elav-GAL4/elav-GAL4 (elav>UAS-*Caz*-IR/UAS-*Caz*-IR), w; UAS-*Caz*-IR363−399/*ter94*^{K15502}; *elav-GAL4/+* (elav> UAS-*Caz*-IR/*ter94*^{K15502}), w; UAS-*Caz*-IR363−399/ UAS-*GFP*; *elav-GAL4/+* (elav>UAS-*Caz*-IR/UAS-*GFP*), w; UAS-*Caz*-IR363−399/UAS-*ter94*; *elav-GAL4/+* (elav>UAS-*Caz*-IR/ UAS-*ter94*).

Immunohistochemistry

Rabbit anti-Caz antibodies were raised against amino acid residues 29−45 and 383−399 of Caz and were produced previously (19). For immunohistochemical analysis, CNS tissues were dissected from third instar larvae and fixed in 4% paraformaldehyde/phosphate buffered saline (PBS) for 15 min at 25°C. These tissue samples were washed with PBS containing 0.3% Triton X-100; fixed samples were then incubated with Alexa 488-conjugated phalloidin (1 unit/200 μl) in PBS containing 0.3% Triton X-100 for 20 min at 25°C. The samples were then blocked with blocking buffer (PBS containing 0.15% Triton X-100 and 10% normal goat serum) for 30 min at 25°C, and then incubated with 1 : 1000 diluted rabbit anti-Caz antibody in the blocking buffer for 20 h at 4°C. After extensive washing with PBS containing 0.3% Triton X-100, samples were incubated in the dark with secondary antibodies labeled with Alexa 546 (1 : 400; Invitrogen) diluted in the blocking buffer for 3 h at 25°C. After washing with PBS containing 0.3% Triton X-100, the samples were stained with DAPI (0.5 μg/ml)/PBS/ 0.1% Triton X-100. After extensive washing with PBS containing 0.1% Triton X-100 and PBS, the samples were mounted in Vectashield (Vector Laboratories-Inc.) and observed under a confocal laser scanning microscope (OLYMPUS FLUOVIEW FV10i). Images were analyzed with the program MetaMorph Imaging System 7.7 (Molecular Devices Inc.). The use of this program made it possible to quantify the average and the standard error of fluorescence emission from nuclei of each fly strain.

For NMJ staining, third instar larvae were dissected in HL3 saline (49), and then fixed in 4% paraformaldehyde/PBS for 30 min. The blocking buffer contained 2% bovine serum albumin and 0.1% Triton X-100 in PBS. Fluorescein isothiocyanate-conjugated goat anti-horseradish peroxidase (HRP) (1:1000, MP Biochemicals) was used as the detection antibody. The samples were mounted and observed under a confocal laser scanning microscope (Carl Zeiss LSM510, Jena, Germany). MN 4 (Ib) in muscle 4 in abdominal segment 2 was quantified. Images were acquired using a Zeiss LSM 510 confocal laser scanning microscope by merging 1 μm interval z-sections onto a single plane. The MetaMorph imaging system was used to measure nerve terminal branch lengths and Ib bouton sizes.

Immunoblotting analysis

Protein extracts from the CNS of *Drosophila* carrying *elav/+*, elav>UAS-*Caz*-IR, elav>UAS-*Caz*-IR/*ter94* ^{K15502}, elav> UAS-*Caz*-IR/UAS-*GFP* and elav>UAS-*Caz*-IR/UAS-*ter94* larvae were prepared as described previously (19). Briefly, the CNS was excised from third instar larvae and homogenized in a sample buffer containing 50 mM Tris−HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.1% bromophenol blue and 1.2% β-mercaptoethanol. The homogenates were boiled at 100°C for 5 min and then centrifuged. The supernatants (extracts)

were electrophoretically separated on SDS-polyacrylamide gels containing 12% acrylamide and then transferred to polyvinylidene difluoride membranes (Merck, Millipore, MA, USA). The blotted membranes were blocked with tris-buffered saline/ 0.05% Tween containing 5% skim milk for 1 h at 25°C, followed by incubation with rabbit polyclonal anti-Caz at a 1:5000 dilution for 16 h at 4°C. After washing, the membranes were incubated with HRP-conjugated anti-rabbit IgG (Thermo Scientific, IL, USA) at 1:10 000 dilution for 2 h at 25°C. Antibody binding was detected using ECL Western blotting detection reagents (Thermo Scientific) and images were analyzed using an ImageQuant^{TM} LAS 4000 image analyzer (GE Healthcare Bioscience, Tokyo, Japan). To compare Caz protein levels in the CNS extracts of those larvae, densitometric quantification of the 45-kDa Caz protein bands was carried out. The relative band intensities were quantified and normalized to Coomassie Brilliant Blue staining, then expressed as the percentage of the band intensity derived from larvae carrying *elav/+*.

Scanning electron microscopy

Adult flies were anesthetized with 99% diethyl ether, mounted on stages and observed under an SEM V-7800 (Keyence Inc.) in the low vacuum mode (50). In every experiment, at least five adult flies were chosen from each line for scanning electron microscopy to assess the eye phenotype. For each experiment, there was no significant variation in eye phenotype among the five individuals from the same strain.

Longevity assay

Longevity assays were carried out in a humidified, temperature-controlled incubator set at 25°C and 60% humidity on a 12-h light and 12-h dark cycle; flies were maintained on standard fly food. Flies carrying *elav/+* ($n = 151$), elav>UAS-*Caz*-IR ($n = 123$), elav>UAS-*Caz*-IR/*ter94*^{K15502} ($n = 120$), elav> UAS-*Caz*-IR/UAS-*GFP* ($n = 140$) or elav>UAS-*Caz*-IR/ UAS-*ter94* ($n = 140$) were placed at 28°C, and newly eclosed adult male flies were separated and placed in vials at a low density (20 flies per vial). Every 3 days, they were transferred to new tubes containing fresh food and deaths were scored. The survival rate was determined by plotting a graph of the percentage of surviving flies among total flies at the starting point of each experiment versus days.

Climbing assay

Climbing assays were performed as described previously (29). Flies carrying *elav/+*, UAS-*Caz*-IR/+, *ter94*^{K15502}/+, elav> UAS-*Caz*-IR, elav>UAS-*Caz*-IR/*ter94*^{K15502}, elav>UAS-*Caz*-IR/UAS-*GFP* and elav>UAS-*Caz*-IR/UAS-*ter94* were placed at 28°C, and newly eclosed adult male flies were separated and placed in vials at a density of 20 flies per vial. Flies were transferred, without anesthesia, to a conical tube. The tubes were tapped to collect the flies to the bottom, and they were then given 30 s to climb the wall. After 30 s, the flies were collected at the bottom by tapping of the tube and were again allowed to climb for 30 s. Similar procedures, all of which were videotaped, were repeated five times in total. For each climbing experiment, the height to which each fly

climbed was scored as score (height climbed); 0 (less than 2 cm), 1 (between 2 and 3.9 cm), 2 (between 4 and 5.9 cm), 3 (between 6 and 7.9 cm), 4 (between 8 and 9.9 cm) or 5 (greater than 10 cm). The climbing index for each fly strain was calculated as follows; each score was multiplied by the number of flies for which that score was recorded, and the products were summed up, then divided by five times the total number of flies examined. These climbing assays were carried out every 7 days until the 28th day after eclosion.

Data analysis

GraphPad Prism version 6.0 was used to perform each statistical analysis. The Mann–Whiney test was used for the assessment of the statistical significance of comparisons between two groups of data. For other assays, one-way analysis of variance (ANOVA) was used to determine the statistical significance of comparisons between groups of data. When the two-way ANOVA showed significant variation among groups, a subsequent Dunnett's test was used for pairwise comparisons between groups. All data are shown as mean $\pm$ standard error (SE).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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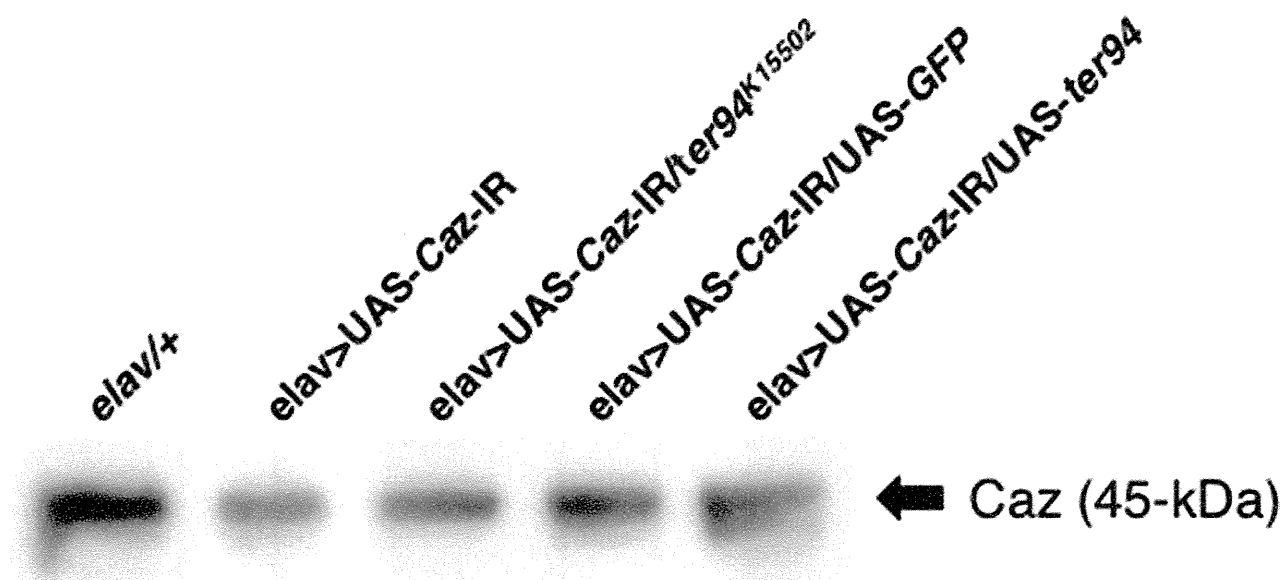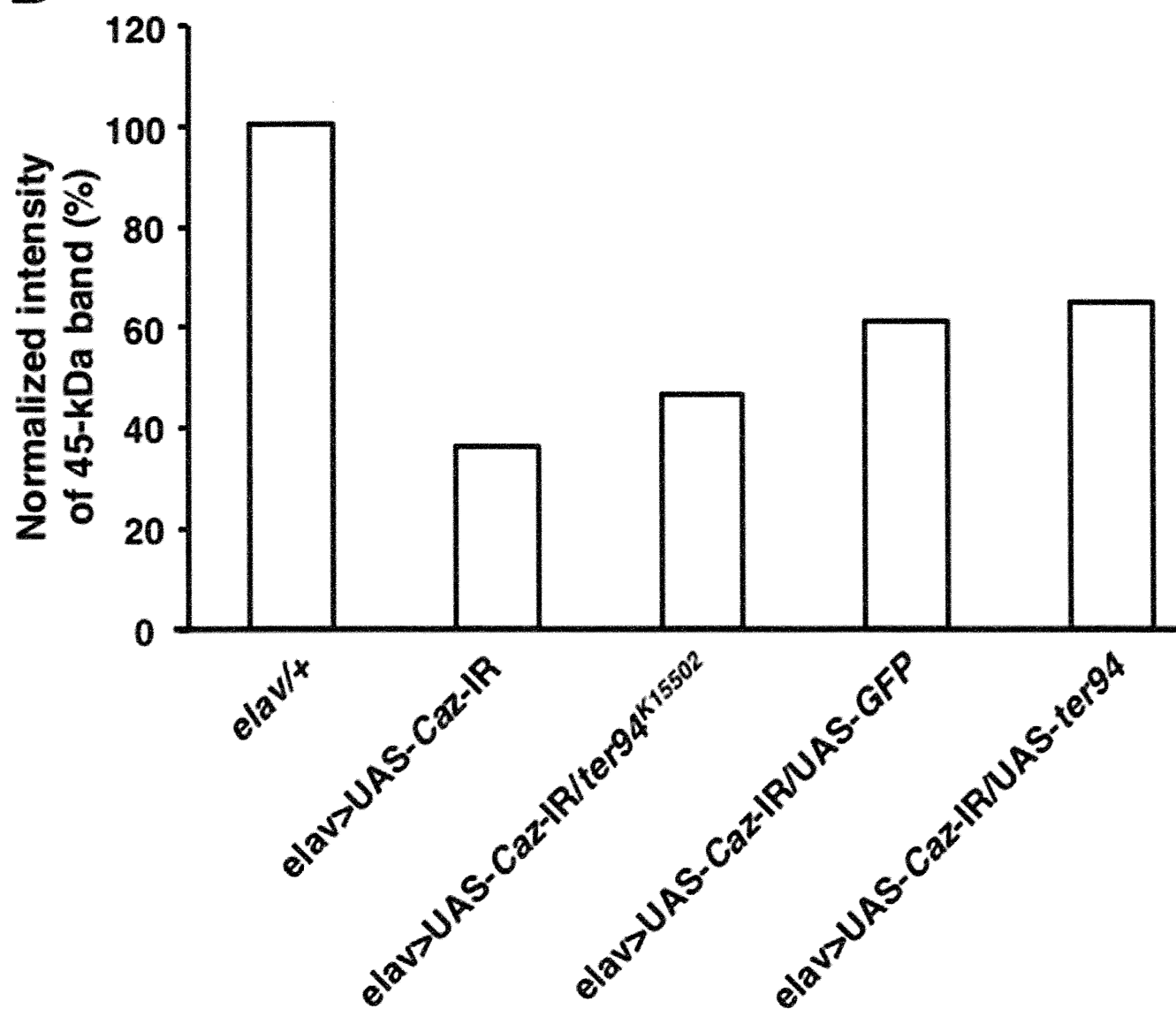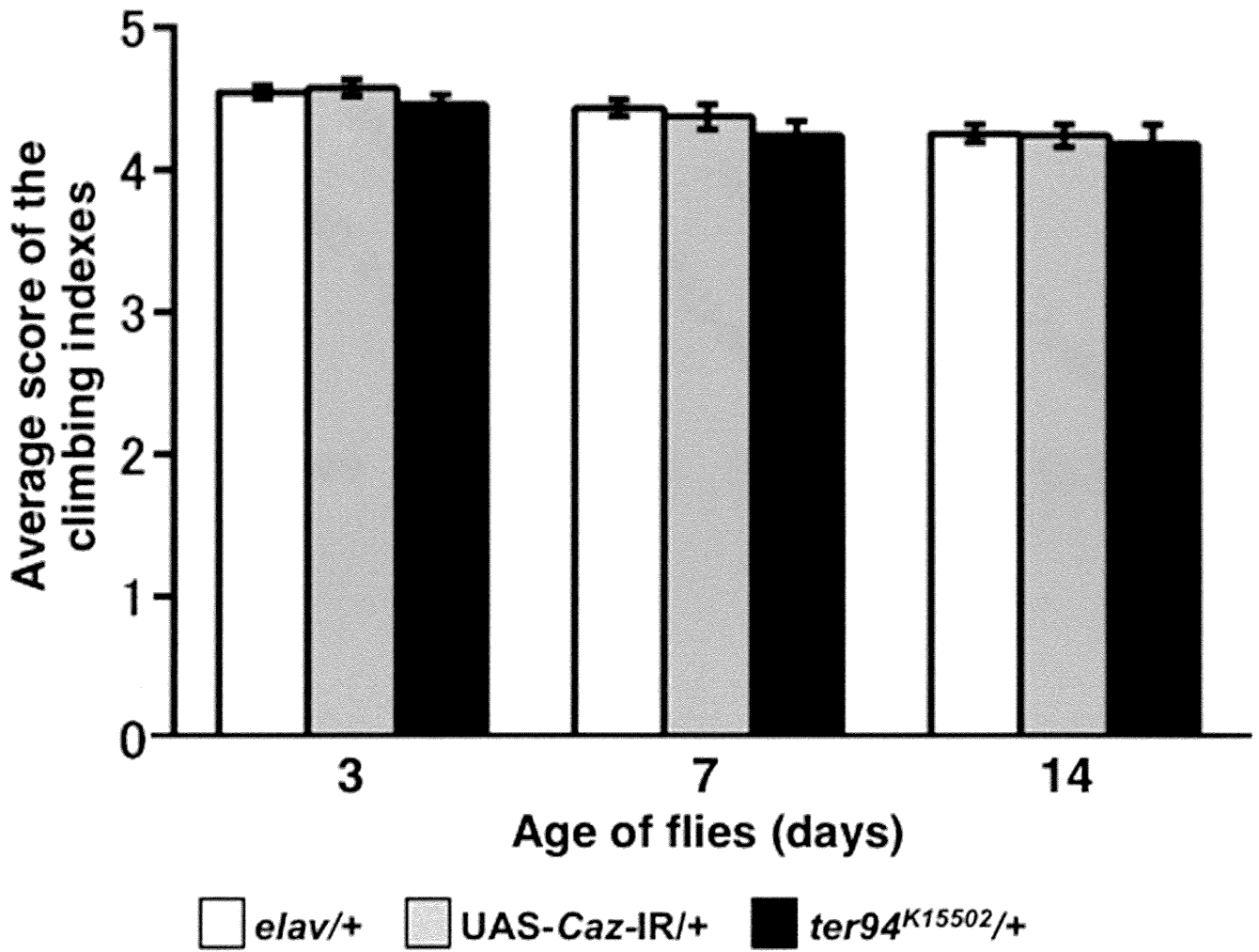
Supplementary Materials

Supplementary Figure 1. Immunoblotting analysis of the CNS extracts of third instar larvae. (A) A representative result of the analysis of protein extracts from the CNS of the *elav/+,* elav>UAS-*Caz*-IR, elav>UAS-*Caz*-IR/*ter94*^{K15502}, elav>UAS-*Caz*-IR/UAS-*GFP* and elav>UAS-*Caz*-IR/UAS-*ter94* larvae (n = 5, each). The blots are probed with the polyclonal anti-Caz antibody used in the previous study (19). A 45-kDa band (arrow) corresponds to the Caz protein. (B) Densitometric quantification of the 45-kDa bands in each fly strain used in (A). The intensity of the 45-kDa band which indicates the expression level of Caz protein is much weaker in larvae carrying elav>UAS-*Caz*-IR than in the larvae carrying *elav/+*. Besides, there is no apparent difference in the intensity of the Caz protein band between the larvae carrying elav>UAS-*Caz*-IR and elav>UAS-*Caz*-IR/*ter94*^{K15502}. Similarly, there is no apparent difference in the intensity of the band between the larvae carrying elav>UAS-*Caz*-IR/UAS-*GFP* and elav>UAS-*Caz*-IR/UAS-*ter94*.

Supplementary Figure 2. The locomotive ability of each control flies, which carry *elav/+* (a driver control, n = 255), UAS-*Caz*-IR/+ (a responder control, n = 250), *ter94*^{k15502}/+ (n = 235). There are no significant differences in climbing abilities among those fly lines in each day after eclosion that was monitored until 14 days.

Supplementary Figure 3. Life-span analyses of flies of each genotype. Percentage survival of adult male flies of the indicated genotype is shown. (A) There are no significant differences in life spans among the control flies carrying *elav/+* (n = 151), neuron-specific *Caz*-knockdown flies carrying elav>UAS-*Caz*-IR (n = 123), and flies

carrying elav>UAS-*Caz*-IR/*ter94*^{K15502} (n = 120). (B) Similarly, there are no significant differences in life spans between flies carrying elav>UAS-*Caz*-IR/UAS-*GFP* (n = 140) and those carrying elav>UAS-*Caz*-IR/UAS-*ter94* (n = 140).

A

elav/+ elav>UAS-Caz-IR elav>UAS-Caz-IR/ter94^{K15502} elav>UAS-Caz-IR/UAS-GFP elav>UAS-Caz-IR/UAS-ter94

← Caz (45-kDa)

B

elav/+ *elav>UAS-Caz-IR* *elav>UAS-Caz-IR/ter94^{K15502}* *elav>UAS-Caz-IR/UAS-GFP* *elav>UAS-Caz-IR/UAS-ter94*

Normalized intensity of 45-kDa band (%)

Average score of the climbing indexes vs Age of flies (days)

Legend: □ *elav/+*   ▨ UAS-*Caz*-IR/+   ■ *ter94*[K15502]/+

A
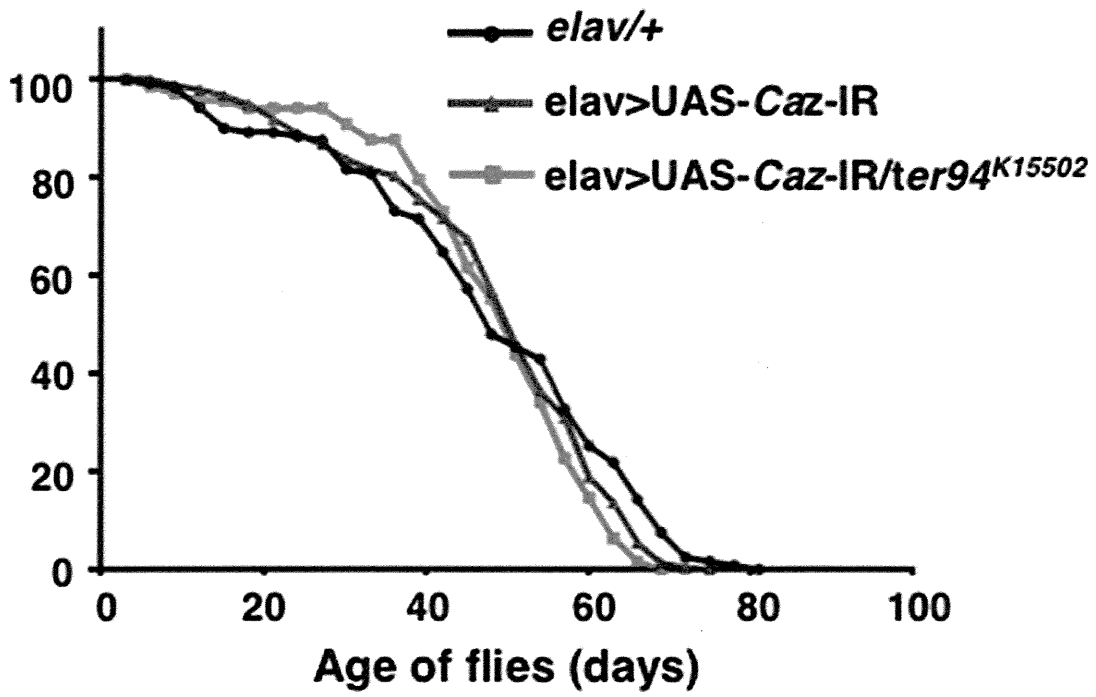
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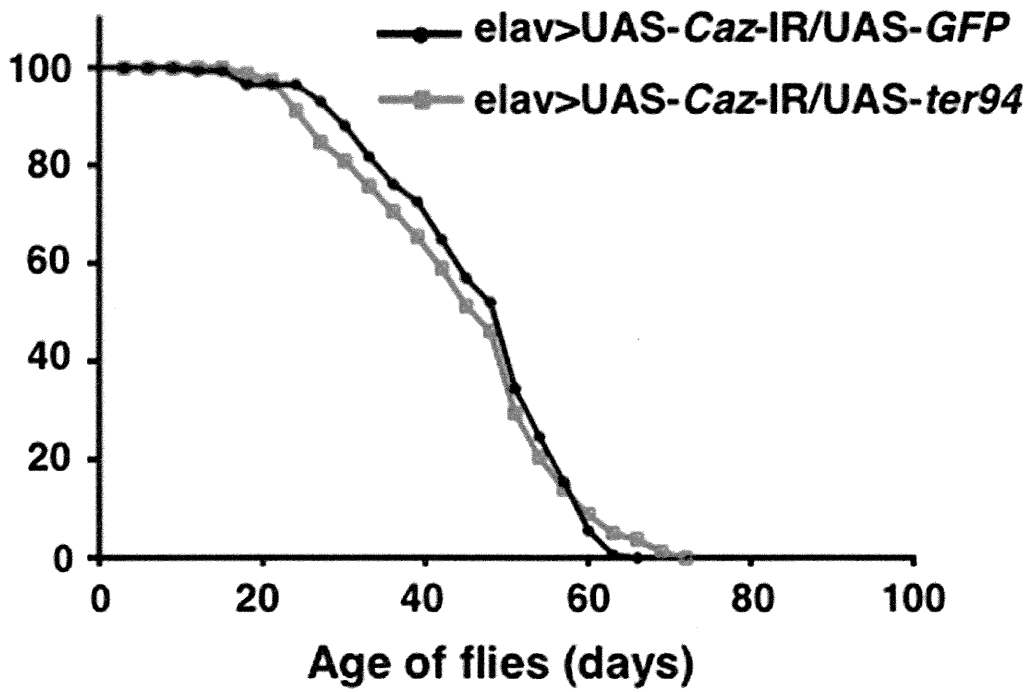
Peptide-Based Therapeutic Approaches for Treatment of the Polyglutamine Diseases

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Abstract: The polyglutamine (polyQ) diseases including Huntington's disease and spinocerebellar ataxias are a group of inherited neurodegenerative diseases that are caused by an abnormal expansion of the polyQ stretch in disease-causative proteins. The expanded polyQ stretches are intrinsically unstable and are prone to form insoluble aggregates and inclusion bodies. Recent studies have revealed that the expanded polyQ proteins gain cytotoxicity during the aggregation process, which may possibly cause detrimental effects on a wide range of essential cellular functions leading to eventual neuronal degeneration. Based on the pathogenic mechanism of the polyQ diseases, several therapeutic approaches have been proposed to date. Among them, here we focus on peptide-based approaches that target either aggregate formation of the polyQ proteins or abnormal cellular processes induced by the expanded polyQ proteins. Although both approaches are effective in suppressing cytotoxicity of the abnormal polyQ proteins and the disease phenotypes of animal models, the former approach is more attractive since it targets the most upstream change occurring in the polyQ diseases, and is therefore expected to be effective against various downstream functional abnormalities in a broad range of polyQ diseases. One of the major current problems that must be overcome for development of peptide-based therapies of the polyQ diseases is the issue of brain delivery, which is also discussed in this article. We hope that in the near future effective therapies are developed, and bring hope to many patients suffering from the currently untreatable polyQ diseases.

Keywords: Neurodegeneration, peptide, polyglutamine diseases, protein aggregation, therapy.

THE POLYGLUTAMINE DISEASES

The polyglutamine (polyQ) diseases are a group of inherited neurodegenerative disorders characterized by a common genetic mutation in the coding sequence of each disease-causative gene, in which a trinucleotide CAG repeat encoding a polyQ stretch is abnormally expanded (>35-40 repeats) [1-3]. So far, nine disorders have been recognized as such diseases, including Huntington's disease (HD), spinal and bulbar muscular atrophy (SBMA), several types of spinocerebeller ataxias (SCAs) [2]. These diseases are all characterized by the progressive degeneration and loss of neurons in various regions of the brain, resulting in progressive neurological and psychiatric symptoms such as cognitive impairment and motor disturbance. No effective treatment for the polyQ diseases has been established to date.

The molecular basis of the polyQ diseases is the abnormal expansion of a polyQ stretch in each host protein. In most cases, the threshold polyQ length for disease manifestation is around 35-40 repeats, as polyQ expansions longer than 40 repeats typically result in the polyQ diseases [3].

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For example, the polyQ length in huntingtin (Htt), the causative protein of HD, ranges in size from 5 to 35 repeats in normal subjects, but is expanded to more than 40 repeats in patients with HD [4]. The polyQ length in the disease-causative protein can also affect the disease progression, as it correlates tightly with the age at onset and severity of disease [5, 6]. Animal studies have demonstrated that typical disease phenotypes such as progressive degeneration and loss of neurons in the brain can be caused by expression of the expanded polyQ stretch alone, further supporting the pathological importance of the abnormal expansion of the polyQ stretch [7-11]. These facts strongly indicate that the polyQ diseases are caused by a gain of toxic function mechanism of the expanded polyQ stretch, and are considered to be unrelated with the specific functions of each host protein.

The expanded polyQ stretches are intrinsically unstable, and are likely to form insoluble aggregates and inclusion bodies, which are a common pathological characteristic observed in the brain of polyQ disease patients as well as animal models [12, 13]. The mechanisms as to how expanded polyQ proteins form aggregates and the relationship between aggregate formation and cytotoxicity have been extensively studied [14-18]. Recent accumulating evidence strongly indicate that abnormal intermediate species such as oligomeric intermediates and even misfolded monomers of the expanded polyQ proteins which form prior to aggregates/inclusion bodies could be more toxic to neurons compared with in-

soluble aggregates/inclusion bodies [19-23]. Although it is still unclear which intermediate species are responsible for polyQ disease pathogenesis, these facts indicate that the expanded polyQ protein gains cytotoxicity during the aggregation process.

THERAPEUTIC APPROACHES FOR THE POLY-GLUTAMINE DISEASES

Since proteins with an abnormally expanded polyQ stretch gain cytotoxicity during their aggregation process, suppression of misfolding and aggregate formation could be a potential therapeutic approach for treatment of the polyQ diseases [3]. Several studies have actually demonstrated in polyQ disease models that increasing levels of molecular chaperones [24-28] and expression of intracellular antibodies (intrabodies) [29-31] successfully reduce the eventual toxicity in neurons through suppression of polyQ protein accumulation and inclusion body formation [32, 33]. Small molecules [34-38] and peptides [39-41] that interfere with the aggregation process of the expanded polyQ protein were also shown to suppress polyQ-induced neurodegeneration in cell culture and animal models of the polyQ diseases. In addition, activation of protein degradation systems, which accelerate the clearance of the polyQ proteins, has been shown to be quite effective to suppress aggegate formation and eventual cell death [42,43]. Since suppression of polyQ aggregation is expected to broadly correct the functional abnormalities of multiple downstream cellular processes (see below), misfolding and aggregate formation of the expanded polyQ proteins are one of the most ideal therapeutic targets of the polyQ diseases [3] (Fig. 1).

On the other hand, it is well known that polyQ disease patients as well as animal models exhibit dysfunctions in various cellular processes in the cascade of polyQ patho-genesis. This includes abnormalities in essential cellular functions including transcription [44], proteasomal degradation [45], synaptic transmission [46], axonal transport [47] and Ca^{2+} signaling pathways [48], which probably contribute to neuronal dysfunction and eventual loss of neurons in various regions of the brain [12, 49, 50]. Although the exact mechanisms as to how they eventually cause degeneration of neurons in patients of the polyQ diseases have not yet been clarified, these cellular processes that are thought to be eventually impaired in the pathogenic cascade are also potential therapeutic targets for treatment of the polyQ diseases (Fig. 1).

In the following sections, selected examples of peptide-based therapeutic approaches focusing on these targets are introduced (Table 1), and the current problems that must be overcome for the development of peptide-based therapies for the polyQ diseases are discussed.

PEPTIDE-BASED INHIBITORS OF POLYGLU-TAMINE AGGREGATION

Therapeutic approaches targeting the polyQ stretch are particularly attractive because effective inhibitors would be expected to work generally on a broad spectrum of the polyQ diseases. Trottier *et al.* showed that the anti-polyQ monoclonal antibody 1C2 binds preferentially to longer polyQ repeats compared with short repeats [51]. Similar preferential binding to expanded polyQ proteins has been reported for the monoclonal antibodies MW1 [52] and 3B5H10 [53]. These studies led to the idea that expanded polyQ stretches may possess structurally different conformations from the shorter ones, and that potential molecules that specifically recognize and bind to such abnormal conformations could interfere with the aggregation processes of expanded polyQ proteins.
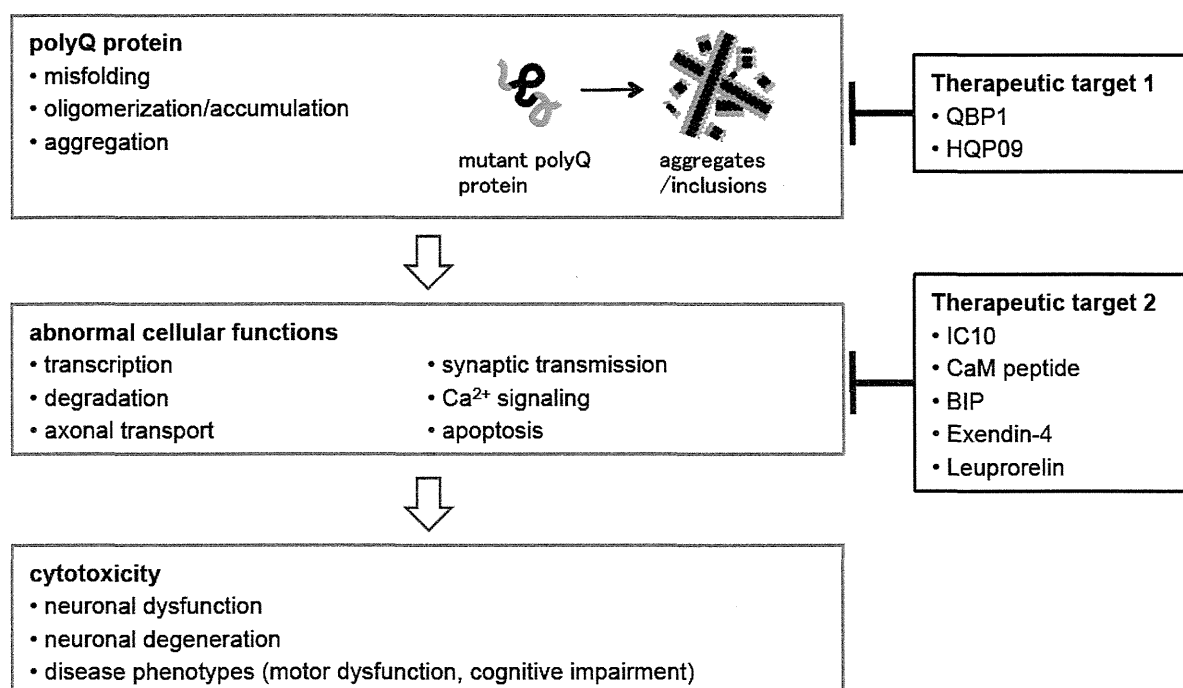

Fig. (1). Proposed mechanism of expanded polyQ protein toxicity and potential therapeutic targets for polyQ disease therapies.

Table 1. Selected examples of peptides potentially effective for the polyQ diseases.

Peptide	Sequence	Therapeutic Target	Effects *In Vitro*	Effects *In Vivo*	Ref
QBP1	SNWKWWPGIFD	polyQ aggregation	Aggregation ↓ Inclusion bodies ↓ Cytotoxicity ↓	Inclusion bodies ↓ Life span ↑ Body weight ↑	[23, 39, 54-60, 62, 63]
HQP09	Npip-Nmba-P-Nmea-Nall-Nlys-Nser[a]	polyQ aggregation	Aggregation ↓ Cytotoxicity ↓	Stabilize Ca²⁺ signaling ↓ Apoptosis ↓ Inclusion bodies	[64]
IC10	A cytosolic C-terminal tail of InsP₃R1 (122 amino acids)	Aberrant interaction between Htt and InsP₃R1	Stabilize Ca²⁺ signaling ↓	Motor function ↑ Neuronal loss ↓	[68]
CaM peptide	MKDTDSEEEIREAFRVFDKDGNGY-ISAAELRHVMTNLGEKLTDEEV (A fragment of CaM, residues 76-121)	Abberant interaction between Htt and CaM	Cytotoxicity ↓	Body weight ↑ Motor function ↑ Inclusion bodies ↓	[69-71]
BIP	VPMLK/VPTLK	Bax-induced apoptosis	Apoptosis ↓	-	[75,76]
Exendin-4	HGEGTFTSDLSKQMEEEAVRLFIEWL KNGGPSSGAPPPS	Abnormal energy metabolism	-	Motor function ↑ Life span ↑ Inclusion bodies ↓	[85]
Leuprorelin	Pyr-HWSY*LL*RP-NHEt[b,c]	Nuclear accumulation of mutant AR	-	Motor function ↑ Life span ↑ Inclusion bodies ↓	[90-92]

[a]N-substituted glycines. Npip, piperonyl; Nmba, methylbenzyl; Nmea, methoxyethyl; Nall, allyl; Nlys, aminobutyl; Nser, hydroxyethyl. [b]Pyr, pyroglutamyl. [c]*d*-amino acids in *Italics*.

QBP1

We previously took a combinatorial screening approach to search for short peptides that selectively and specifically bind to an expanded polyQ stretch, but not to a normal length polyQ stretch, using the phage display technique. Multiple rounds of screening resulted in six peptides that preferentially bind to the abnormally expanded polyQ stretch [39]. One of these peptides, QBP1 (polyQ binding peptide 1), had a particularly high affinity for the abnormal polyQ stretch with a dissociation constant (K_d) of 5.7 μM [54], and also had a suppressive effect on polyQ aggregation *in vitro* [39]. Studies focusing on its structure-activity relationship revealed that the tryptophan-rich sequence is necessary for the inhibitory activity of QBP1 [55-57]. Expression of QBP1 effectively suppressed inclusion body formation and cytotoxicity of expanded polyQ proteins in cell culture [23,39,58,59] and *Drosophila* models of the polyQ diseases [60]. Since QBP1 is poorly membrane permeable, we employed protein transduction domains (PTDs) [61] to improve the bioavailability of QBP1 by its efficient intracellular delivery. We found that the delivery efficiency of QBP1 was dramatically improved by conjugation with a PTD, leading to successful suppression of polyQ inclusions as well as polyQ-induced premature death in *Drosophila* by its oral administration [62]. The therapeutic potential of PTD-QBP1 was further investigated using a mouse model of HD. However, the therapeutic effect of PTD-QBP1 was limited to neither inhibition of body weight loss with any improvement in the other disease phenotypes nor inhibition of aggregate formation in the brains, probably due to low efficiency of PTD-QBP1 delivery to the mouse brain by intraperitoneal injections [63].

HQP09

Chen *et al.* also performed combinatorial screening to search for potential inhibitors of polyQ aggregation [64]. In contrast to our approach using peptide-based phage display libraries, they used a combinatorial library consisting of peptoids as scaffolds. Peptoids, which are oligomers of N-substituted glycines, have an advantage in developing therapeutic molecules since they are considered to be superior in stability to protease degradation, cell permeability, and structural diversity [65, 66]. They prepared a peptoid library containing 60,000 unique compounds, and screened for molecules that specifically bind to the Htt fragment with an expanded polyQ stretch. The peptoid HQP09, which was isolated from this screening process, was found to bind with high specificity to the expanded polyQ forms of Htt and ataxin-3, which is the causative protein of SCA3, and to effectively suppress polyQ aggregation *in vitro*. Interestingly, although HQP09 and QBP1 had comparable binding affinity to mutant Htt proteins, HQP09 did not show any competition with QBP1 in binding, possibly indicating that these two inhibitors recognize the abnormally expanded polyQ stretches in a different manner. The authors also tested the therapeutic activity of this peptoid, and confirmed that HQP09 reduced cytotoxicity in primary cultured neurons and decreased polyQ inclusion bodies in a mouse model of HD upon its intracerebroventricular injection. Importantly, they successfully identified the pharmacophore of HQP09 based on a structure-activity relationship study, and developed the minimal derivative peptoid HQP09-9 (4-mer, MW = 585) without significant loss of activity. Although HQP09-9 failed to exert therapeutic effects on a mouse model upon its subcu-

taneous injection probably due to poor blood-brain barrier permeability, this could be a promising lead compound for the development of drugs against a broad spectrum of the polyQ diseases.

PEPTIDE-BASED MODULATORS OF POLYGLU-TAMINE TOXICITY

Another therapeutic approach is to target the various cellular dysfunctions occurring in the cascade of polyQ pathogenesis. Although the mechanisms as to how these abnormalities contribute to eventual neurodegeneration in various regions of the brain are not known, normalizing such dysfunctions has been shown to effectively reduce the toxicity of the expanded polyQ proteins and improve disease phenotypes in polyQ disease animal models.

IC10 peptide

Since aberrant interactions between mutant Htt and various proteins often cause abnormalities in downstream cellular functions [50], disruption of such interactions could be a promising therapeutic approach. Bezprozvanny and coworkers found that the polyQ expanded Htt protein specifically binds to type 1 inositol 1,4,5-triphosphate receptor (InsP$_3$R1) and facilitates its activity, indicating that abnormal neuronal Ca^{2+} signaling may play an important role in HD pathogenesis [48, 67]. Since mutant Htt specifically binds to the C-terminal cytosolic region of InsP$_3$R1 (IC10 fragment), they hypothesized that introduction of the IC10 peptide into neurons would normalize Ca^{2+} signaling and eventual neurodegeneration by interfering with the abnormal interaction between mutant Htt and InsP$_3$R1[48]. They indeed found that viral vector-mediated expression of the IC10 peptide effectively stabilized neuronal Ca^{2+} signaling, improved motor dysfunctions and reduced neuronal loss in a mouse model of HD [68].

CaM-peptide

Mutant Htt also associates with calmodulin (CaM) with a higher affinity than wild-type Htt, and this interaction facilitates a wide range of downstream cellular functions. Muma and coworkers prepared several deletion mutants of CaM and found that a fragment corresponding to 76-121 amino acids of CaM (CaM-peptide) is responsible for binding with mutant Htt [69]. They demonstrated that expression of CaM-peptide reduced cytotoxicity by disrupting the abnormal interaction between endogenous CaM and mutant Htt in cellular models [69, 70] and improved disease phenotypes including body weight loss and motor dysfunctions in a mouse model of HD [71]. The studies on both IC10 and CaM peptides strongly indicate that abnormal interactions of the expanded polyQ proteins is critical for polyQ disease pathogenesis, and that molecules targeting these abnormal interactions may be promising lead compounds for polyQ disease treatment.

BIP

It has been reported that expanded polyQ proteins directly induce apoptosis. Mutant Htt with expanded polyQ stretch was shown to activate p53 and increase the expression level of Bax, a proapoptotic member of the Bcl-2 family

of proteins that play a key role in programmed cell death in neurons [72]. Similarly, activation of Bax and subsequent cell death has also been shown in cells expressing polyQ-expanded ataxin-3 and ataxin-7, causative proteins of SCA3 and SCA7, respectively [73, 74]. Matsuyama, Yokota and coworkers found that the proapoptotic activity of Bax is normally suppressed by Ku70, a cytoprotective protein that interacts with Bax and prevents its mitochondrial translocation, while in SCA3, mutant ataxin-3 abnormally stimulates the acetylation of Ku70, which results in dissociation of Bax from Ku70 and promotes the subsequent activation of apoptosis [75]. Importantly, expression of Ku70 effectively blocked mutant ataxin-3-induced cell death, which strongly indicates that approaches targeting the activation process of Bax could be effective for suppression of the eventual apoptosis induced by expanded polyQ proteins [75]. To develop peptide-based suppressors of Bax-induced apoptosis, they identified the Bax-binding domain of Ku70 and designed a penta-peptide, Bax-inhibiting peptide (BIP) derived from this domain [76]. BIP is particularly promising since this is cell-permeable and effectively suppresses the mitochondrial translocation of Bax and subsequent apoptotic cell death [75].

Exendin-4

Although the polyQ diseases are considered primarily as neurological disorders, patients also exhibit peripheral symptoms. In HD, it is known that patients suffer from various metabolic abnormalities including progressive weight loss, appetite dysfunction and poor glycemic control [77-80]. Similarly, mouse models of HD also exhibit these symptoms, together with impaired glucose metabolism in both brain and periphery and elevated blood glucose levels [81, 82]. This is probably due to the significant toxicity caused by high levels of mutant Htt in peripheral tissues including the pancreatic islet cells, leading to decrease in β-cell mass and impaired insulin release capacity [81, 82]. Since molecules that improve abnormal energy metabolism such as creatine have been shown to work as a neuroprotective agent and to delay the onset of motor dysfunction in a mouse model of HD [83], therapeutic approaches targeting this diabetic-like condition may be promising. Exendin-4 (Ex-4) is an agonist for glucagon-like peptide-1 receptor, and is used as a peptide drug for diabetes to improve glucose regulation [84]. Martin *et al.* tested the effects of Ex-4 on a mouse model of HD, and found that daily administration of Ex-4 by subcutaneous injection improved motor dysfunction and extended the life span of HD mice [85]. They also found that Ex-4 injection significantly promoted pancreatic β-cell growth and reduced Htt aggregates in the pancreas as well as in the brain cortex [85]. This study strongly indicates that therapeutic approaches targeting not only the central pathophysiologies but also the peripheral symptoms could be an effective strategy for treatment of the polyQ diseases.

Leuprorelin

SBMA is an adult-onset motor neuron disease, which is caused by the expansion of a polyQ stretch in the androgen receptor (AR) [86]. Although the specific pathogenic mechanisms of SBMA still remain unclear, the nuclear accumulation of abnormal AR proteins is thought to be respon-

sible for neuronal toxicity. Since testosterone binds to AR as a ligand and induces its nuclear translocation, reduction of the testosterone level would lead to a decrease in the eventual nuclear accumulation of mutant AR and to lower cytotoxicity [87, 88]. This idea is actually supported by the experimental fact that surgical castration significantly improved motor dysfunctions of a mouse model of SBMA [87, 89]. Katsuno *et al.* tested the effects of leuprorelin, a lutenizing hormone-releasing hormone (LHRH) peptide agonist that reduces testosterone release from the testis, and found that subcutaneous injection of leuprorelin reduced the nuclear accumulation of mutant AR in muscle and spinal cord, and improved the motor dysfunctions and extended the life span of a SBMA mouse model [90]. Furthermore, they conducted a series of clinical trials of leuprorelin including a randomized, placebo-controlled trial in a large cohort of 204 SBMA patients from 14 hospitals in Japan [91, 92]. Although clinical outcomes of leuprorelin administration for 48 weeks were limited to suppression of nuclear AR accumulation and decreased serum levels of testosterone with no significant improvement of motor functions, there is a possibility that leuprorelin could be effective in long-term trials in early-phase SBMA patients.

FUTURE DIRECTIONS

In this review, we introduced selected studies focusing on the development of peptide-based therapies for treatment of the polyQ diseases. Among them, the therapeutic approach focusing on aggregate formation of the expanded polyQ stretch, which targets the most upstream change occurring in the polyQ diseases, is considered to be most attractive because potential inhibitors are expected to suppress a large number of downstream functional abnormalities in a broad range of the polyQ diseases. However, efficient delivery into brains is always problematic in developing peptide-based drugs [93], as aggregation inhibitors developed by us [63] and by Chen *et al.* [64] both failed to demonstrate therapeutic effects on mouse models via their subcutaneous or intraperitoneal administration. Since both QBP1 and HQP09 have been shown to possess high potential to specifically and selectively suppress mutant polyQ-induced cytotoxicity, it is highly likely that they would be promising leads for development of polyQ disease drugs if given the ability to efficiently translocate across the blood-brain barrier (BBB). Therefore, it is quite clear that one of the future directions that we should progress toward is to re-design these potential peptide inhibitors into BBB-permeable molecules. Elucidating the structural basis as to how QBP1 and HQP09 inhibit aggregate formation of the expanded polyQ stretch would be helpful towards designing their small chemical analogues with high BBB permeability without loss of its inhibitory activity. Another direction is to develop effective delivery systems using carrier molecules which would efficiently deliver cargoes to the brain. Potential carriers include cell-penetrating peptides (CPPs, protein transduction domains/PTDs) [94-96], viral vectors [97, 98] and liposomes [99, 100], which may enable these peptide inhibitors to translocate through the BBB and to perform their therapeutic activities in specific regions of the brain. We hope that in the near future therapeutic approaches that are widely effective against the polyQ diseases are developed, and bring hope to many patients suffering from the currently untreatable polyQ diseases.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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ABBREVIATIONS

AR	=	Androgen receptor
Bax	=	Bcl-2 associated X protein
BBB	=	Blood-brain barrier
Bcl2	=	B-cell lymphoma 2
BIP	=	Bax-inhibiting peptide
CaM	=	Calmodulin
Ex-4	=	Exendin-4
HD	=	Huntington's disease
Htt	=	Huntingtin
InsP$_3$R1	=	Type 1 inositol 1,4,5-triphosphate receptor
K_d	=	Dissociation constant
LHRH	=	Lutenizing hormone-releasing hormone
MW	=	Molecular weight
PolyQ	=	Polyglutamine
PTD	=	Protein transduction domain
QBP1	=	PolyQ binding peptide 1
SBMA	=	Spinal and bulbar muscular atrophy
SCA	=	Spinocerebeller ataxia

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その他のFTLD — 異常蛋白の視点から

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はじめに

前頭側頭葉変性症（FTLD：frontotemporal lobar degeneration）は前頭葉と側頭葉の神経が進行性に変性・脱落する神経変性疾患である．病理型としては，多くの神経細胞あるいはグリア細胞内に封入体が認められる FTLD と，封入体が認められない FTLD-ni（no inclusions）に大別され，封入体が認められるものは，蓄積する蛋白質により FTLD-tau，FTLD-TDP，FTLD-FUS，および FTLD-UPS（ubiquitin proteasome system）の4型に分類されている[1]．本稿では FTLD-tau 以外の封入体が認められる FTLD-U について異常蛋白質の視点から紹介する．また，近年 FTLD の原因として同定された *C9orf72* 遺伝子変異による異常蛋白質の蓄積についても最新の知見を紹介する．

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FTLD-TDP

タウ陰性でユビキチン陽性の封入体を伴った FTLD-U において，封入体を構成する主要蛋白質は不明であったが，2006 年に RNA 結合蛋白質である TAR DNA-binding protein of 43（TDP-43）が蓄積していることが明らかにされた[2,3]．FTLD-U のうち TDP-43 陽性封入体の病理所見を示すものは FTLD-TDP と分類され，FTLD の 45％を占める[4]．さらに，筋萎縮性側索硬化症（ALS：amyotrophic lateral sclerosis）においても TDP-43 が蓄積していることが明らかにされたことから，FTLD と ALS 両疾患の病態には共通の分子基盤があると考えられるようになった．TDP-43 は FTLD-TDP 患者脳内において疾患特異的なリン酸化や断片化を受けており，特に C 末断片の凝集・蓄積が観察される[5,6]．TDP-43 の C 末端には凝集性に富むプリオン様ドメインが存在し（図1），断片化により凝集性が増すことから，この C 末断片が患者脳における凝集・蓄積
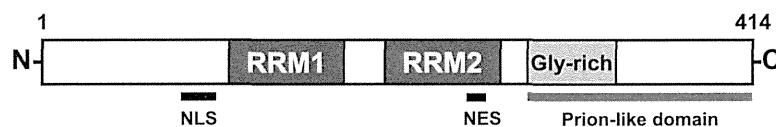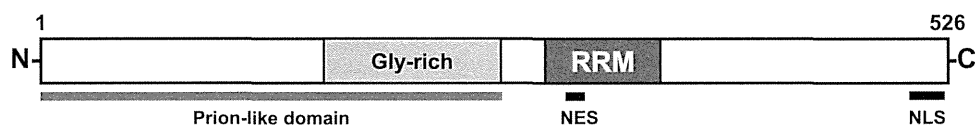

図 1　TDP-43 と FUS の蛋白質構造
　TDP-43 は 414 残基，FUS は 526 残基のアミノ酸からなり，その分子内には RNA 結合に関わる RNA 認識モチーフ（RRM：RNA recognition motif），蛋白質-蛋白質相互作用に関わるグリシンリッチ領域（Gly-rich）を含むプリオン様ドメイン（Prion-like domain），核移行シグナル（NLS：nuclear localization signal），および核外移行シグナル（NES：nuclear export signal）が存在する．

に関与すると予測される．異常な TDP-43 の凝集・蓄積メカニズムおよび細胞間伝播は未解明であったが，2013 年に Nonaka らは，患者脳由来の不溶性 TDP-43 を培養細胞内に導入すると，本来は可溶性であった細胞内の TDP-43 が凝集体を形成することを示し，異常 TDP-43 の凝集性が正常 TDP-43 に伝播すること，さらにこの TDP-43 の凝集性は細胞間でも伝播することを明らかにした．これらの結果から，患者脳の不溶性 TDP-43 はプリオン様の特性を保持していると結論付けている[7]．培養細胞を用いた *in vitro* の実験ではあるが，TDP-43 の凝集・蓄積モデルを樹立し，細胞間伝播までを示した上記の研究は興味深いものであり，今後，動物モデルや患者病理の解析から TDP-43 のプリオン様の性質獲得と病態の進行との関連性の解明が期待される．

FTLD-FUS

2008 年に家族性 ALS（ALS6）の原因として FUS 変異が同定され，ALS6 患者の病理学的解析から FUS が神経細胞質内の封入体の構成成分であることが明らかにされた．続いて，TDP-43 陰性封入体を伴った aFTLD-U（atypical FTLD-U），NIFID（neuronal intermediate filament inclusion disease），と BIBD（basophilic inclusion body disease）において，神経細胞およびグリア細胞の細胞質に FUS 陽
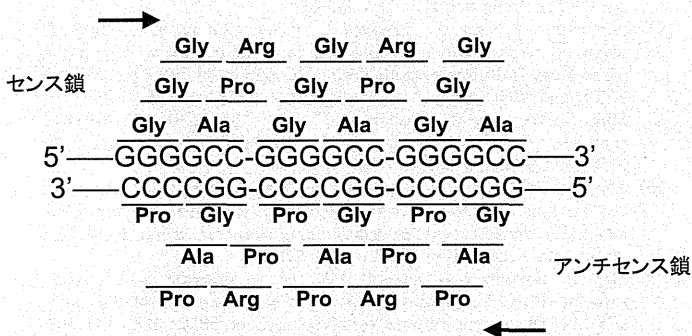

図 2　*C9orf72* 遺伝子 GGGGCC 異常伸長リピート由来のジペプチドリピート蛋白質

C9-FTLD/ALS 患者において *C9orf72* 遺伝子非翻訳領域の 6 塩基（GGGGCC）異常伸長リピート配列はセンス鎖およびアンチセンス鎖の両方向に転写される．さらに，ATG に依存しない翻訳（RANT：repeat-associated non-ATG translation）により 5 種類のジペプチドリピート（DPR：dipeptide repeat）蛋白質が産生される．Gly：グリシン，Arg：アルギニン，Pro：プロリン，Ala：アラニン

性の封入体が認められることが明らかになり[8〜10]，これらはまとめて FTLD-FUS と分類された．RNA 結合蛋白質である FUS は TDP-43 と同様に，分子内に凝集性に富むプリオン様ドメインを持っていることから（図1），FTLD-TDP で認められている異常蛋白質の伝播が FTLD-FUS でも生じる可能性が示唆される．しかし，FUS が凝集・蓄積するメカニズムは未解明であり，今後の研究が必要である．

FTLD-UPS

FTLD-U のうち TDP-43 陰性および FUS 陰性で，未だその主要構成成分の不明な封入体を伴うものは FTLD-UPS と分類されている．そのうちの一部において *CHMP2B*（charged multivesicular body protein 2B）の遺伝子変異が明らかにされた[11]．CHMP2B はエンドソーム輸送選別複合体を構成する分子で，リソソームにおけるユビキチン化蛋白質の分解に関与することが知られており，CHMP2B の機能不全によりユビキチン化蛋白質が細胞質内に残留し，封入体を形成していると考えられる．

最近の話題 — C9-FTLD/ALS

2006 年に ALS-FTD 家系の連鎖解析から第 9 番染色体に原因遺伝子座があると報告された．その遺伝子変異は長らく不明であったが，2011 年にこの家族性 ALS-FTD の原因遺伝子変異として染色体 9p21 領域に存在する *C9orf72* 遺伝子の非翻訳領域に 6 塩基（GGGGCC）リピート配列の異常伸長が発見された[12,13]．この *C9orf72* 遺伝子変異はヨーロッパおよび北米の家族性と孤発性 FTLD/ALS の最も高頻度な発症原因であり，本邦でも紀伊半島の FTLD/ALS 家系を中心に報告されている．*C9orf72* 遺伝子変異による FTLD/ALS（C9-FTLD/ALS）患者では TDP-43 陽性を示す封入体が脳の広範囲でみられ，FTLD-TDP に分類されているが，小脳，海馬，および前頭側頭新皮質では TDP-43 陰性の封入体が認められる[14]．C9-FTLD/ALS と同様な遺伝子非翻訳領域にあるリピート配列の異常伸長に起因する SCA8，DM1，および FXTAS などの一群の疾患では，転写された異常伸長リピート RNA を鋳型として開始コドン ATG に依存しない翻訳（RANT：repeat-associated non-ATG translation）により，

異常伸長したポリアミノ酸リピート蛋白質が産生されることが知られている．C9-FTLD/ALS においても，異常伸長GGGGCC リピート RNA から RANT により翻訳されたポリ（グリシン-アルギニン），ポリ（グリシン-プロリン），ポリ（グリシン-アラニン），ポリ（プロリン-アラニン），およびポリ（プロリン-アルギニン）の 5 種類のジペプチドリピート（DPR：dipeptide repeat）蛋白質（図2）の存在が示され，これらが TDP-43 陰性封入体に蓄積していることが明らかになった[15,16]．続いて，DPR 蛋白質のうちポリ（グリシン-アラニン），ポリ（グリシン-アルギニン），およびポリ（プロリン-アルギニン）は，培養細胞に発現させると細胞死を引きおこすことが示され[17~19]，さらにショウジョウバエモデルにおいても，ポリ（グリシン-アルギニン）とポリ（プロリン-アルギニン）が神経変性を引きおこすことが示され[20]，DPR 蛋白質の一部は神経細胞毒性を持つことが明らかにされた．以上のことから，DPR 蛋白質の蓄積がC9-FTLD/ALS 発症の原因として考えられるようになり，病理型として新たに FTLD/ALS-DPR として分類することが提唱されている[15]．しかしながら，各 DPR の細胞毒性はモデルによって一致しておらず，今後の検証が必要である．また，すべての封入体が DPR 陽性ではなく，TDP-43 陽性を示す封入体もみられる理由は不明であり，今後の研究が必要である．

むすび

近年の遺伝学的・生化学的研究の発展により，FTLD において封入体を構成する主要蓄積蛋白質が次々と同定された．しかし，未だに FTLD-UPS において蓄積する異常蛋白質は不明であり，蓄積蛋白質が解明された FTLD-TDPや FTLD-FUS においても凝集・蓄積および伝播のメカニズムは様々な仮説が提唱されているが，多くは未解明である．今後，FTLD-TDP については，細胞モデルを用いた研究から得られた異常 TDP-43 の凝集・蓄積および伝播に関する知見を，動物モデルで検証することが期待される．また，他の FTLD についても FTLD-TDP の研究が起因となり，異常蛋白質の凝集・蓄積，伝播メカニズムの解明が進むことが期待されている．

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ポリグルタミン病における神経変性

4

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ポリグルタミン病は，CAGリピート配列の異常伸長という特徴的な遺伝子変異に起因する遺伝性疾患であるため，分子生物学を基盤とした病態研究が他疾患に先んじて進展してきた．遺伝子改変マウスの解析から，神経症状は神経細胞死に至る前の可逆性神経機能障害に起因することが明らかになった．その神経機能障害は，個々の神経細胞の自律的（cell-autonomous）な障害だけでなく，グリアなどを含む非細胞自律的（non-cell-autonomous）なネットワーク障害に起因すると考えられるようになった．その中でも，シナプスを介する神経伝達の障害は発症前から存在することが示唆され，発症後からでも神経症状を改善できる治療標的となる可能性がある．

はじめに

アルツハイマー病，パーキンソン病，筋萎縮性側索硬化症（ALS），ハンチントン病，脊髄小脳失調症などに代表される神経変性疾患は，それぞれ特定の領域の神経細胞群が進行性に変性・脱落した結果，さまざまな神経・精神症状を呈する原因不明の疾患群と元来定義されていた．しかしながら，遺伝性を示す神経変性疾患においては，分子遺伝学的解析から大多数の原因遺伝子変異が同定され，アミロイド前駆蛋白質，タウ，α-シヌクレイン，SOD1，TDP-43，huntingtin，ataxinなど多くの遺伝子変異により構造異常（ミスフォールディング）・凝集を起こしやすい変異蛋白質が産生されることが明らかにされた．一方，孤発性の神経変性疾患においても，これらの蛋白質が凝集して神経細胞内外に封入体として蓄積することが従来から知られていた．

以上のことから，異常蛋白質のミスフォールディング・凝集により，共通に神経変性が引き起こされるという普遍的な発症分子メカニズムが想定され，これらの疾患はコンフォメーション病もしくはミスフォールディング病と総称されている[1]．本稿では，コンフォメーション病のモデル疾患としてポリグルタミン病に着目し，その神経変性メカニズムの解明を目指した研究から明らかになった最新の知見を紹介する．

1 ポリグルタミン病

ポリグルタミン（PolyQ）病とは，ハンチントン病，脊髄小脳失調症（SCA）1，2，3，6，7，17型，歯状核赤核淡蒼球ルイ体萎縮症，球脊髄性筋萎縮症など9疾患の総称であり，これらの疾患は，それぞれ別の原因遺伝子内にあるグルタミンをコードするCAGリピート配列の異常伸長という