direct neurotoxic effects driving neurodegeneration. Moreover, it underscores the therapeutic potential of restoring mitochondrial function, particularly through targeted reversal of oxidative phosphorylation defects, as a promising strategy for treating NIID.

IPSC-DERIVED MODELS OF NIID

iPSCs, generated through reprogramming of patient-derived somatic cells, have revolutionized disease modeling by enabling the study of pathogenic processes within human-specific genomic and cellular contexts. Unlike traditional animal models, iPSC-based platforms retain the full genomic context of the donor, providing an essential tool for investigating diseases linked to human-specific genes. This is particularly significant for NIID, whose causative gene, *NOTCH2NLC*, is exclusively present in the human genome and likely plays a critical role in brain development (Fiddes et al., 2018; Suzuki et al., 2018). As such, iPSC-derived models are essential for elucidating the molecular and developmental pathogenesis of NIID.

Yu et al. (2023) successfully reprogrammed skin fibroblasts from NIID patients into iPSCs and subsequently differentiated them into forebrain organoids and neurons. The authors observed p62- or ubiquitin-positive uN2CpolyG inclusions in GGC-expanded forebrain organoids (90 days *in vitro*, 90DIV) and neurons (30DIV), accompanied by enhanced autophagic flux and activation of the integrated stress response, as evidenced by increased levels of ATF4 and phosphorylated eIF2α (p-eIF2α). Bulk and single-cell RNA sequencing (scRNA-seq) revealed reduced expression of ribosomal and translation-related mRNAs, indicating impaired ribosome biogenesis and translation. Notably, although overall expression levels of the NOTCH2NL gene family were unchanged, scRNA-seq analysis revealed that GGC-expanded organoids exhibited a reduced proportion of immature neurons and an increased proportion of radial glial cells, a type of neural progenitor cell (Fan et al., 2023).

CONCLUSIONS AND FUTURE PERSPECTIVES

Mechanistic insights into NIID

Investigations using diverse NIID models have uncovered multiple pathogenic mechanisms driven by GGC repeat expansion in *NOTCH2NLC*, including both protein- and RNA-mediated toxic GOF pathways (Figure 1). Among these, polyG protein toxicity has emerged as the most consistently implicated mechanism, associated with widespread disturbances in RNA splicing (Liu et al., 2022; Zhong et al., 2024), impaired ribosome biogenesis (Fan et al., 2023), disrupted nucleocytoplasmic transport (Zhong et al., 2021), compromised mitochondrial function (Pan et al., 2023; Tu et al., 2024; Yu et al., 2022), and aberrant myelination (Tu et al., 2024). PolyG aggregates can also sequester the transcription factor p65, leading to inhibition of the NF-κB-NLRP3 pathway and suppression of autophagy (Shen et al., 2025), potentially impairing the cellular clearance of misfolded proteins. In addition to neuronal pathology, glial dysfunction contributes to disease progression, as evidenced by microglial activation in both patient tissues and mouse models (Liu et al., 2022; Zhong et al., 2024), while environmental stressors may further exacerbate disease severity (Fan et al., 2023; Wang et al., 2024).

RNA-mediated toxicity may also play a role in NIID pathogenesis. Deng et al. (2022) reported that GGC repeat-containing RNAs form p62-positive nuclear foci in patient skin biopsies, where they sequester RNA-binding proteins, including Sam68, MBNL1, and hnRNP A/B, into intranuclear inclusions. Similarly, Fukuda et al. (2021) identified G-quartet RNA foci in lymphoblastoid cell lines derived from NIID patients. In contrast, although Zhong et al. (2024) observed p62-positive inclusions in a skin biopsy sample from one patient and in the brains of AAV-based mouse models, corresponding RNA foci were absent, possibly due to differences in FISH probe design, sample size, tissue source, or species specificity. Moreover, the lack of overt phenotypes

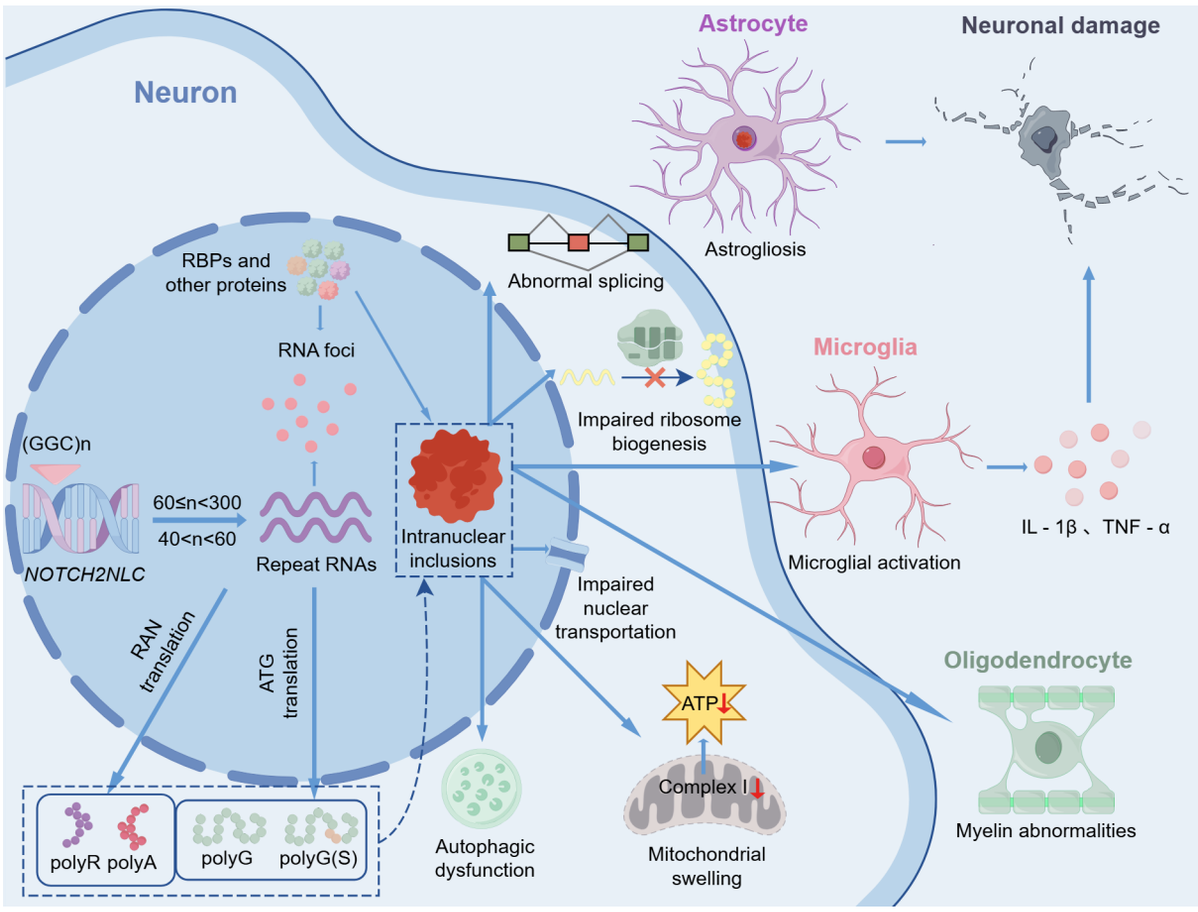

Figure 1 Proposed pathogenic mechanisms of NIID

Expansion of GGC repeats within *NOTCH2NLC* represents the genetic basis of NIID, with pathogenic repeat size typically ranging from 60 to 300. Transcription of the expanded repeat tract can give rise to RNA foci that sequester RNA-binding proteins (RBPs), contributing to RNA-mediated toxicity. In parallel, the expanded GGC repeats undergo translation through both canonical ATG-dependent initiation and non-canonical repeat-associated non-ATG (RAN) translation, generating aberrant polypeptides, including polyG—occasionally interspersed with polyS—as well as polyA and polyR. These polypeptides predominantly accumulate as intranuclear inclusions, though extranuclear aggregates may also form, with both types frequently immunopositive for p62 and ubiquitin. The resulting inclusions are thought to disrupt cellular homeostasis by sequestering essential interacting proteins, leading to mitochondrial structural and functional abnormalities, dysregulation of RNA splicing, impaired ribosome biogenesis, and disrupted nucleocytoplasmic transport. PolyG species may also interfere with autophagic processes, reducing clearance of misfolded proteins and ultimately exacerbating neuronal dysfunction and degeneration. In addition to neuron-intrinsic mechanisms, non-neuronal mechanisms have been implicated in NIID pathogenesis. Activated microglia may amplify neuroinflammation via the release of proinflammatory cytokines, while disrupted oligodendrocyte myelination and reactive astrogliosis have likewise been observed. Notably, astrocytic intranuclear inclusions are also frequently present, although their precise role in disease progression remains to be elucidated.

in TAG-GGC100 (RNA-only) mice following AAV-mediated expression does not preclude the possibility that other repeat RNA may exert toxicity in patients. Hence, the precise contribution of RNA toxicity to NIID pathogenesis remains an open question warranting further investigation.

Although most studies have reported no changes in the methylation status of the *NOTCH2NLC* upstream region or in its expression levels (Ishiura et al., 2019; Sone et al., 2019; Tian et al., 2019; Yau et al., 2020a), several cases have described hypermethylation of the CpG island in *NOTCH2NLC* and decreased mRNA levels in asymptomatic fathers with GGC repeats exceeding 300 (Deng et al., 2022; Fukuda et al., 2021). Therefore, whether larger GGC repeats and the precise threshold at which they occur affects CpG island methylation and alters *NOTCH2NLC* expression requires further investigation in larger patient cohorts. In parallel, quantification of NOTCH2NLC protein levels across a broader range of tissues and patients is needed to clarify disease-relevant expression dynamics.

Collectively, these findings underscore the complex and multifactorial nature of NIID pathogenesis. Importantly, several therapeutic strategies have emerged from these mechanistic insights. For example, idebenone treatment rescues mitochondrial dysfunction and neurodegeneration in transgenic flies (Yu et al., 2022); microglial depletion using the colony-stimulating factor 1 receptor (CSF1R) inhibitor PLX5622 alleviates neurodegeneration and behavioral deficits in mouse models (Zhong et al., 2024); and overexpression of the splicing regulator hnRNP M rescues abnormal alternative splicing and mitigates cellular toxicity in cellular models (Liu et al., 2022).

Development of new models and future directions in NIID

Current genetic mouse models have limitations in faithfully recapitulating the full spectrum of disease phenotypes observed in NIID. Transgenic mice carrying large GGC repeat

expansions (e.g., 98 repeats) exhibit early death around two months of age, while mice harboring intermediate repeat sizes (e.g., 45 repeats) display no overt abnormalities even at 18 months. This stands in contrast to the human disease course, where symptom onset typically occurs between 30 and 60 years of age, and patients may survive for decades post-diagnosis (Tian et al., 2022). Generation of mouse lines with appropriate GGC repeat lengths (e.g., 60–80 repeats) may better mimic the temporal dynamics of disease onset and progression. Furthermore, models incorporating AGC interruptions have suggested that such interruptions may contribute to the clinical heterogeneity of NIID. However, the impact of other interruption motifs, such as GGA, remains unclear, underscoring the need for expanded allelic series in animal models to dissect the influence of repeat structure on clinical heterogeneity.

Another limitation of current models is the insufficient recapitulation of hallmark neuroimaging features. White matter lesions and hyperintense signals at the corticomedullary junction on diffusion-weighted imaging are considered radiological hallmarks of NIID (Tai et al., 2023; Tian et al., 2022), yet have been largely overlooked in preclinical models. Although white matter abnormalities have been reported in a recent mouse study (Zhong et al., 2024), the underlying histopathological basis remains undefined. Given the reduced complexity of murine white matter and lower glial cell density relative to primates, large-animal models, particularly non-human primates, may offer superior anatomical and cellular fidelity for modeling these magnetic resonance imaging (MRI) features (Li at al., 2024; Pan at al., 2024; Yin et al., 2022). Establishing such models, combined with high-resolution neuroimaging platforms, represents a promising avenue for advancing NIID research.

Existing animal studies have predominantly focused on the intrinsic pathogenic mechanisms, with limited exploration of encephalopathic episodes triggered by infection or metabolic stress. Therefore, incorporation of stress paradigms, such as dietary manipulation or exposure to chemical agents to mimic metabolic or inflammatory insults, may help uncover the underlying mechanisms of these encephalopathic episodes and facilitate the development of potential therapeutic strategies.

A further limitation of current transgenic and AAV-based models is the reliance on exogenous promoters to drive repeat expansions, which may not fully recapitulate the intrinsic spatiotemporal expression pattern of *NOTCH2NLC*. Establishing bacterial artificial chromosome (BAC) or yeast artificial chromosome (YAC) mouse models may represent a promising approach. Complementarily, recent advances in organoid technology offer alternative platforms for disease modeling. However, conventional brain organoids face challenges in long-term culture and maturation due to size-related nutrient deprivation. Thus, vasculature-integrated organoid-on-a-chip systems have emerged as a strategy to overcome these limitations. Incorporating microglia into such vascularized organoids may enable modeling of disease-driven neuroinflammation-neurodegeneration cascades, a hallmark of NIID pathology. Furthermore, multi-organoid microfluidic systems can maintain organ-specific nutrient profiles and microenvironments for target organs, thereby establishing a highly promising organoid model for translational research and drug screening.

CONCLUSION

In summary, both animal and human-derived cellular models have substantially advanced our understanding of NIID pathogenesis. The diverse and intersecting molecular mechanisms revealed through these systems highlight the biological complexity of this neurodegenerative disorder. Continued development of refined and physiologically relevant models will be essential for elucidating unresolved disease mechanisms and accelerating the discovery of effective therapeutic interventions.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

X.H.W. conducted the literature review and wrote the draft. Y.C.P. and Q.L. designed and revised the manuscript. Y.J., H.J., L.S., and B.S.T. provided critical suggestions. All authors read and approved the final version of the manuscript.

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