DISEASES AND DISORDERS

Expression of expanded GGC repeats within NOTCH2NLC causes behavioral deficits and neurodegeneration in a mouse model of neuronal intranuclear inclusion disease

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GGC repeat expansions within NOTCH2NLC have been identified as the genetic cause of neuronal intranuclear inclusion disease (NIID). To understand the molecular pathogenesis of NIID, here, we established both a transgenic mouse model and a human neural progenitor cells (hNPCs) model. Expression of the NOTCH2NLC with expanded GGC repeats produced widespread intranuclear and perinuclear polyglycine (polyG), polyalanine (polyA), and polyarginine (polyR) inclusions, leading to behavioral deficits and severe neurodegeneration, which faithfully mimicked the clinical and pathological features associated with NIID. Furthermore, conserved alternative splicing events were identified between the NIID mouse and hNPC models, among which was the enrichment of the binding motifs of hnRNPM, an RNA binding protein known as alternative splicing regulator. Expanded NOTCH2NLC-polyG and NOTCH2NLC-polyA could interact with and sequester hnRNPM, while overexpression of hnRNPM could ameliorate the cellular toxicity. These results together suggested that dysfunction of hnRNPM could play an important role in the molecular pathogenesis of NIID.

INTRODUCTION

Polymorphic CGG repeats are more prevalent in the human genome than currently appreciated, and they can have a role in both neurodevelopmental and neurodegenerative disorders. In addition to paradigmatic CGG repeat expansions at the FMR1 locus associated with fragile X syndrome, several neuromuscular and neurodegenerative disorders with overlapping symptoms and similar pathological features have recently been shown to be caused by CGG repeat expansions in distinct loci (1, 2). Among them, neuronal intranuclear inclusion disease (NIID), a rare neurodegenerative disease characterized by widespread intranuclear inclusions in the nervous system as well as multiple visceral organs (3, 4), was found to be caused by expanded GGC repeats in the 5′ untranslated region (5′UTR) of the human-specific NOTCH2NLC gene by three independent research groups (1, 5, 6). Furthermore, the same mutation was then found in an increasing number of neurodegenerative and neuromuscular diseases, including essential tremor, Alzheimer’s disease, Parkinson’s disease, amyotrophic lateral sclerosis (ALS), frontotemporal dementia, leukoencephalopathy, multiple system atrophy, and oculopharyngodistal myopathy type 3 (5, 7–14).

NOTCH2NLC belongs to one of three NOTCH2 N-terminal-like (NOTCH2NL) paralogs (NOTCH2NLA, NOTCH2NLB, and NOTCH2NLC), which are only found in the human genome. Evolutionarily, the NOTCH2NL genes are derived from gene duplication and gene conversion of NOTCH2 and function in cortical neurogenesis exclusively in the human species (15, 16). Genetic analyses have shown that the extent of pathogenic GGC repeats in NOTCH2NLC ranges from about 60 to 500 (7). On the basis of the age of onset, NIID can be categorized into three subgroups: infantile, juvenile, and adult. To date, expanded GGC repeats in NOTCH2NLC have been reported in both juvenile and adult NIID (1, 5, 6, 17). Clinically, the manifestation of NIID is highly heterogeneous and may include dementia, muscle weakness, peripheral neuropathy, cerebellar ataxia, parkinsonism, seizure, and encephalitic episodes (5, 18–21). Immunohistochemically, the eosiophilic intranuclear inclusions in NIID are positive for p62 and ubiquitin. Although these findings in tissues of patients provide important clues for pathogenesis, the underlying molecular mechanism remains unclear due to the lack of genetic models. Current NIID mouse models were generated by overexpression of NOTCH2NLC with artificial pathogenic GGC repeats in the adult mouse brain via retro-orbital adeno-associated virus (AAV) injection (22) or by in utero electroporation in the mouse neocortex (23),...
making it difficult to define the specific effect of pathogenic GGC repeat expansions at different ages and in various types of tissues.

Here, we report the first transgenic mouse model in which the exon 1 of NOTCH2NLC gene is expressed, with either normal GGC repeats (17 GGC repeats) or expanded GGC repeats (98 GGC repeats) cloned from the genomic DNA of control and NIID patient, respectively. We show that GGC repeat expansions in NOTCH2NLC produced multiple polypeptides [polyglycine (polyG), polyalanine (polyA), or polynarginine (polyR) containing protein] through AUG-dependent translation, which together could cause neuronal toxicity. The NOTCH2NLC-(GGC)98 mice displayed severe neurodegeneration, motor dysfunction, and cognitive deficits, faithfully recapitulating the clinical and pathological features associated with NIID. Gene expression profiling revealed multiple pathways altered in different brain regions of the NOTCH2NLC mouse model, including the prefrontal cortex, cerebellum, and hippocampus. We further established human induced pluripotent stem cells (iPSCs) from patients with NIID and differentiated them into neural progenitor cells (NPCs). We also performed gene expression profiling of NPCs and compared it with that of our NOTCH2NLC mouse model. We identified overlapping patterns of alternative splicing (AS) events shared between human NPCs (hNPCs) and mouse models. Among the AS sites, motif analyses revealed the enrichment of the RNA binding motifs of heterogeneous nuclear ribonucleoprotein M (hnRNPM). Mechanistically, we show that hnRNPM could bind to NOTCH2NLC-polyG and NOTCH2NLC-polyA and be sequestered into inclusions containing NOTCH2NLC-polyG and NOTCH2NLC-polyA. Furthermore, overexpression of hnRNPM could ameliorate cellular toxicity caused by expanded GGC repeats within NOTCH2NLC. These results together suggest that dysfunction of hnRNPM-mediated regulation could play a vital role in the molecular pathogenesis of NIID.

RESULTS
Expression of NOTCH2NLC-(GGC)98 produces polyG and polyA in an AUG-dependent manner and causes neuronal toxicity in vitro
The human NOTCH2NLC gene has two different splicing isoforms, transcript variant 1 (TV1) and transcript variant 2 (TV2) (fig. S1A). We analyzed expression levels of the transcript variants using published human brain RNA sequencing (RNA-seq) datasets (24) and found that TV1 was the dominant transcript in different brain regions (fig. S1B). A similar pattern was also observed in human peripheral blood samples (fig. S1, C and D). Therefore, we focused on NOTCH2NLCTV1 and referred to it as NOTCH2NLCTV1 unless otherwise stated. The GGC repeat expansions reside in the 5′ UTR of NOTCH2NLCTV1. Notably, an unconventional AUG (uAUG) codon lies upstream and a TAA codon downstream, both in the same reading frame as the GGC repeat, which could produce a short polyG-containing protein named uN2CpolyG or N2NLcpolyG (Fig. 1A and fig. S1A) (22, 23). In addition, GGC repeat expansions could be translated into polyA or polyR via repeat-associated non-AUG (RAN) translation or ribosomal frameshifting (Fig. 1A), which had been reported in FMR1 with CGG repeat expansions (25, 26) or in C9ORF72 with GGC repeat expansions (27).

To test this possibility, we first cloned exon 1 of NOTCH2NLCTV1 harboring 17 GGC repeats or 98 GGC repeats from genomic DNA of control and NIID patient and fused the repeats with a green fluorescent protein—hemagglutinin (GFP-HA) tag (start codon removed) in each of three reading frames (Fig. 1B and fig. S2, A to C). As expected, when overexpressed in human embryonic kidney (HEK) 293 cells, homogeneous cytoplasmic GFP fluorescence was detected with the NOTCH2NLCTV1-(GGC)98 construct in the polyG frame, while abundant intranuclear or perinuclear GFP fluorescent aggregates were found for NOTCH2NLCTV1-(GGC)98 in the polyG frame (Fig. 1C). Immunoblotting using anti-GFP antibody showed that NOTCH2NLCTV1-(GGC)98 overexpression led to the appearance of bands around 35 kDa. Soluble NOTCH2NLCTV1-(GGC)98–polyG appeared at about 40 kDa, while aggregated NOTCH2NLCTV1-(GGC)98–polyG remained in the stacking gel (Fig. 1E). We developed a rabbit polyclonal antibody (PEP122), whose epitope was identical to that of a 4D12 antibody (Fig. 1D). The 4D12 antibody was validated in both animal model and patients with NIID in a previous report (22). Immunoblotting using PEP122 antibody presented similar results with that using anti-GFP antibody (Fig. 1F).

Unexpectedly, a small quantity of intranuclear polyA inclusions were observed when the polyA frame NOTCH2NLCTV1-(GGC)98 construct was expressed, while cytoplasmic GFP fluorescence was also detected with NOTCH2NLCTV1-(GGC)98 in the polyA frame. The origin of this protein may be translation starting with an ATG initiation codon downstream of GGC repeats but upstream of the GFP sequence (Fig. 1C and fig. S2B). This was further confirmed by immunoblotting with anti-GFP antibody because both NOTCH2NLCTV1-(GGC)98 and NOTCH2NLCTV1-(GGC)98 constructs generated the same band size around 32 kDa. Same as polyG, NOTCH2NLCTV1-(GGC)98–derived polyA had a soluble form around 48 kDa and also formed aggregates that remained in the stacking gel (Fig. 1G). To further confirm the existence of polyA-containing protein, we developed a rabbit antibody named PEP123, which was designed to recognize the C terminus of polyA (Fig. 1D). Immunoblotting showed that anti-PEP123 antibody could identify the same smeared polyA bands as anti-GFP (Fig. 1H). Moreover, liquid chromatography–tandem mass spectrometry analysis on HEK293 cells transfected with NOTCH2NLCTV1-(GGC)98 identified partial peptide sequences of polyG and polyA proteins (fig. S2E). These results suggested that polyA-containing proteins were being produced. The production of polyA may result from at least two mechanisms: RAN translation or AUG-initiated ribosomal frameshifting translation. Because the production of NOTCH2NLCTV1-polyG was uAUG dependent (22, 23), mutation of the uAUG codon should disturb the translation of polyA, if polyA was produced from ribosomal frameshifting. To this end, the uAUG codon of polyG frame in the NOTCH2NLCTV1-(GGC)98–polyA–GFP construct was mutated to AAG (Fig. 1I). Immunoblotting showed that the polyA inclusion, which stayed in the stacking gel with the uAUG construct, disappeared when the uAUG was mutated (Fig. 1I). These results indicated that the translation of polyA was uAUG codon dependent.

In addition, immunoassaying showed that both polyG and polyA inclusions colocalized with ubiquitin and P62 (fig. S3A), reminiscent of what has been observed in patients with NIID (5). The colocalization of polyG and polyA with ubiquitin and P62 led us to check whether polyG and polyA appeared in the same inclusions. To check this possibility, the GFP-HA tag of NOTCH2NLCTV1-(GGC)98–GFP was replaced by a 3*HA/3*Myc/3*Flag (3TAG) tag, which was in reading frame with polyA, polyR, and polyG, respectively (Fig. 1B and fig. S2D). Seventy-two hours after transfection in HEK293 cells, coimmunostaining using anti-Flag and anti-PEP122 showed immunofluorescence of polyG (Fig. 1K); polyA also showed coimmunofluorescence when using anti-HA and anti-PEP123 antibodies.
Fig. 1. Expression of NOTCH2NLC-(GGC)₉₈ produces multiple polypeptides and causes cellular toxicity in vitro. (A) Schematic diagram of the putative mutant NOTCH2NLC proteins. Glycine, Gly; alanine, Ala; arginine, Arg. (B) Schematic diagram of NOTCH2NLC constructs. NOTCH2NLC exon 1 with 17 GGC repeats [(GGC)₁₇ for short] and 98 GGC repeats [(GGC)₉₈ for short] were fused with a GFP tag (start codon removed) or 3*HA/3*Myc/3*Flag (3Tag) tag. (C) Direct GFP fluorescence of HEK293 cells transfected with NOTCH2NLC-GFP constructs. The magenta arrows indicate polyA aggregates. Green, GFP; blue, 4′,6-diamidino-2-phenylindole (DAPI). Scale bar, 100 μm. (D) Putative amino acid sequences of polyG-containing and polyA-containing proteins. The underlined sequences indicated the epitope of antibodies against polyG (PEP122) and polyA (PEP123). Ab, antibody. (E and F) Immunoblotting (IB) of HEK293 cells transfected with NOTCH2NLC-GFP (polyG frame) constructs using anti-GFP and PEP122 antibodies. (G and H) Immunoblotting of HEK293 cells transfected with NOTCH2NLC-GFP (polyA frame) constructs using anti-GFP and PEP123 antibodies. (I) Frameshifting hypothesis for polyA. The production of polyA may result from AUG-initiated ribosomal frameshifting translation. (J) Immunoblotting of HEK293 cells transfected with NOTCH2NLC-GFP (polyA frame with uAUG mutation) construct. (K to M) Coimmunostaining of HEK293 cells transfected with NOTCH2NLC-3Tag construct using anti-Flag and anti-PEP122 (K) or using anti-HA and anti-PEP123 (L) or using anti-Flag and anti-HA (M). Scale bars, 10 μm. (N) Cell viability of Neuro-2a cells transfected with NOTCH2NLC-GFP constructs was analyzed by the Cell Counting Kit-8 (CCK-8) method. Data are presented as means ± SEM. (N = 5 independent experiments, **P = 0.0036, two-tailed t test). (O and P) Cell viability of cultured mouse primary cortical neurons transfected with NOTCH2NLC-GFP constructs. Data are presented as means ± SEM (N = 4 independent experiments, at least 120 cells were counted per experiment, ****P < 0.0001, two-tailed t test). Green, GFP; magenta, Tuj1. Scale bar, 10 μm.
(Fig. 1L). Coimmunostaining showed that polyG inclusions colocalized with polyA inclusions, and polyG-containing protein was aggregation prone while polyA-containing protein was more soluble (Fig. 1M). These results together indicated that GGC repeat expansions at the NOTCH2NLC locus could be translated into polyG- and polyA-containing proteins. Although two previous studies reported no polyA and polyR products coming from NOTCH2NLC in vitro, here, we identified the detection of polyA protein. The reasons for this discrepancy may be complicated. One possible reason is that our patient DNA-derived constructs harbor GGA interruptions within the GGC repeats, while the constructs Boivin et al. and Zhong et al. (22, 23) used contained homogeneous synthetic GGC repeats.

Next, we examined whether the products of NOTCH2NLC with GGC repeat expansions were toxic in vitro. Both NOTCH2NLC-(GGC)17- and NOTCH2NLC-(GGC)98-containing constructs were transiently transfected into Neuro-2a cells, followed by a cell viability assay. NOTCH2NLC-(GGC)98-transfected cells showed a significant decrease in viability when compared to NOTCH2NLC-(GGC)17-transfected cells (Fig. 1N). Transfected Neuro-2a cells were also treated with 25 mM retinoic acid for 72 hours to induce differentiation and promote neurite outgrowth. NOTCH2NLC-(GGC)98-expressing cells grew shorter neurites than NOTCH2NLC-(GGC)17-expressing cells (Fig. S3, B and C). We further assessed cellular toxicity in cultured mouse primary cortical neurons by analyzing the proportion of GFP-positive neurons with neurites 48 hours after transfection. NOTCH2NLC-(GGC)98 caused more severe cell death when compared to NOTCH2NLC-(GGC)17 (Fig. 1O and P). These results demonstrated neuronal toxicity associated with the expression of NOTCH2NLC-(GGC)98 in vitro.

**Expression of NOTCH2NLC-(GGC)98 produces multiple polypeptides in NOTCH2NLC transgenic mice in an age-dependent manner**

To systematically explore the pathogenic role of the GGC repeat expansions in the NOTCH2NLC gene underlying NIID, we generated two conditional transgenic NOTCH2NLC mouse models by inserting NOTCH2NLC exon 1 with 17 GGC repeats or 98 GGC repeats (with GGA interruption; fig. S2D) into the Rosa26 site. Contrasting with a previously reported AAV-injected mouse model in which only polyG was expressed (22), our transgenic mouse models may express both polyG and polyA, based on our in vitro observations (Fig. 1). Hence, the 3′Tag tag was used for convenient detection of translated products of NOTCH2NLC in mice. Because intranuclear inclusions were found in the nervous system and peripheral tissues in patients with NIID, the conditional transgenic mice were crossed with Eli1a-Cre mice to express NOTCH2NLC-(GGC)17 or NOTCH2NLC-(GGC)98 ubiquitously (figs. S4A and S5A). Genotyping was confirmed by polymerase chain reaction (PCR) and Sanger sequencing (fig. S4, B and C). The mRNA levels of NOTCH2NLC-(GGC)17 or NOTCH2NLC-(GGC)98 were comparable in two transgenic mice (fig. S4D). Because NOTCH2NLC is only present in the human genome, it was difficult to compare its expression level between the transgenic mice and human samples. By examining the fragments per kilobase of exon model per million mapped fragments (FPKM) of NOTCH2NLC, we found that the expression level of NOTCH2NLC in our mouse model was comparable to or lower than hNPCs, human differentiated neurons, and forebrain cortex from human postmortem brains (fig. S4E).

From birth to postnatal 40 days (P40), the wild-type (WT), NOTCH2NLC-(GGC)17, and NOTCH2NLC-(GGC)98 mice showed no overt anomalies in appearance. However, after 40 days of age, the NOTCH2NLC-(GGC)98 mice gradually developed a progressive phenotype characterized by inactivity, hunchback, aberrant posture, and ataxia (movie S1). NOTCH2NLC-(GGC)98 mice died at around 2 months, regardless of gender. Immunofluorescent staining of transgenic mouse brains at P50 showed that NOTCH2NLC-(GGC)98 formed a large amount of polyG inclusions throughout the brain, including the striatum, hippocampus, cerebellum, and cortex, while comparable inclusions were hard to detect in the NOTCH2NLC-(GGC)17 mice (Fig. 2A and fig. S5B). Furthermore, small amounts of polyA and polyR inclusions were also seen in the hippocampus and, to a lesser extent, in the striatum and cortex of the NOTCH2NLC-(GGC)98 mice (Fig. 2A); confocal images further indicated the presence of intranuclear or perinuclear polyG, polyA, and polyR inclusions in the mouse brains (Fig. 2A). PEP122 and PEP123 antibodies were also used to examine the inclusions. As expected, PEP122- and PEP123-positive inclusions colocalized with Flag and HA, respectively; most of them colocalized with P62 and ubiquitin as well (fig. S6). Electron microscopy images of NOTCH2NLC-(GGC)98 mice cerebellum showed obvious neuronal intranuclear inclusions exhibiting a pile of round dense filamentous materials without membrane structure (Fig. 2B), which were similar to those observed in patients with NIID (4, 5, 7). Meanwhile, Western blotting using anti-Flag, anti-HA, and anti-Myc antibodies showed abundant polyG, polyA, and polyR aggregates in the NOTCH2NLC-(GGC)98 mouse brains and soluble form of polyG and polyA with expected size (Fig. 2C). PolyG and polyA aggregates were further confirmed by anti-PEP122 and anti-PEP123 antibodies, respectively (Fig. 2D). Double immunostaining using anti-Flag and anti-HA antibodies showed the colocalization of the polyG inclusions with polyA inclusions (Fig. 2E).

Because NOTCH2NLC mice died at around 2 months old, a point at which major brain development in mice is complete (28), we then examined expression patterns of polyG inclusions at earlier stages. Immunofluorescent staining of NOTCH2NLC-(GGC)98 mouse brain showed clearly distinct intranuclear polyG inclusions mainly in the striatum, hippocampus (CA1) and cortex, and less in the cerebellum at P12 (Fig. 2F and fig. S7). At P32, however, polyG inclusions had accumulated across the entire brain—not only in the nucleus but also outside the nucleus (Fig. 2F and fig. S7); Western blotting analysis of Flag (polyG) and HA (polyA) in the cortex at P12 and P32 of NOTCH2NLC-(GGC)98 and NOTCH2NLC-(GGC)17 mice demonstrated that polyG and polyA aggregates became more abundant over time (Fig. 2G). These results together suggested that NOTCH2NLC-(GGC)98-derived polypeptides accumulate in an age-dependent manner in vivo.

**Expression of NOTCH2NLC-(GGC)98 causes neurodegeneration in mice**

To determine the impact of widespread expression of NOTCH2NLC-(GGC)98, we first performed anatomical examinations on NOTCH2NLC-(GGC)98 mice and control mice. In general, we observed no remarkable abnormalities in the morphology of visceral organs from NOTCH2NLC-(GGC)98 mice at P50, regardless of gender. We then investigated whether there was neuronal loss in different brain regions of NOTCH2NLC-(GGC)98 mice. Immunofluorescent staining clearly showed that the numbers of NeuN-positive cells in the hippocampi of NOTCH2NLC-(GGC)98 mice were reduced significantly when compared to the control mice at P50 (Fig. 3, A and C, and fig. S8A). The numbers of calbindin-labeled Purkinje cells in the cerebellum were markedly reduced in NOTCH2NLC-(GGC)98 mice at P32 (Fig. 3,
Fig. 2. Expression of NOTCH2NLC-(GGC)₉₈ produces multiple polypeptides in NOTCH2NLC transgenic mice in an age-dependent manner. (A) Immunofluorescent staining against Flag (polyG), HA (polyA), and Myc (polyR) in the brain of NOTCH2NLC-(GGC)₉₈ mice at P50. The intranuclear or perinuclear polyG, polyA, and polyR inclusions were observed under higher magnification in the hippocampus of NOTCH2NLC-(GGC)₉₈ mouse. Green, Flag/HA/Myc; blue, DAPI. Scale bar, 50 µm. (B) Electron microscopy of cerebellar neurons from the NOTCH2NLC-(GGC)₁₇ and NOTCH2NLC-(GGC)₉₈ mice at P50. Yellow arrow indicates the intranuclear inclusions. Scale bar, 2 µm. (C and D) Western blotting against Flag (polyG), HA (polyA), and Myc (polyR) tag (C) and PEP122 and PEP123 (D) in the cortex from control and NOTCH2NLC-(GGC)₉₈ mice at P50. (E) Immunofluorescent staining against Flag (polyG) and HA (polyA) in cortex of NOTCH2NLC-(GGC)₉₈ mice at P50. Green, Flag; magenta, HA. Scale bar, 10 µm. (F) Immunofluorescent staining against Flag (polyG) in the different brain regions of NOTCH2NLC-(GGC)₉₈ mice at P12 and P32. Green, Flag; blue, DAPI. Magenta arrows indicate the intranuclear inclusions in the cerebellum. Scale bar, 50 µm. (G) Western blotting against Flag (polyG) and HA (polyA) in the cortex from NOTCH2NLC-(GGC)₁₇ and NOTCH2NLC-(GGC)₉₈ mice at P12 and P32.
Fig. 3. Expression of NOTCH2NLC-(GGC)₉₈ causes neurodegeneration in mice. (A and B) Immunofluorescence against NeuN in the hippocampus (A) and calbindin in the cerebellum (B) from NOTCH2NLC mice. Red, NeuN; green, calbindin. Scale bars, 100 μm. (C and D) Quantification of the staining intensity of NeuN (C) and the number of Purkinje neurons (D) from NOTCH2NLC-(GGC)₁₇ and NOTCH2NLC-(GGC)₉₈ mice at P50. Data are presented as means ± SEM. (N = 3 mice per group, *P = 0.0152 (C), **P = 0.0086 (D), two-tailed t test). (E) Nissl staining of the cortex and cerebellum from NOTCH2NLC-(GGC)₁₇ and NOTCH2NLC-(GGC)₉₈ mice at P50. Red arrowheads indicate dark shrunken neurons; red arrows indicate the Purkinje neurons. Scale bar, 50 μm. (F and G) Quantification of the number of Purkinje neurons in the cerebellum (F) and the number of normal neurons in the cortex (G). Data are presented as means ± SEM. (N = 3 mice per group, ***P = 0.0001 (F) and **P = 0.0033 (G), two-tailed t test). (H and I) Western blotting against NeuN in the hippocampus (HIP) and against calbindin in the cerebellum (CB) from NOTCH2NLC-(GGC)₁₇ and NOTCH2NLC-(GGC)₉₈ mice at P50. Normalized relative expressions of NeuN and calbindin are present in (I). Data are presented as means ± SEM. (N = 3 mice per group, *P = 0.0434 and **P = 0.0038, two-tailed t test). (J and K) Immunofluorescence against GFAP and Iba1 in the HIP and CB from NOTCH2NLC-(GGC)₁₇ and NOTCH2NLC-(GGC)₉₈ mice at P50 (J). (K) Quantification of GFAP and Iba1 relative staining intensity. Data are presented as mean ± SEM. (N = 3 mice per group, ****P < 0.0001 (GFAP in HIP), **P = 0.0055 (GFAP in CB), **P = 0.0023 (Iba1 in HIP), and **P = 0.0048 (Iba1 in CB), two-tailed t test). Green, GFAP; red, Iba1. Scale bar, 100 μm.
B and D). Nissl staining also confirmed that most of the Purkinje cells were lost at P50 (Fig. 3, E and F). In the cortex, a large number of dark shrunken neurons and a significantly reduced number of normal neurons indicated widespread neuronal death in NOTCH2NLC-(GGC)98 mice at P50 (Fig. 3, E and G). We also performed Western blotting of NeuN and calbindin in the hippocampus and cerebellum, respectively, which allowed for quantitative analysis of the relative expression level of NeuN and calbindin, and found a significant reduction in NOTCH2NLC-(GGC)98 mice (Fig. 3, H and I).

Next, we examined gliosis, another marker of neurodegeneration, which can be assessed by glial fibrillary acidic protein (GFAP) (astrocyte marker) and ionized calcium binding adapter molecule 1 (IBA1) (microglia marker) staining. As expected, activation of astrocytes and microglia was seen in the hippocampus, cerebellum, and striatum of NOTCH2NLC-(GGC)98 mice at P50 (Fig. 3, J and K, and fig. S8B). Together, our results suggested that the expression of NOTCH2NLC-(GGC)98 could cause neurodegeneration and inflammation in the nervous system.

Expression of NOTCH2NLC-(GGC)98 causes muscle degeneration in mice
Because muscle weakness is one of the major clinical manifestations of NIID, we wanted to know whether the expression of NOTCH2NLC-(GGC)98 would influence muscle function in the mouse model. Immunoblotting of gastrocnemius muscle showed aggregated polyG and polyR in the NOTCH2NLC-(GGC)98 mouse (Fig. 4A). Then, we analyzed the distribution of NOTCH2NLC-derived polypeptides in the gastrocnemius muscle. Resembling the pattern observed in the brain, polyG and polyR also accumulated around the nuclei of muscle cells (Fig. 4B). Hematoxylin and eosin staining of gastrocnemius muscle

![Fig. 4. Expression of NOTCH2NLC-(GGC)98 causes muscle degeneration in mice.](image)

(A) Western blotting against polyG (Flag), polyA (HA), and polyR (Myc) in the gastrocnemius muscle from WT, NOTCH2NLC-(GGC)17, and NOTCH2NLC-(GGC)98 mice. (B) Immunofluorescent staining against Flag (polyG) and Myc (polyR) in the gastrocnemius muscle of NOTCH2NLC-(GGC)17 and NOTCH2NLC-(GGC)98 mice at P50. Magenta arrows indicate the perinuclear inclusions. Scale bar, 20 μm. (C) Representative images of gastrocnemius muscle sections from NOTCH2NLC-(GGC)17 and NOTCH2NLC-(GGC)98 mice at P50 using hematoxylin and eosin staining. Scale bar, 100 μm. (D to F) EMG recording for gastrocnemius muscle from NOTCH2NLC-(GGC)17 and NOTCH2NLC-(GGC)98 at P50. Spontaneous activity (fibrillation potentials and positive sharp waves) of the gastrocnemius muscle was observed in NOTCH2NLC-(GGC)98 mice (D). Representative images of motor unit potential (MUP) (E) and quantification of average amplitude and duration of MUP (F) in NOTCH2NLC-(GGC)17 and NOTCH2NLC-(GGC)98 mice. Data are presented as means ± SEM. N = 5 mice per group, **P = 0.0086 and *P = 0.0246.
revealed normal morphology in the NOTCH2NLC-(GGC)\textsubscript{17} mice, presented as well-defined muscle cells in which the nuclei were localized peripherally (Fig. 4C). However, fragmented muscle morphology and multiple internalized or centralized nuclei, a feature of regenerated muscle cells after degeneration, were found in the NOTCH2NLC-(GGC)\textsubscript{98} mice (Fig. 4C). The notable pathology we observed raised the possibility that the degeneration could also degrade muscle function. Electromyography (EMG) recordings of gastrocnemius muscle revealed fibrillation potentials and positive sharp waves, indicating denervated fibers (Fig. 4D). Moreover, a reduced average amplitude and duration of motor unit potential were observed in the NOTCH2NLC-(GGC)\textsubscript{98} mice (Fig. 4, E and F), indicating muscle degeneration caused by expanded GGC repeats.

**NOTCH2NLC-(GGC)\textsubscript{98} mice display behavioral phenotypes associated with NIID**

Because patients with NIID mainly display dementia and motor symptoms, we wanted to determine whether such deficits could be recapitulated in our mouse models. After 40 days of age, the NOTCH2NLC-(GGC)\textsubscript{98} mice gradually developed progressive inactivity, hunchback, aberrant posture, ataxia (Fig. 5A and movie S1), and premature death (Fig. 5B), regardless of gender. Because the body weight of NOTCH2NLC-(GGC)\textsubscript{98} mice decreased at the terminal stage (Fig. 5C), most behavioral tests were performed before P40 to avoid the effects of reduced body weight on locomotion (Fig. 5D).

We first performed the open-field test to measure physical/motor activity. The NOTCH2NLC-(GGC)\textsubscript{98} mice showed markedly reduced total duration of activity and total distance traveled when compared to the control mice (Fig. 5, E to G), suggesting decreased motivation or motor ability. Meanwhile, the percentage of time spent and distance traveled in the central area displayed no significant differences, indicating that the NOTCH2NLC-(GGC)\textsubscript{98} mice did not exhibit a heightened level of anxiety-like behaviors (Fig. 5, H and I).

The novel object recognition test is commonly used to evaluate cognitive function (29, 30). Because decreased activity of NOTCH2NLC-(GGC)\textsubscript{98} mice was observed in the open-field test, the exploration ratio (time spent exploring one object over time spent exploring both objects) was analyzed. In familiarization sessions, two identical objects were placed in the box (Fig. 5I), and both NOTCH2NLC mice and control mice displayed a comparable preference for one object (Fig. 5K). However, when the less-preferred object was replaced by a novel object, both NOTCH2NLC-(GGC)\textsubscript{17} mice and WT mice spent significantly more time exploring the novel object, while the NOTCH2NLC-(GGC)\textsubscript{98} mice spent more time on the familiar object (Fig. 5L), which indicated impaired object recognition.

To evaluate motor functions, we conducted the rotarod test, wire-hang test, and cylinder test. In the rotarod test, the NOTCH2NLC-(GGC)\textsubscript{98} mice exhibited notably poorer performance on the rod compared to the control mice (Fig. 5M). We also found that the length of time of NOTCH2NLC-(GGC)\textsubscript{98} mice hanging on the inverted cage lid was shorter than that of control mice (Fig. 5N). In the cylinder test, the NOTCH2NLC-(GGC)\textsubscript{98} mice could barely stand up to touch the cylinder with their forepaws (Fig. 5O). These results indicated locomotor deficits in the NOTCH2NLC-(GGC)\textsubscript{98} mice.

**Molecular signature associated with the expression of NOTCH2NLC-(GGC)\textsubscript{98} in NOTCH2NLC transgenic mice**

Both brain-preferential (22) and E1a-Cre-induced ubiquitous expression of NOTCH2NLC-(GGC)\textsubscript{98} led to NIID-like motor symptoms, suggesting that the brain is the main tissue involved in the pathogenesis of NIID. Therefore, we wanted to determine whether a molecular signature is associated with the expression of NOTCH2NLC-(GGC)\textsubscript{98} in the brain. RNA-seq was performed on P50 tissue samples from major brain regions related to cognitive and motor functions: prefrontal cortex, cerebellum, and hippocampus. In total, 2465, 1132, and 1826 significantly differentially expressed genes (DEGs) were identified in the cerebellum, hippocampus, and cerebral cortex, respectively, when comparing NOTCH2NLC-(GGC)\textsubscript{98} mice to the NOTCH2NLC-(GGC)\textsubscript{17} mice (Fig. 6A and tables S1 to S3). These three major brain regions shared a large number of DEGs (Fig. 6, B and C). The commonly down-regulated genes were mainly associated with neuronal activities, such as modulation of synaptic transmission, long-term potentiation, and signal transduction, and especially involved in calcium signaling and adenosine 3’,5’-monophosphate (cAMP) signaling (Fig. 6, D and E, and fig. S9). The commonly up-regulated genes were particularly enriched in immune and cellular defense-related pathways (fig. S9A), which was consistent with increased gliosis seen in the NOTCH2NLC-(GGC)\textsubscript{98} mouse brain (Fig. 3, I and K).

A limitation of bulk RNA-seq is that it typically measures average gene expression across many molecularly diverse cell types. Recent studies have shown that the proportions of different cell types in the brain might show correlations with various phenotypes, based on certain pathophysiological conditions (31). Hence, we investigated the proportion of each cell types in the prefrontal cortex, cerebellum, and hippocampus of NOTCH2NLC mice, using a cellular deconvolution approach that we developed previously (32–35). In all investigated brain regions, there were several prominent cell type alterations in NOTCH2NLC-(GGC)\textsubscript{98} mice, affecting the proportion of cell types such as neurons, astrocytes, and microglia (Fig. 6F and fig. S10). A large proportional increase in astrocytes and microglial cells was consistent with the pathophysiological phenotypes of gliosis and excessive neuronal cell death (Fig. 3 and fig. S8A). Intriguingly, in the cerebellum, Bergmann glial cells appeared to decrease in proportion in NOTCH2NLC-(GGC)\textsubscript{98} mice (Fig. 6F). Bergmann glial cells are proposed to contribute to plasticity and information processing in the cerebellum by actively interacting with Purkinje cells, through modulation of calcium signaling (36, 37). A decreased proportion of Bergmann glial cells was consistent with significant Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Gene Ontology (GO) enrichment in calcium signaling pathway genes in NOTCH2NLC-(GGC)\textsubscript{98} mice (Fig. 6, D and E, and fig. S9, A and B).

**Dysregulated A5 is a common molecular alteration in both NOTCH2NLC-(GGC)\textsubscript{98} mice and hNPCs**

Because the NOTCH2NLC gene is only present in the human genome and not in mouse, we established iPSCs from patients with NIID and control individuals. These iPSC lines showed no obvious differences in proliferation and pluripotency gene expression (fig. S11, A and B; details about iPSCs lines were provided in table S4). The iPSC lines were then differentiated into hNPCs, which displayed ubiquitin and NOTCH2NLC double-positive aggregates (Fig. 7A). In NIID hNPCs, expression of NOTCH2NLC TV1 was dominant, which was similar to what was observed in human brain tissue (fig. S11C). We performed gene expression profiling and compared these results with the findings from the NOTCH2NLC mice. Although hundreds of genes were differentially expressed in the hNPCs of NIID (Fig. 7B and table S5), we only observed a small number of
overlapping DEGs between hNPCs and the NOTCH2NLC mouse model (4.2 to 8.6%; Fig. 7C and fig. S11D).

We next investigated alterations of AS in mouse cerebellum, cortex, hippocampus, and hNPCs upon expression of expanded GGC within NOTCH2NLC (Fig. 7D and fig. S12A). AS events were compared between mice and hNPCs (Fig. 7E, fig. S12B, and tables S6 to S9). Unlike the low overlapping rate of DEGs between them, about 15.9 to 17.3% of differential AS events in the hippocampus, cerebellum, and cortex of NOTCH2NLC-(GGC)98 mice overlapped with those in the hNPCs. To further evaluate AS changes, five types of AS events, including skipped exons (SEs), retained introns, alternative 5’ splice sites (A5SS), alternative 3’ splice sites (A3SS), and mutually exclusive exon (MEX), were analyzed by replicate multivariate analysis of transcript splicing (rMATS) on the RNA-seq data of the NOTCH2NLC mice. In total, 1125 genes in the cerebellum, 839 genes in the hippocampus, and 856 genes in the cortex showed 1532, 1089, and 1147 differential AS events, respectively (fig. S12C). In our deconvolution analysis, most of the cell population changes occurred in astrocytes and microglial cells (Fig. 6F and fig. S10, A and B). Approximately 30% of 856 genes with
Fig. 6. Differential gene expression profiles in NOTCH2NLC-(GGC)$_{98}$ mice. (A) Shown are volcano plots of gene expression changes in the cerebellum, hippocampus, and prefrontal cortex of NOTCH2NLC-(GGC)$_{98}$ mice compared to NOTCH2NLC-(GGC)$_{17}$ mice. Colored dots indicate statistically significant DEGs [false discovery rate (FDR) < 0.05]. Blue dots are down-regulated genes [log$_2$(fold change) < −0.8] and red dots are up-regulated genes [log$_2$(fold change) > 0.8]. Cuffdiff. N = 3 biological replicates per genotype per condition. (B and C) The numbers of down-regulated (B) and up-regulated (C) overlapping genes between the cerebellum, hippocampus, and cortex DEGs are shown in the Venn diagrams. (D and E) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (D) and Gene Ontology (GO) biological process (E) enrichment analyses for the overlapping down-regulated DEGs identified from RNA-seq of NOTCH2NLC-(GGC)$_{98}$ mice. The numbers on the bars are two-sided P values using Fisher’s exact. ECM, extracellular matrix; cGMP-PKG, cyclic guanosine 3',5'-monophosphate-dependent protein kinase G. (F) The cellular proportions of neurons, Bergmann glial cells, astrocytes, and microglial cells were estimated using MuSiC in the cerebellum samples from NOTCH2NLC-(GGC)$_{98}$ mice and NOTCH2NLC-(GGC)$_{17}$ mice. N = 3 mice per group, two-tailed t test, $P = 0.1360$ (neuron), $P = 0.0540$ (astrocyte), $P = 0.0368$ (microglia), and $P = 0.1232$ (Bergmann glia).
significantly altered AS were differentially expressed in \textit{NOTCH2NL}-(GGC)\textsubscript{98} mouse cortex.

Because the interaction partners of expanded repeat-related polypeptides have been reported to contribute to pathogenesis across several repeat expansion disorders (38), we then looked for proteins that interact with \textit{NOTCH2NL}-derived polypeptides. We overexpressed \textit{NOTCH2NL}-(GGC)\textsubscript{17} or \textit{NOTCH2NL}-(GGC)\textsubscript{98} in SH-SY5Y cells using lentivirus infection and then performed immunoprecipitation and mass spectrometry to identify associated proteins. A total of 41 proteins were identified exclusively in \textit{NOTCH2NL}-(GGC)\textsubscript{98} mice compared to control hNPCs. Cuffdiff. N = 4 for NID group; N = 3 for control group. (C) The overlaps of up-regulated and down-regulated DEGs between hNPCs and the hippocampus of \textit{NOTCH2NL}-(GGC)\textsubscript{98} mouse are shown in the Venn diagrams (P value = 9.56 × 10\textsuperscript{-5} for up-regulated DEG overlaps and P value = 1.18 × 10\textsuperscript{-12} for down-regulated DEG overlaps. The P value was obtained by chi-square test). (D) Shown are plots of differential AS events in hNPCs and the hippocampus of \textit{NOTCH2NL}-(GGC)\textsubscript{98} mice compared to the corresponding controls. N = 4 for NID group; N = 3 for control group. P value < 0.05, inclusion level difference > 0.2, rMATS. (E) The prediction of hnRNPM binding on SE events was performed using RBPmap with high stringency. (G) The proportions of differential AS events of each sample [the cerebellum, hippocampus, or cortex of \textit{NOTCH2NL}-(GGC)\textsubscript{98} mice and hNPCs] bound by hnRNPM are shown in the pie charts.
with translation and RNA processing (fig. S13B), especially mRNA splicing. In this, group of 41 is the hnRNPM.

HnRNPM has been reported to be a component of the spliceosome complex and a splicing regulator for multiple genes (39–41). To gain insight into its possible role in altered patterns of AS, we examined the previously published large-scale cross-linking immunoprecipitation sequence (CLIP-seq) dataset (42). HnRNPM binding sites were enriched in UGUUG and ACAAC motifs, based on CLIP-seq data from human K562 and HepG2 cells (43). Because the exons in human and mouse are highly conserved, we used published hnRNPM CLIP-seq data from human K562 and HepG2 to look for hnRNPM binding sites flanking AS sites in mouse. The hnRNPM CLIP-seq peak files were downloaded from ENCODE (42). The hnRNPM binding sites reported in all replicates were selected by “bedtools intersect” and converted to mm9 by LiftOver. In cerebellum, about 9% of splice-out events labeled as red dots with higher percent spliced-in (PSI) in control than NIID were associated with hnRNPM, while only 5% of the splice-in events, which were labeled as blue dots in fig. S12A, were associated with hnRNPM, suggesting that hnRNPM-promotes slightly more splice-out than splice-in (chi-square test P value = 0.024). Similar trends were also observed in cortex and hippocampus with no statistical significance (Fig. 7D and fig. S12A).

The predicted binding motifs of hnRNPM were highly enriched in genes with altered AS events at average 79% in all samples including the hippocampus, cerebellum, cortex of NOTCH2NL-(GGC)48 mice, and NIID hNPCs by the analysis using RBPmap with high stringency and three consensus motifs, GGUUGGUGU, UGUUGU, and UGUUGU (Fig. 7F) (44). Moreover, we observed that about 34 to 43% of genes with altered AS events identified in NIID mice and as many as 56% of genes with altered AS events in hNPCs overlapped with the previously reported mRNAs bound by hnRNPM (Fig. 7G) (42). The high enrichment of hnRNPM binding sites/motifs regardless of species and consistent overlapping percentage of AS events across the samples suggested that dysregulated AS could be a common molecular alteration in both NIID mice and hNPCs.

**Expanded NOTCH2NL polypeptides interact with and sequester hnRNPM, while hnRNPM overexpression alleviates cellular toxicity**

To verify the interaction of hnRNPM and NOTCH2NL polypeptides, we performed coimmunoprecipitation and found that hnRNPM was associated with both NOTCH2NL-polyG and NOTCH2NL-polyA (Fig. 8A and fig. S14, A and B). Immunostaining also showed that hnRNPM was colocalized with NOTCH2NL-polyG– and NOTCH2NL-polyA–positive inclusions in the NOTCH2NL-(GGC)48 mice (Fig. 8, B and C). In addition, hnRNPM was also colocalized with ubiquitin-positive inclusions in both mice and the hNPCs (Fig. 8D and fig. S14C). These results suggested that the inclusions of NOTCH2NL-polyG and NOTCH2NL-polyA might sequester hnRNPM from its normal function.

If the sequestration of hnRNPM could affect its physiological functions and cause neuronal toxicity, then overexpression of hnRNPM might mitigate toxicity associated with the expanded GGC repeats. We tested this possibility using our established neuronal models. HnRNPM was transiently cotransfected with NOTCH2NL-(GGC)17 or NOTCH2NL-(GGC)48 constructs into cultured primary mouse neurons and Neuro-2a cells using the same evaluation criteria (Fig. 1, N to O, and fig. S3, B and C). The expression of hnRNPM with NOTCH2NL-(GGC)48 could increase cellular viability and significantly improve neurite outgrowth as compared to expressing the NOTCH2NL-(GGC)48 alone (Fig. 8, E to G). These results together confirmed the involvement of hnRNPM in neurodegeneration associated with the expression of expanded GGC repeats in NOTCH2NL.

**DISCUSSION**

NIID, a progressive neurodegenerative disorder characterized by widespread intranuclear inclusions in the nervous system and multiple visceral organs, is caused by the expansion of GGC repeats in the 5′UTR of the human-specific NOTCH2NL gene. The underlying molecular mechanism of NIID remains unclear. With the first transgenic mouse model of NIID, we have demonstrated that the expression of expanded GGC repeats leads to widespread intranuclear inclusions, severe neurodegeneration, motor dysfunction, and cognitive deficit. Furthermore, we established an hNPC cell model of NIID and identified a pattern of overlapping AS events between the NIID mouse and hNPC cell models by transcriptome analysis. Motif analyses revealed the enrichment for the binding motifs of hnRNPM among the AS sites. Mechanistically, we showed that NOTCH2NL polypeptides might sequester hnRNPM and disturb its function, while overexpression of hnRNPM could ameliorate cellular toxicity caused by expanded GGC repeats within NOTCH2NL. These results together suggested that dysfunction of hnRNPM could play a vital role in the molecular pathogenesis of NIID.

In this study, we developed an exon 1 but not the full gene model based on the following factors. Although NOTCH2NL has two different splicing isoforms, our results suggested that TV1 was the dominant isoform in human brain, hNPCs, and peripheral blood, while the 5′UTR (exon 1 plus partial exon 2) and coding sequences (CDS) of TV1 may produce two separate proteins (figs. S1 and S11C). Furthermore, evidence from our study and others indicated that the 5′UTR sequence of TV1 restrained the expression of CDS of NOTCH2NL (23). Therefore, we hypothesized that the 5′UTR of TV1 may produce the dominant product of the NOTCH2NL gene. Although partial exon 2 sequences (36 bp) were not included in the model, our models faithfully mimicked the clinical manifestation and pathological features associated with NIID patients.

Compared to the previously reported AAV-injected mouse model (22), our transgenic mouse model displayed more severe pathological and behavioral phenotypes and premature death (around 2 months old), which may occur for several reasons. First, the expression of NOTCH2NL with pathogenic GGC repeats began at 2 months of age in the AAV-injected mouse model, whereas expression started during embryonic development in the genetic mouse model, which was more consistent with the expression timing of NOTCH2NL genes in humans (15, 16). Second, in our genetic mouse model, NOTCH2NL with pathogenic GGC repeats produced not only polyG- but also polyA- and polyR-containing proteins, which might contribute to the phenotypes of the mice. Third, NOTCH2NL is expressed more broadly in the genetic mouse model. Consequently, NOTCH2NL polypeptide inclusions could cause lesions in other tissues besides the brain such as the muscle, a tissue linked to NOTCH2NL-related GGC repeat disorders (45, 46). Last, the constructs we used were directly cloned from a patient with NIID and thus harbor GGA interruptions, while the constructs Boivin et al. (22) used were homogeneous synthetic GGC repeats. It has been reported that different expansion compositions may cause different
Fig. 8. Expanded NOTCH2NLC polypeptides interact with and sequester hnRNPM while hnRNPM overexpression alleviates cellular toxicity. (A) Interaction of NOTCH2NLC-polyG and endogenous hnRNPM in HEK293 cells transfected with NOTCH2NLC-(GGC)_{17}-GFP-HA or NOTCH2NLC-(GGC)_{98}-GFP-HA was determined by coimmunoprecipitation. (B and C) Shown are the images of immunofluorescence staining against hnRNPM and NOTCH2NLC-polyG (B) or NOTCH2NLC-polyA (C) in the hippocampus of NOTCH2NLC-(GGC)_{17} and NOTCH2NLC-(GGC)_{98} mice. Green, NOTCH2NLC-polyG/polyA; magenta, hnRNPM; blue, DAPI. Scale bars, 20 μm. (D) Shown are representative images of immunofluorescence staining against hnRNPM in hNPCs. Green, hnRNPM; magenta, ubiquitin; blue, DAPI. Scale bar, 20 μm. (E) Cell viability of Neuro-2a cells transfected with NOTCH2NLC-GFP plus control or hnRNPM constructs was analyzed by the CCK-8 method. Data are presented as means ± SEM. (N = 4 independent experiments, one-way ANOVA followed with Tukey’s multiple comparisons test was performed, *P = 0.0327). n.s., not significant. (F and G) Cell viability of cultured mouse primary cortical neurons transfected with NOTCH2NLC-GFP plus control or hnRNPM constructs. Data are presented as means ± SEM (N = 4 independent experiments, at least 120 cells were counted per experiment, one-way ANOVA followed with Tukey’s multiple comparisons test was performed, ***P < 0.0005, ****P < 0.0001). (F) Representative immunofluorescence image. Green, GFP; magenta, Tuj1; gray, His. Scale bar, 10 μm.
in intracellular Ca\(^{2+}\), contributing to cellular and especially neuronal signaling and cAMP signaling pathways. It is well documented that shared a large number of DEGs and enriched GO and KEGG pathways, learning, and memory. Intriguingly, these brain regions hippocampus, the major brain regions involved in movement, co-

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with neurodegenerative diseases, such as Huntingtin and C9ORF72.

The level of cellular toxicity, as is seen with other proteins associated are known to accumulate with age and this accumulation amplifies be ascribed to age-related cellular stress, because misfolded proteins tide inclusions precede neurological symptoms. These results might at P32, indicating that the generation and accumulation of polypeptide inclusions were not detected at P2 and began to appear in the striatum and hippocampus at P12, while obvious neuronal loss was observed at P32, indicating that the generation and accumulation of polypeptide inclusions precede neurological symptoms. These results might be ascribed to age-related cellular stress, because misfolded proteins are known to accumulate with age and this accumulation amplifies the level of cellular toxicity, as is seen with other proteins associated with neurodegenerative diseases, such as Huntingtonin and C9ORF72 dipeptide repeat proteins (47, 48).

Using the NIID mouse model developed here, we performed de-
tailed transcriptome analysis on the cerebellum, cerebral cortex, and hippocampus, the major brain regions involved in movement, coordination, learning, and memory. Intriguingly, these brain regions shared a large number of DEGs and enriched GO and KEGG pathways among dysregulated genes, converging on calcium signaling and CaMP signaling pathways. It is well documented that subtle changes in calcium homeostasis can cause aberrant alterations in intracellular Ca\(^{2+}\), contributing to cellular and especially neuronal dysfunction, and the onset of a range of human diseases like ALS and Huntingtonin’s disease (49). These results suggested the potential involvement of the abnormal calcium signaling pathway in NIID pathogenesis.

Our cellular and mouse models—based on NPCs and p50 mouse brain—reflect early and mature stages of brain development, respectively. Comparative transcriptomic analyses of our NIID mouse model and hNPCs revealed a small number of overlapping DEGs, but they shared a higher proportion of aberrant AS events. The high percentage of overlapping AS events in two models represent-
ing different developmental stages and from different species im-
plies that dysregulated AS may play a vital role in the pathogenesis of NIID.

The spliceosome machinery that regulates AS is composed of a large number of elements, such as small nuclear ribonucleoproteins and RNA binding proteins like serine and arginine-rich proteins and hnRNPs, including hnRNPM (50). Here, we found that hnRNPM could bind to the AS sites that we identified in NIID hNPCs and mice. Meanwhile, hnRNPM was colocalized with polyG and polyA inclusions in the NIID mice and interacted with both polyG and polyA in vivo. These data suggested that polypeptides produced by \( \text{NOTCH2NLC} \) with expanded GGC repeats could potentially se-

quetter hnRNPM and affect hnRNPM-mediated RNA splicing. Consistent with this model, coexpression of hnRNPM could ame-
liorate cellular toxicity caused by expanded GGC repeats within \( \text{NOTCH2NLC} \). These findings together indicated a key role for hnRNPM in NIID pathogenesis.

In summary, we have developed both mouse and hNPCs models for NIID, which faithfully mimicked the clinical manifestations and pathological features of patients with NIID. Our molecular analyses revealed that dysregulated AS was involved in the neurodegeneration and identified hnRNPM as the fulcrum molecule in the pathogene-
sis of NIID, providing a promising therapeutic target for the treat-
mint of NIID.

**MATERIALS AND METHODS**

**Human samples**

This study was approved by the Ethics Committee of Xiangya Hos-
pital of the Central South University in China. Human skin samples used for generation of fibroblast lines and human peripheral blood were collected in a previous study (5). Written informed consent was obtained from all individuals.

**Animals**

The conditional transgenic \( \text{NOTCH2NLC}-(GGC)_{17} \) and \( \text{NOTCH2NLC}-(GGC)_{98} \) mice were generated by a CRISPR-Cas9 system in a C57BL/6j mouse background. Briefly, Cas9 mRNA was in vitro–transcribed with the mMESSAGE mMACHINET7 Ultra Kit (Ambion, TX, USA) according to the manufacturer’s instructions. 5’-GGG GAC GAC CTA AGG GAG CT-3’ was chosen as the Cas9-targeted single guide RNA (sgRNA) and in vitro–transcribed using the MEGas-

hortsKit (Thermo Fisher Scientific, USA). The Rosa26 targeting vector, sgRNA, and Cas9 mRNA were microinjected into C57BL/6j fertilized eggs. The microinjection was performed by Shanghai Model Organisms Center Inc. F0 generation mice positive for homologous recombination were crossed with C57BL/6j mice to obtain germline conditional transgenic mice. To express \( \text{NOTCH2NLC}-(GGC)_{17} \) or \( \text{NOTCH2NLC}-(GGC)_{98} \) ubiquitously, conditional transgenic mice were crossed with EIIa-Cre mice. The EIIa-Cre mice was obtained from The Jackson Laboratory and backcrossed into the C57BL/6 background for 13 generations. Primers used for genotyping are listed in the Supplementary Materials. All mice were bred and maintained in the animal facility under specific pathogen–free conditions in accordance with the institutional guidelines of the Animal Care and Use Committee at Central South University.

**Derivation of iPSCs and differentiation to NPCs**

The generated fibroblasts from patient skin biopsies were repro-
grammed into iPSCs using a CytoTune-iPS 2.0 Sendai reprogramming kit (Thermo Fisher Scientific, A16517) to derive integration-free iPSCs. Briefly, three Sendai virus–based reprogramming vectors, includ-
ing Yamanaka factors, klf4, oct4, sox2, hc-Myc, and hKlf4, were propor-
tionally mixed and applied to the fibroblasts (51). The transduced cells were cultured in fibroblast medium [Dulbecco’s modified Eagle’s medium (DMEM) medium supplemented with...
10% fetal bovine serum (FBS) and 1% minimum essential medium (MEM) nonessential amino acid solution] for 8 days, and transduced fibroblasts were passed to mouse embryo fibroblast (MEF) dishes with fibroblast medium on day 7. Medium was switched to iPSC medium [DMEM/F-12 with GlutaMAX supplemented with 20% KnockOut Serum Replacement, 1% MEM nonessential amino acid solution, 100 μM β-mercaptoethanol, 1% penicillin/streptomycin, and basic fibroblast growth factor (10 ng/ml)] from day 8. The emergence of iPSC colonies was monitored, and they were manually picked and passed to 24-well MEF plates. After that, iPSCs were expanded to Matrigel-coated six-well plates and maintained on Matrigel in mTeSR1 medium (STEMCELL Technologies, 85850) and passaged with ReLeSR (STEMCELL Technologies, 05873) to selectively pass the undifferentiated iPSCs. Then, iPSCs were differentiated to NPCs using the STEMdiff SMADi Neural Induction Kit (STEMCELL Technologies, 08581) via a monolayer protocol. The generated NPCs were maintained in neural progenitor medium (STEMCELL Technologies, 05833) on Martigel and used for the immunocytochemistry, RNA-seq, and Western blot experiments.

HEK293 and Neuro-2a cells were cultured in DMEM medium supplemented with 10% FBS and penicillin/streptomycin (100 U/ml) at 37°C with 5% CO2.

**Constructs**

The human exon 1 of NOTCH2NLC with 17 GGC repeats or 98 GGC repeats were cloned into pCDNA3.1(+) vector using polyG frame–, polyA frame–, and polyR frame–specific primers, respectively. A GFP-HA tag was then fused to the aforementioned three frames to obtain NOTCH2NLC-(GGC)17-GFP and NOTCH2NLC-(GGC)98-GFP constructs (polyG, polyA, and polyR frames, respectively). Primers used for constructing NOTCH2NLC constructs are listed as follows: Exon 1_Common_F: ATA GGT ACC ACC GGT GCT GAG GCG GCC GAG GAG CG, Exon 1_polyG frame_R: ATA GGA TTC CGG GGG TCG CCG AGC AGC AC, Exon 1_polyA frame_R: ATA GGA TTC TGC GGG GGG GTG GCC GAG CAC, Exon 1_polyR frame_R: ATA GGA TTC ATG CGC GGG GGT CGC GCA, polyG frame_ATG to AAG_F: TCC CCA AGT GGG GTA GCC GCC CA, and polyG frame_ATG to AAG_R: GGG GGT CCC GAT AGA AGA GC. To make the Rosa26 donor vector, the GFP-HA tag of NOTCH2NLC-GFP-HA (polyA frame) was replaced with a 3′HA/3′Myc/Flag (3TAG) tag. Then, NOTCH2NLC-(GGC)17–3TAG or NOTCH2NLC-(GGC)98–3TAG were digested by Age I and Ecor V and inserted into the Rosa26 targeting vector, which contains cytomegalovirus immediate enhancer/β-actin (CAG)–loxP–stop–loxP–Age I–Ecor V–woodchuck hepatitis virus posttranscriptional regulatory element–bovine growth hormone (bGH) poly(A).

The hnRNPM construct was generated by cloning complementary DNA (cDNA) of human hnRNPM (NM_005968) from human first-strand cDNA into a pCDNA3.1-3*Flag vector with the following primers: hnRNPM_F: CG GGA TTC ATG GCG GCA GGA GGT GCA A; and hnRNPM_R: GC TTA AGC GTC TGT ATC AA. All constructs were confirmed by sequencing.

**Antibodies**

Primary antibodies used in this study include the following: Flag (Sigma-Aldrich, F1804), HA (Cell Signaling Technology, 3724S), Myc (Cell Signaling Technology, 2276S), calbindin (Sigma-Aldrich, C9848), vinculin (Sigma-Aldrich, V9131), NeuN (Cell Signaling Technology, 24307s), GFAP (Cell Signaling Technology, 3670S), IBA1 (Wako, 019-19741), ubiquitin (Sigma-Aldrich, 05-1307), P62 (BD Biosciences, 610832), GFP (Invitrogen, A-11122), β-actin (Cell Signaling Technology, 3700S), TuJ1 (Abcam, ab18207), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Proteintech, 60004-1-lg), NOTCH2NLC (Gentex, GTX117659), and hnRNPM (Santa Cruz Biotechnology, sc-20002). Secondary antibodies were donkey anti-rabbit and donkey anti-mouse Alexa Fluor 488 or 594 from Jackson ImmunoResearch.

**Generation of anti-NOTCH2NLC polypeptide antibodies**

Rabbit polyclonal anti–NOTCH2NLC-polyG (Pep212) and anti–NOTCH2NLC-polyA (Pep213) antibodies were developed commercially. Briefly, rabbits were sequentially immunized with synthetic peptide CAARPPRMH (polyG) and EEAATEKPALRSGAVG (polyA) for three times. One week after the third injection, the sera were tested by enzyme-linked immunosorbent assay. Rabbits with eligible titer were immunized again, and the sera were purified through affinity purification and further validated by Western blot. The sera from the rabbit (Pep212) and rabbit (Pep213) were collected and purified to generate anti–NOTCH2NLC-polyG (Pep212) and anti–NOTCH2NLC-polyA (Pep213) antibodies.

**Cell culture, transfection, and cell viability assay**

HEK293 cells, Neuro-2a cells, and SH-SY5Y cells were obtained from the National Collection of Authenticated Cell Culture of China (HEK293 accession number: GNFHu43; Neuro-2a accession number: TCM29; SH-SY5Y accession number: SCSP-5014). HEK293 cells and Neuro-2a cells were cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin (100 U/ml) at 37°C with 5% CO2. Plasmid transient transfection on HEK293 cells and Neuro-2a cells was performed using Lipofectamine 2000 reagent following the manufacturer’s instruction. Cell viability assay was performed using the Cell Counting Kit-8 (CCK-8) following the manufacturer’s instruction. Twenty-four hours after transfection, Neuro-2a cells were cultured in DMEM with 1% FBS and supplemented with retinoic acid at final concentration of 25 mM for another 72 hours to induce neurite outgrowth. The Neuro-2a cells were fixed with 4% paraformaldehyde and stained with TuJ1. The neurite length of GFP-positive cells was measured by ImageJ software using a previously reported method (52). SH-SY5Y cells were cultured in DMEM/F-12 supplemented with 10% FBS and penicillin/streptomycin (100 U/ml) at 37°C with 5% CO2. Twenty-four hours after seeding, lentivirus infection was performed at a multiplicity of infection (20) for 12 hours. For mouse primary cortical neuron culture, the cortex from embryonic day 16 (E16) and E17 mice was dissected and digested by 0.025% trypsin (Thermo Fisher Scientific) at 37°C for 25 min. After washing twice with Hanks’ balanced salt solution, the cortical tissue was triturated with Pasteur pipettes, and cells were plated on Lab-Tek chamber slides (Thermo Fisher Scientific) in Neurobasal medium (Thermo Fisher Scientific) supplemented with B27, GlutaMAX, and penicillin/streptomycin. For cell viability and morphology, primary cortical neurons were transfected at days in vitro 2 using Lipofectamine 2000 reagent following the manufacturer’s instruction. After 48 hours, neurons were fixed with 4% paraformaldehyde and stained with TuJ1.

**Mass spectrometry analysis**

Forty-eight hours after transfection, protein was extract from HEK293 cells (72 hours transfection for SH-SY5Y cells), treated with dithiothreitol, alkylated with iodoacetamide, reconstituted in Triethylammonium bicarbonate (TEAB), hydrolyzed with trypsin, and
Lastly desalted by C18 SPE column. The tryptic peptides were dissolved and directly loaded onto a reversed-phase analytical column. Peptides were separated with a gradient formic acid and acetonitrile on an EASY-nLC 1200 ultra performance liquid chromatography system (Thermo Fisher Scientific). The separated peptides were analyzed in Q Exactive HF-X (Thermo Fisher Scientific) with a nano-electrospray ion source. The resulting tandem mass spectrometry data were processed using MaxQuant search engine (v.1.6.15.0). Tandem mass spectrometry was searched against the human SwissProt database (20,422 entries) and homemade database of three potential translated peptides from exon 1 of NOTCH2NLC concatenated with reverse decoy database. The mass tolerance for precursor ions was set at 20 parts per million (ppm) in the first search and 5 ppm in the main search, and the mass tolerance for fragment ions was set at 0.02 Da. Carbamidomethyl on Cys was specified as fixed modification, and oxidation on Met was specified as variable modifications. False discovery rate was adjusted to <1%

Mouse behavioral analysis
Mouse body weight was measured once every 2 days, and survival was monitored regularly. There was no gender difference in the survival time. At least eight male mice per genotype were included for the behavioral tests. Open field test was performed in a perspex box (70 cm by 70 cm by 31 cm) with the floor divided into 16 equal squares. The movement of mice during a 10-min test was filmed and analyzed by an automated behavior analysis system. Total moving duration and distance and the moving duration and distance in the central four squares were compared between different groups. Novel object recognition test was performed using a modified method (29, 30). Briefly, in habituation session, mice were habituated for 5 min in an empty perspex box (30 cm by 30 cm by 30 cm) for three consecutive days (habituation session). On the fourth day (familiarization session), two identical objects were placed at the opposite ends of the box. Mice were allowed free exploration for 10 min, and the amount of time exploring each object was recorded. Twenty-four hours later (test session), the less preferred object was replaced by a novel object, and again, the exploratory time to each object was recorded. The exploratory ratio was calculated as the ratio of time spent exploring one object over time spent exploring both objects. In the wire hang test, mice were placed on a wire lid of a mouse cage, which was then inverted. During a 3-min test, the latency to fall was recorded. Each mouse was tested three times, and the longest hang time was recorded. In the rotorod test, mice were trained for 3 min on the rotorod for three trials a day for three consecutive days (habituation session). On the testing day, a fixed-speed test at 20 rpm for 3 min was performed three times for each mouse (10-min intertrial interval), and latency to fall was recorded for each trial. In the cylinder test, mice were placed in a transparent cylinder and filmed with video from the side. The times of standing and touching the cylinder wall with forepaws for each mouse were recorded.

Immunofluorescent staining, Nissl staining, and electron microscopy
Mice were anesthetized and perfused intracardially with 0.9% saline solution, followed by 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.2. Isolated mouse brains were dehydrated in 30% sucrose at 4°C and sectioned at 30 μm for subsequent immunofluorescence study: Brain sections were blocked in 3% bovine serum albumin in 0.3% Triton X-100/phosphate-buffered saline (PBS) for 60 min, followed by incubation with primary antibodies overnight at 4°C. After incubation with the fluorescent secondary antibodies and 4',6-diamidino-2-phenylindole (DAPI), the brain sections were mounted to coated glass slides and examined using a Leica DMi8 fluorescence microscope and a Zeiss LSM-880 confocal microscope. For hNPC immunostaining, cells grown on coverslips were fixed with 4% paraformaldehyde for 15 min at room temperature, and 5% normal goat serum and 0.5% Triton X-100 in PBS were used for whole staining processes. Incubation with primary antibodies was overnight at 4°C and followed by fluorescent secondary antibody incubation for 45 min at room temperature. Pictures were taken by a Zeiss AX10 fluorescence microscope. For Nissl staining, the paraffin sections were dewaxed with xylene and washed with graded ethanol, followed by incubation with 1% toluidine blue for 40 min and then washed with ultrapure water. After dehydration with gradient ethanol and vitrification by xylene for 5 min (twice), the slices were sealed with neutral gum. Images were taken by Leica DMi8. Electron microscopic study was performed as previously reported (5); prepared sections were examined and photographed on a Hitachi HT-7700 electron microscope.

Coimmunoprecipitation and Western blot
Methods for coimmunoprecipitation and Western blot were described previously (38). Cells were harvested in 1% NP-40 lysis buffer [150 mM NaCl, 1% NP-40, 2 mM EDTA, and 50 mM tris (pH 8.0)] with protease inhibitor and phosphatase inhibitor cocktails 48 hours after transfection. Approximately 500 μg of protein was incubated overnight at 4°C with the primary antibody. Protein A beads (20 μl) were used to capture the antibody-protein complex, which was then analyzed by Western blot.

Electrophysiology
Five male mice per genotype were included for EMG. The concentric needle electrode was inserted without anesthesia into the quadriiceps, and a ground electrode was placed on the tail. The EMG signal was amplified and recorded by DANTEC Keypoint 9033A07 machine (sweep speed = 10 ms/division; sensitivity = 0.2 mV/division), and 10 different motor unit potentials (MUPs) per mouse were recorded. Average MUP amplitude and duration of motor unit action potential were calculated.

Reverse transcriptase PCR and quantitative real-time PCR
Total RNA was extracted from mouse brain tissue using a TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. cDNA was synthesized from total RNA (1 μg) using the RevertAid First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific, K1622) and oligo dT primers. Quantitative real-time PCR was analyzed with the comparative cycle threshold method. Primers used for quantitative real-time PCR were listed as follows: for NOTCH2NLC, NOTCH2NLC_3Tag_F: GGG GAG TCG AGG CAT TTG and NOTCH2NLC_3Tag_R: CAC TCT CTC CTC CCC CCC TC; for GAPDH, GAPDH_F:
RNA-seq and analysis

Tissues from P50 male mice or hNPCs were used for RNA extraction. An amount of 1 μg of total RNA per sample was processed for library preparation using the NEBNext UltraTM RNA Library Prep Kit for Illumina (New England Biolabs, USA) following the manufacturer’s recommendations. Libraries were sequenced on a HiSeq with a read length configuration of 150 paired end (PE), targeting 150 M total reads per sample.

All total reads were mapped to hg38/mm9 by TopHat2 with default parameters, followed by gene expression quantification and differential expression analysis using Cuffdiff. Genes with an adjusted P value < 0.05 and |log2 (fold change)| > 0.8 were assigned as significantly differentially expressed. KEGG pathways and GO enrichment analysis were performed by DAVID or PANTHER online. The cel-motifs, GGUUGGU, UGUUGU, and UGUGU (44) were converted to mm9 by LiftOver. The prediction of hnRNPM binding flanking region of AS sites in a mouse. The hnRNPM CLIP-seq peak data from human K562 and HepG2 as hnRNPM binding sites in a mouse. The hnRNPM CLIP-seq peak data from human K562 and HepG2 as hnRNPM binding sites in a mouse. Splicing events with a P value of less than 0.05 and |log2 (fold change)| > 0.8 were assigned as significantly differentially expressed. KEGG pathways and GO enrichment analysis were performed by DAVID or PANTHER online. The cel-motifs, GGUUGGU, UGUUGU, and UGUGU (44) were converted to mm9 by LiftOver. The prediction of hnRNPM binding flanking region of AS sites in a mouse. The hnRNPM CLIP-seq peak data from human K562 and HepG2 as hnRNPM binding sites in a mouse. Splicing events with a

Quantification and statistical analysis

To eliminate bias, images or animal analyses were either completely automated or blinded. All statistical analyses were performed with two-tailed Student’s t test or the one-way analysis of variance (ANOVA) analysis (Tukey’s multiple comparisons test). All quantification data were presented as means ± SEM. A P value of less than 0.05 was considered statistically significant.

SUPPLEMENTARY MATERIALS

Supplemental material for this article is available at https://science.org doi/10.1126/sciadv.aba6391

View/request a protocol for this paper from Bio-protocol.

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