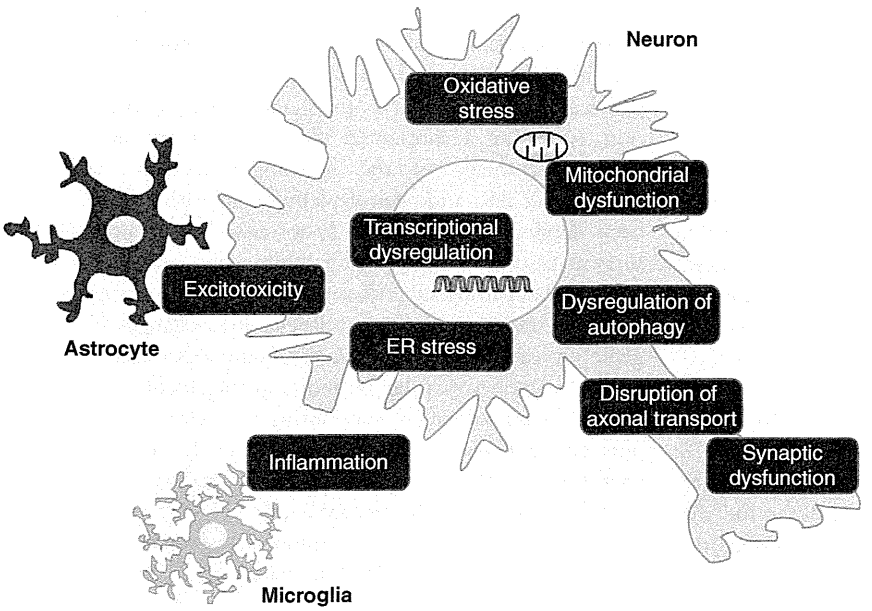

Figure 2. Therapeutic targets of polyglutamine diseases. The accumulation of polyglutamine-expanded proteins instigates a variety of downstream molecular events including transcriptional dysregulation, mitochondrial dysfunction, oxidative stress, axonal transport deficits and ER stress. Non-neuronal cells, particularly astrocytes and microglia, appear to modify the neurodegenerative process. All the alterations have been demonstrated in cellular and/or animal models of Huntington's disease. Transcriptional dysregulation, mitochondrial dysfunction and ER stress have also been reported in animal models of spinal and bulbar muscular atrophy and spinocerebellar ataxias.
ER: Endoplasmic reticulum.

polyglutamine diseases [23]. Also, the abnormal interaction between soluble polyglutamine-expanded proteins and transcriptional co-activators is demonstrated in animal models of polyglutamine diseases as well as in the postmortem tissues of patients [24]. Because cAMP binding protein functions as a histone acetyltransferase that regulates gene transcription and chromatin structure, decreased histone acetyltransferase activity and eventual histone hypoacetylation are likely to underlie the pathogenesis of neurodegeneration in polyglutamine diseases.

Mitochondrial function is also affected by the abnormal polyglutamine proteins through direct interaction and/or transcriptional dysregulation of mitochondrial proteins. The pathogenic huntingtin with an expanded polyglutamine induces mitochondrial fragmentation and defects in anterograde and retrograde mitochondrial transport through abnormal interactions with the mitochondrial fission GTPase dynamin-related protein-1 [25]. The transcription of PPAR-γ coactivator-1α (*PGC-1α*), a transcriptional co-activator of mitochondrial genes, is also suppressed by the pathogenic huntingtin [26]. Mitochondrial failure further leads to defective energy metabolism as well as disturbances in calcium homeostasis [27]. Roles of mitochondrial dysfunction are also suggested in SCA3 and SBMA [28,29].

Activation of *N*-methyl-D-aspartic acid receptors (NMDARs) is another molecular mechanism underlying the pathogenesis of polyglutamine diseases [30]. In mouse models of HD, glutamate uptake by astrocytes is decreased, partially due to the down-regulation of glutamate transporter 1 [31]. It also has been shown that the pathogenic huntingtin increases the extrasynaptic localization of GluN2B-containing NMDARs, leading to excitotoxicity [32]. The activity of extrasynaptic NMDARs is enhanced by the pathogenic huntingtin-mediated down-regulation of *PGC-1α*, which disrupts cAMP response element binding protein-meditated transcription, suggesting the link among these downstream molecular events in the pathogenesis of HD [33].

The abnormal polyglutamine proteins have also been shown to disrupt axonal transport, although the precise molecular mechanism underlying this phenomenon is still a matter of debate. Abnormal interaction between the pathogenic huntingtin and huntingtin-associated protein 1 disrupts axonal transport of brain-derived neurotrophic factor (BDNF), which is also the target of polyglutamine-mediated transcriptional dysregulation [20]. Disrupted axonal transport results in decreased trafficking of organelles such as mitochondria and autophagosomes [34]. Retrograde axonal trafficking is also disrupted in certain animal models of SBMA, although no deficit of axonal transport was detected in another mouse model of this disease [35].

DNA damage also plays a causative role in the pathogenesis of polyglutamine-mediated neurodegeneration. The pure polyglutamine introduced in cultured cells activates ataxia telangiectasia mutated kinase, and thereby induces a DNA damage response through accumulation of reactive oxygen species [36]. Expression of the pathogenic form of huntingtin and ataxin-2 leads to reactive oxygen species-induced DNA damage [37]. The polyglutamine-expanded huntingtin intensifies DNA damage by interaction with Ku70, a component of the DNA damage repair complex [38]. Similarly, the pathogenic AR interferes with the Pax transactivation-domain interacting protein, which functions in DNA repair [39]. An even more intriguing aspect of DNA damage in polyglutamine diseases emerges from the observation that the causative proteins of HD, SBMA and SCAs mitigate DNA double-stranded break repair by aberrant interaction with transitional endoplasmic reticulum ATPase/valosin-containing protein/p97 [40].

Non-cell autonomous toxicity

Although neurons are the target of polyglutamine-mediated toxicity, non-neuronal cells also play critical roles in the pathogenesis of neurodegeneration in polyglutamine diseases. Expression of the pathogenic ataxin-7 in Bergmann glia of the cerebellum is

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sufficient for the development of neurodegeneration in Purkinje cells [41]. Astrocyte-specific expression of polyglutamine-expanded huntingtin also gives rise to age-dependent motor deficits in mice, although there is no detectable neuronal cell death [42]. Overexpression of wild-type *AR* in the skeletal muscle induces motor axon loss in an androgen-dependent manner, suggesting a pathogenic role of muscle in SBMA [43]. Inhibition of the pathogenic *AR* expression in skeletal muscle results in amelioration of motor neuron degeneration [44]. These observations suggest that AR-mediated myopathy might contribute to non-cell autonomous degeneration of spinal motor neurons.

Native function of polyglutamine proteins

The key targets of polyglutamine toxicity have not been fully elucidated, but recent studies suggest that enhanced native functions of the host protein may underlie the neurodegeneration in polyglutamine diseases. Among polyglutamine diseases, the native functions are well understood for AR. In a fly model of SBMA, native interaction between AR and its co-regulators is strongly associated with the polyglutamine-mediated cytotoxicity [45]. This finding is in accordance with the observation that overexpression of wild-type *AR* induces neurodegenerative changes [43]. The interaction between ataxin-1 and RNA-binding motif protein 17, a splicing factor, is enhanced by the expansion of polyglutamine [46]. The accumulation of insoluble polyglutamine-expanded proteins sequestrates many interacting molecules into the protein complex and thereby inhibits the physiological function of these molecules, leading to pathological cellular events such as transcriptional dysregulation, altered heat shock response and DNA damage [47]. On the other hand, several lines of evidence suggest that expanded polyglutamine repeats may impair the physiological function of host proteins. For example, ataxin-3 possesses a deubiquitinating activity, which is potentially suppressed by polyglutamine elongation [48]. Taken together, these observations indicate that the altered native function of host proteins plays a role in the pathogenesis of polyglutamine-mediated neurodegeneration.

Symptomatic therapies for polyglutamine diseases

Although there is currently no therapy that halts neurodegeneration in polyglutamine diseases, several medicines are used for ameliorating neurological symptoms in clinical settings. Tetrabenazine is an US FDA-approved dopamine-depleting agent that suppresses involuntary movement including chorea in HD [49]. Anti-psychotic drugs have also been used for chorea and psychiatric symptoms such as aggression [49]. Selective serotonin reuptake inhibitors may be the treatment of choice for depression, but close attention should be paid to relatively high frequencies of suicidal ideation in HD patients. Ataxia in polyglutamine-mediated SCAs may be controlled through medications such as tandospirone, although the efficacy of such therapies has not been shown in large-scale, randomized, controlled trials [50]. Medications for essential tremor and muscle cramps may be used as symptomatic therapies for SBMA and SCAs [51].

Disease-modifying therapies for polyglutamine diseases

Agents inhibiting abnormal protein accumulation

The abnormal accumulation of polyglutamine-expanded protein has been implicated in the pathogenesis of neurodegeneration, providing a substantial target for therapy development. Taking advantage of the hormone-dependent intracellular dynamics of AR, androgen-depleting/inactivating therapies have been developed for SBMA. Testosterone deprivation through surgical castration substantially inhibits the nuclear accumulation of the pathogenic AR and suppresses the progression of motor dysfunction and neurodegenerative changes in a transgenic mouse model of SBMA [15]. Leuprorelin acetate, a luteinizing hormone-releasing hormone analog, depletes the serum levels of testosterone by suppressing the release of gonadotropins and has been used for various sex hormone-dependent conditions including prostate cancer. Leuprorelin is also shown to inhibit the nuclear accumulation of the polyglutamine-expanded AR, resulting in a profound improvement of neuromuscular phenotypes as well as histopathological findings in SBMA mice [52]. In a Phase II clinical trial, treatment for 12 months with leuprorelin acetate significantly decreased the serum level of creatine kinase and suppressed the nuclear accumulation of the polyglutamine-expanded AR in the scrotal skin of patients with SBMA. A noteworthy observation was that the frequency of neurons bearing nuclear accumulation of the pathogenic AR within the anterior horn and brainstem was less in an autopsied patient who received leuprorelin for 2 years than in untreated SBMA patients [53]. Nevertheless, no definite effects on motor functional scores or the 6-min walk test were shown in a large-scale, multicentric, randomized, placebo-controlled, clinical trial of leuprorelin acetate, for which 204 patients were enrolled. However, there was an improvement of swallowing function, measured by the pharyngeal barium residue in videofluorography, in a subgroup of patients whose disease duration was less than 10 years [54]. Also, the effectiveness of dutasteride, a 5α-reductase inhibitor that inactivates testosterone, was not clearly shown in a Phase II clinical trial [55]. Several factors may underlie the lack of positive results, insensitiveness of the outcome measure utilized, anti-anabolic effects of tested drugs, a relatively short observation period and placebo-related improvement of motor function. The disease duration of the patients might also influence the effects of the androgen-depleting therapy.

Several compounds that directly inhibit abnormal polyglutamine proteins have also been identified. For example, a green tea polyphenol, (-)-epigallocatechin-3-gallate, inhibits the oligomerization of the pathogenic huntingtin, resulting in amelioration of photoreceptor degeneration in a fly model of HD [56]. Similarly, methylene blue is shown to suppress the abnormal huntingtin accumulation and thereby attenuate neurodegenerative changes in a mouse model of HD [57]. Inhibitors of caspase attenuate the pathogenic huntingtin toxicity by preventing proteolytic cleavage of the protein [58]. Transglutaminase, which may play a role in the formation of polyglutamine aggregation, is another target of therapy development, and inhibitors of this

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enzyme are shown to mitigate neurodegeneration in animal models of HD and DRPLA [54]. On the other hand, an agent that promotes aggregate formation is shown to alleviate the polyglutamine-mediated neurotoxicity in animal models of HD and SBMA, possibly by facilitating the formation of less-toxic visible inclusion bodies [59,60]. These results again indicate the need for identification of toxic species of polyglutamine-expanded proteins for determining the precise target of disease-modifying therapies.

Restoration of protein homeostasis is another promising approach to polyglutamine diseases, and several strategies activating cellular machinery such as chaperones, the ubiquitin–proteasome system and autophagy have been tested in cellular and animal models of polyglutamine diseases [61]. Certain agents that induce the expression of HSPs or their co-activators are shown to inhibit the accumulation of abnormal polyglutamine proteins and thereby mitigate neurodegenerative processes in animal models of SBMA [62–64]. The complex of HSP90–client proteins, such as AR, is stabilized when it is associated with p23, a co-chaperone interacting with HSP90, whereas inhibition of HSP90 facilitates proteasomal degradation of its client proteins by altering the components of the protein complex. An HSP90 inhibitor, 17-*N*-allylamino-17-demethoxygeldanamycin (17-AAG), dissociates p23 from the HSP90–AR complex and thus facilitates proteasomal degradation of the polyglutamine-expanded AR in cellular and mouse models of SBMA [65]. Nuclear accumulation of this protein is thereby inhibited by 17-AAG, leading to marked mitigation of motor impairment in SBMA mice. The 17-AAG is also a potent inducer of HSPs and is shown to ameliorate neurodegeneration caused by the pathogenic ataxin-3 in a fly model of SCA3 [66]. Oral administration of 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin, a derivative of 17-AAG, also shows similar effects in the mouse model of SBMA [67]. The amiloride derivative, benzamil, is shown to attenuate the pathogenic huntingtin accumulation by increasing the activity of the ubiquitin–proteasome system in cellular and animal models of HD [68].

Interferon β facilitates the proteasomal degradation of polyglutamine-expanded ataxin-7 by up-regulating promyelocytic leukemia protein, which associates with various causative proteins of polyglutamine diseases [69].

Induction of autophagy, another proteostasis controlling system, is also shown to mitigate the toxicity of the polyglutamine-expanded AR [66,67,70,71]. Pharmacological activators of autophagy, including trehalose and rapamycin analogs, suppress neuronal damage mediated by the polyglutamine-expanded huntingtin and ataxin-3 proteins [72–74]. The HSP90 inhibitors, 17-AAG and 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin, are shown to activate both the ubiquitin–proteasome system and autophagy [75]. Similarly, paeoniflorin, a major component of *Paeonia* plants, and Rho kinase inhibitors also facilitate the degradation of the pathogenic polyglutamine proteins through the ubiquitin–proteasome system and autophagy [76,77]. Although these studies suggest beneficial therapeutic effects of autophagy-modulating therapies on polyglutamine diseases, opposing results are shown in experiments using a mouse model of DRPLA [78]. In addition, inhibition of the unfolded protein response, an endoplasmic reticulum stress response, exacerbates the muscle pathology of SBMA by activation of autophagy, suggesting that fine tuning of protein quality control systems may be necessary for coping with the toxicity of polyglutamine-expanded proteins [79].

Therapies targeting downstream molecular events

The histone acetylation level is determined by the interplay between histone acetyltransferase and histone deacetylase (HDAC). Because suppression of HDAC activity results in augmentation of histone acetylation and subsequent restoration of gene transcription, HDAC inhibitors have been considered to be therapeutically beneficial in polyglutamine diseases [80]. Sodium butyrate, the first HDAC inhibitor to be discovered, has been shown to ameliorate the symptomatic and histopathological phenotypes of mouse models of HD, including SBMA, SCA3 and DRPLA, through up-regulation of histone acetylation in nervous tissues, although the therapeutic window of this treatment is limited by the toxicity [81–84]. Other HDAC inhibitors such as suberoylanilide hydroxamic acid and sodium valproate also show similar effects in animal models of HD and SCA3, respectively [85,86].

Several compounds are shown to rescue neurons from polyglutamine-mediated neuronal dysfunction and cell death. For instance, c-Jun N-terminal kinase (JNK) appears to play a causative role in the pathogenesis of polyglutamine diseases. CEP-1347, an inhibitor of mixed lineage kinases, suppresses the JNK pathway and improves motor performance as well as *BDNF* expression in HD mice [87]. In SBMA, the pathogenic AR activates the JNK pathway by increasing the expression levels of the calcitonin gene-related peptide α (*CGRP1*) gene [88]. The genetic depletion of *CGRP1* inactivates JNK and ameliorates neurodegeneration in SBMA mice. Naratriptan, a 5-hydroxytryptamine receptor (5-HT) 1B/1D agonist, suppresses *CGRP1* expression by the induction of mitogen-activated protein kinase phosphatase 1, attenuates JNK activity, and mitigates the pathogenic AR-mediated neuronal damage in cellular and mouse models of SBMA. Caspase inhibitors are also shown to attenuate the apoptotic pathway induced by the abnormal polyglutamine proteins [89]. As for excitotoxicity, treatment with low-dose memantine inhibits extrasynaptic NMDARs and ameliorates the neuropathological and behavioral phenotypes of HD mice [90]. Aminopyridines, which are potassium channel blockers, ameliorate cerebellar ataxia in SCA1 mice by improving the electrophysiological functions of Purkinje cells [91]. Lithium is shown to ameliorate neuropathology in animal models of polyglutamine diseases by restoring the Wnt pathway and inducing autophagy [92], although the beneficial effects of this agent on motor function were not shown in a randomized trial of SCA3 patients [93].

Neurotrophic factors are alternative targets of disease-modifying therapies. Chronic systemic administration of BDNF results in the attenuation of polyglutamine-dependent neurodegeneration in HD mice [94]. Treatment with LM22A-4,

a small molecule ligand of the BDNF receptor TrkB, results in alleviation of HD-related pathology in mouse models of HD. This therapy suppresses intranuclear huntingtin aggregates, restores the expression of *DARPP-32*, mitigates degeneration of parvalbumin-containing interneurons and attenuates neuroinflammation in HD mice [95]. Pharmacological inhibition of calcineurin by FK506 facilitates the phosphorylation of mutant huntingtin at S421, and thus restores BDNF transport [96]. Ampakine also up-regulates BDNF and ameliorates neurological phenotypes in a knock-in mouse model of HD [97]. Supplementation of IGF-1 augments Akt-mediated AR phosphorylation, leading to suppression of AR accumulation and eventual amelioration of neuromuscular deficits in a transgenic mouse model of SBMA [98,99].

Mitochondrial dysfunction is a common therapeutic target of neurodegenerative disorders [100]. A pan-PPAR agonist, bezafibrate, restores the expression levels of *PGC-1α*, PPARs and downstream genes and ameliorates motor phenotypes of HD mice [101]. Rosiglitazone, a PPAR-γ agonist, suppresses the pathogenic huntingtin-induced neurodegeneration and improves motor function and glucose metabolism in HD mice [102]. Nicotinamide, an activator of sirtuin, is also shown to ameliorate HD-mediated neurodegeneration by up-regulating the expression of *PGC-1α* and *BDNF* [103]. Mitochondria-targeted compounds such as coenzyme-Q10 and creatine show neuroprotective effects in animal models of HD, and their efficacy has been tested in large-scale clinical trials [104].

Although animal experiments suggest promising therapies for polyglutamine diseases, the preclinical success has not been reproduced in clinical trials. For example, studies using valproate sodium or riluzole have failed to show any benefit in HD [105–107]. Similar failures have been noted with other potential neuroprotective studies in symptomatic HD, including with the use of eicosapentaenoic acid, donepezil, ethyl eicosapentaenoic acid and minocycline [108]. These discrepancies reflect a number of unsolved problems: limited knowledge of the exact pathways of neurodegeneration, safety and delivery issues of compounds, limitations of using animal studies to predict benefit in humans, sensitive outcome measures, and limited tools to diagnose and treat presymptomatic patients.

Other experimental therapies

Gene silencing is an ideal approach to the gain-of-toxicity of abnormal polyglutamine proteins. Nonallele-specific silencing of huntingtin through siRNA decreases the protein levels of huntingtin and ameliorates motor phenotypes of mouse models of HD and SCAs [109,110]. Similar approaches using short hairpin RNA antisense oligonucleotides have also been examined in animal models of polyglutamine diseases [111,112]. Because the possibility that the loss of physiological function of polyglutamine proteins contributes to the pathogenesis of neurodegeneration, allele-specific silencing via siRNA and antisense oligonucleotides has also been developed [113].

Cellular and gene therapies may be the choice for the advanced stage of polyglutamine diseases. Transplant of fetal brain tissues and human embryonic stem cells has been used as a cell resource, although both the safety and efficacy of such therapies are uncertain in clinical trials, indicating the need for further study [114]. Activation of neurogenesis is another avenue of therapy, given that selective serotonin reuptake inhibitors and BDNF potentiate the addition of adult neuronal cells [115–120]. A study whereby BDNF is produced by stem cells is currently in current clinical trials. Adeno-associated virus-mediated delivery of HSP40 and polyglutamine binding peptide into the striatum suppresses inclusion body formation in HD mice [121]. Viral delivery of micro RNA that destabilizes the pathogenic AR has potent therapeutic effects on motor dysfunction in SBMA mice [122].

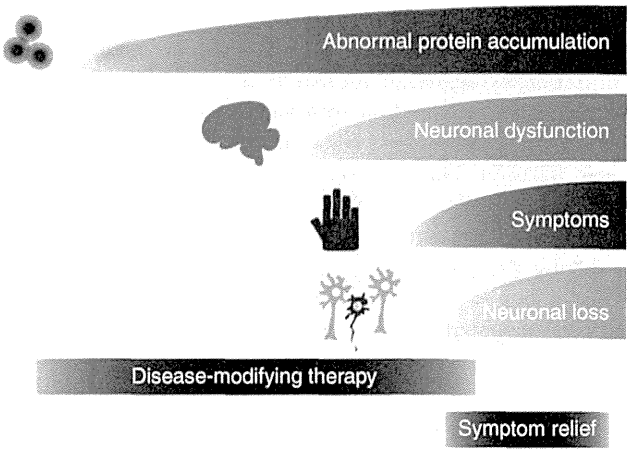

Figure 3. Progression of neurodegeneration and phase-dependent therapeutic approaches. Abnormal protein production and accumulation leads to subclinical dysfunction of the relevant nervous system areas, followed by manifestation of clinical symptoms. Several studies suggest the need for pre-onset/early initiation of disease-modifying therapies for polyglutamine diseases. On the other hand, the development of cell-based therapies and symptom relief is necessary at the later stages of diseases.

Exercise is another promising non-pharmacological therapy for polyglutamine diseases. In mouse models of SCA1 and HD, exercise is shown to attenuate neurological phenotypes [123–127]. The beneficial effects of exercise are also shown in a pilot study of patients with SBMA [128]. As for other modulators of HD pathology, experimental manipulations including environmental enrichment have beneficial effects in mouse models of HD [129–131], although stress is also identified as a negative environmental factor of HD [132].

Presymptomatic therapy for polyglutamine diseases

Polyglutamine diseases are single-gene disorders, which can be diagnosed at preclinical stages by genetic examination. Furthermore, the strong correlation between CAG repeat size and the age of onset enables the estimation of disease onset in preclinical mutation carriers of HD [133]. Based on this estimation, several studies have revealed neuropsychiatric and imaging deficits during a premanifest phase of HD (Figure 3). Two large studies

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following prodromal HD subjects, TRACK-HD and PREDICT-HD, demonstrated subclinical motor and cognitive dysfunction as well as decreased caudate volume in presymptomatic mutant huntingtin carriers [134,135]. These observations are substantially informative for designing clinical trials for early/preventive intervention. For HD, the effects of coenzyme Q10 and creatine have been assessed in preclinical mutation carriers of HD [136,137]. Although these preventive trials warrant further attempts to examine disease-modifying therapies in prodromal subjects with polyglutamine diseases, several ethical and methodological issues surrounding this type of trials should be addressed [138].

Biomarkers of polyglutamine diseases

Conventional neuropsychiatric batteries and motor functional measures are not sensitive enough to evaluate interventions during prodromal periods of polyglutamine diseases. Therefore, for conducting preventive trials, biomarkers that reflect the biological consequences of polyglutamine toxicity and detect premanifest neuronal dysfunction should be developed [2,139]. Currently studied biomarkers for polyglutamine diseases include parameters of neuroimaging, biofluid analysis, physical assessment, gene expression profiles, proteomics, metabolomic analysis and histopathology, some of which have been investigated in clinical trials. The most popular biomarkers of polyglutamine diseases are neuroimaging parameters. For example, the rate of decrease in caudate volumes is faster in preclinical HD carriers than in age-matched controls, even those who are more than 15 years from the estimated age of onset [140]. MRI volumetry also detects regional brain atrophy in patients with SCAs [141]. Diffusion tensor imaging detects microstructural changes in the corticostriatal fibers, which are associated with cognitive and motor performance in pre-HD [142]. Studies using functional MRI demonstrated alteration of blood oxygen level dependent hemodynamic responses in the striatum and oculomotor cortex of presymptomatic HD [143,144]. PET has demonstrated glucose hypometabolism, the degree of which correlates with cognitive deficits in HD patients [145] Reduction in dopamine D2 receptor binding in the striatum is also shown to correlate with cognitive dysfunction in HD individuals [146]. Another approach to visualize microglial activation in PET has also been tested in the brains of HD mice and patients [147].

Biofluid markers have also been developed. Oxidative stress markers, such as 8-hydroxydeoxyguanosine in plasma and urine, have also been widely assessed in polyglutamine diseases and used as an outcome measure in preventive clinical trials of HD [148,149]. The concentrations of 24S-hydroxycholesterol, a cholesterol metabolite produced in the brain, are decreased in presymptomatic HD [150]. There are also studies demonstrating a reduced function of the A2A adenosine receptor in peripheral blood cells of individuals with HD and those with polyglutamine-mediated SCAs, although no correlation was found between the measured variable, B_{max}, and the disease severity in HD [151–153]. The levels of several molecules involved in inflammation and transcription have also been identified as a possible plasma marker of HD [154,155]. Direct measure of soluble huntingtin shows promise as a biomarker of HD which is closely relevant to brain pathology [156,157].

Sensitive measures of motor and cognitive functions are also rigorously tested in patients with polyglutamine diseases. Tongue strength is shown to be reduced in premanifest HD as well as in SBMA patients who report no subjective dysphasia [158,159]. Quantitative measurements of eye movement have also shown that saccade variables, such as velocity and latency, are also potential biomarkers of disease progression in the presymptomatic and early clinical stages of HD, as well as in SCA [160,161]. Given that peripheral neuropathy is often associated with polyglutamine diseases, electrophysiological parameters are alternative potential biomarkers for these diseases [162].

Since the use of biomarkers as primary outcome measures in pivotal trials for regulatory approval has not been accepted, the correlation between the therapy-induced changes in the biological parameters and targeted clinical outcomes should be validated. Reproducibility, sensitivity to therapy-related changes and relevance to clinical phenotype may be the key to the usage of biomarkers as surrogate endpoints.

Expert commentary

Since the discovery of *AR* gene mutations in SBMA, neurobiological studies using cellular and animal models have revealed the molecular pathogenesis of polyglutamine diseases. As clearly shown in SBMA, nuclear accumulation of the polyglutamine-expanded proteins appears to be the pivotal molecular event that instigates myriads of cellular dysfunctions and neuronal cell death. Nevertheless, no therapy has proven effective in clinical trials, despite positive results obtained in preclinical studies using animal models. These facts suggest a need to elucidate the entire disease mechanism, to initiate therapeutic interventions at early/premanifest stages and to develop sensitive outcome measures for evaluating the effects of therapies [163].

As for basic research, several critical issues regarding polyglutamine-mediated cytotoxicity remain unclear. There is a strong need for the identification of toxic species of pathogenic proteins and the development of agents that selectively target such species. Given the potential side effects with long-term use of neuroprotective agents, therapies at the pre-onset or early stages of disease should target the accumulation of abnormal proteins. The molecules that regulate the expression of the polyglutamine-expanded proteins are alternative targets of preventive therapies [164]. How to evaluate the efficacy of compounds in preclinical trials is another critical issue in the development of molecular-targeted therapies. Animal studies are often performed to clarify disease mechanisms, rather than to validate preclinical therapeutic efficacy. The lack of rigorous investigations into dose-finding and side effects in basic studies are potential causes of the failures in translational research [163].

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In exploratory phase clinical trials, biomarkers may be used for evaluating pharmacological and toxicological features of tested agents. Biomarkers are expected to provide valuable information on bioavailability and safety, leading to rational choices of agents to be further investigated in confirmatory-phase clinical trials. Pre-onset diagnosis could be improved by investigating several biomarkers. However, it is still unclear whether the effects of therapies on biomarkers correspond to clinical efficacy such as slowness of progression and delayed onset. Therefore, studies should be carried out to validate biomarkers with regard to the effects of therapies for future development of disease-modifying therapies. Utilization of species-independent biomarkers that are applicable to both animals and humans is another key to bridging the gap between basic and clinical research.

In addition to pharmacological approaches, non-pharmacological therapies such as exercise are potential interventions for polyglutamine diseases. Although the plausibility of physical therapy for neurodegenerative diseases is empirically suggested, there are several limitations in previous trials of exercise, including lack of an appropriate control arm and insufficient regulation of compliance. Therefore, randomized controlled trials are needed to augment the evidence supporting the disease-modifying effects of exercise. The combination of pharmacological and non-pharmacological therapies appears to be an ideal approach to combat polyglutamine-mediated neurodegenerative disorders.

Five-year view

The results of basic and clinical research on polyglutamine diseases suggest that the effects of disease-modifying therapies are likely limited at symptomatic stages due to the progression of neuropathological changes during preclinical periods. In fact, more than 50% of neurons in the striatum are lost even in mildly symptomatic HD patients [165]. It is thus clear that early/premanifest intervention is the key for the future development of therapeutic strategies against polyglutamine diseases. To this end, longitudinal studies on biological and clinometric indices should be rigorously investigated in pre-clinical carriers of expanded CAGs, although such studies may face practical problems, such as feasibility and ethics. Systems of patient registry and international networking will support clinical studies, including subjects at the premanifest and early stages of the disease.

Another developing area in polyglutamine research is the therapeutic approach to the symptomatic stage of neurodegeneration. Although simple suppression of abnormal protein aggregation may not be powerful at symptomatic stages, combination of disease-modifying therapies with various molecular targets may ameliorate the disease progression. Although certain populations of neurons are lost even at the onset of neurological symptoms, the remaining neuronal function may be preserved and enhanced by cellular therapy and non-pharmacological approaches. Given that non-neuronal, dividing cells play crucial roles in the pathogenesis of polyglutamine diseases, therapies that target non-cell autonomous toxicity are alternative strategies against polyglutamine diseases. There is also a need to develop symptomatic therapies that are currently not available for certain symptoms of HD and other polyglutamine-mediated disorders.

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Key issues

- Polyglutamine diseases are a group of inherited neurodegenerative disorders that are caused by an abnormal expansion of a trinucleotide CAG repeat.
- Intraneuronal accumulation of a polyglutamine-expanded protein is the pivotal molecular event that instigates neurodegeneration.
- Suppression of abnormal protein accumulation is a fundamental therapeutic strategy for polyglutamine diseases, but the effects of such therapies have not been clearly shown in clinical trials.
- Elucidation of downstream events, such as transcriptional dysregulation and mitochondrial dysfunction, provides molecular targets of disease-modifying therapy.
- Pathological and functional decline precedes the onset of clinical symptoms, suggesting the need for presymptomatic/early initiation of interventions.
- Development of biomarkers is indispensable for the evaluation of disease-modifying therapy at the prodromal stage of polyglutamine diseases.

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REVIEW

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Masato Yano · Takafumi Ohtsuka · Hideyuki Okano

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Abstract An increasing number of studies have demonstrated that neural RNA-binding proteins (nRNABPs) participate in several steps of neural development through post-transcriptional regulation of their RNA targets (Grabowski Curr Opin Genet Dev 21:388–394, 2011). Classical genetics and in vitro biochemical approaches have identified several important RNA targets of nRNABPs linked to cell-fate decision and neuronal functions. In recent years, new technologies, such as unbiased in vivo protein–RNA interaction approaches, high-throughput sequencing-cross-linked immunoprecipitation (HITS-CLIP), microarrays, RNAseq and others, have been developed. The use of these with genetics has succeeded in defining a dynamic range of RNA targets of RNABPs at the transcriptome-wide level. This new platform also provides the mechanistic insights into a specific biological function of nRNABPs. This review highlights the discoveries and challenges of the interplay between the nRNABPs and their biological functions in neural development.

Keywords RNA-binding protein · HITS-CLIP · Neural development

Introduction

Neural development is achieved by a developmental gene regulation program. Major contributors to this program are DNA-binding proteins, or transcription factors, which control the transcription initiation of genes and maintain the chromatin state and the level of DNA replication and repair. Several transcription factors are expressed in a specific cell lineage and are identified as a cell-fate determinant in the developing brain. Indeed, these unique expression patterns are achieved to control DNA to RNA transcription in the specific cell and cell lineage. In addition, RNA transcribed by actions of DNA-binding factors is also finely regulated to ensure that an appropriate isoform and level of final protein products are present in the right cell at the correct time. Its regulation is modulated by numerous RNA-binding proteins (RNABPs) that play a central role in the post-transcriptional regulation of protein-coding genes, such as alternative splicing, polyadenylation and translation (Richter and Treisman 2011). Hence, similar to cell-type-specific transcription factors, the involvement of neural RNABPs (nRNABPs) is suggested in neural RNA regulation and is dependent on their expression patterns and functions. RNA itself also has crucial roles in neural development. Approximately 80 % of human genomic DNA is transcribed into RNA and includes long non-coding RNAs that could modulate RNABP's function as a sponge (Tripathi et al. 2010). It is increasingly clear that genomic complexity is amplified through RNA regulation, termed "RNA complexity", which would be strongly related to the function of RNABPs. Furthermore, single RNABPs are shown to have multiple roles in post-transcriptional regulation. For example, SR proteins are involved in gene regulation from the transcription level to RNA processing, mRNA export and translation (Zhong et al. 2009). In addition to multiple gene regulation, most RNABPs participate in multiple physiological processes through a very large subset of directly bound transcripts, such as RNA-induced silencing complexes (RISCs) (miRNAs; Bartel 2009). Although many studies using traditional genetics assays and biochemistry have discovered specific roles for RNABP-dependent functions in neural development, these still have technical limitations in generalizing the mechanism of RNABP regulation to their in vivo targets and in predicting accurate biological functions.

M. Yano (✉) · T. Ohtsuka · H. Okano (✉)
Department of Physiology, Keio University School of Medicine,
35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan
e-mail: myano@a2.keio.jp
e-mail: hidokano@a2.keio.jp

In recent years, several trials of the target RNAs of each RNABP have revealed that one such RNABP controls many RNA targets in vivo. Therefore, RNABPs play a role in the generation of RNA complexity, which leads to biological complexity in the higher organism. RNABPs also fine-tune the quality and quantity of RNA and protein generated, in terms of their isoforms, localization and proof of transcriptional error. Recent genome-wide strategies, such as microarrays, RNA-seq, bioinformatics and the detection of in vivo protein–RNA interactions, provide global insights into generalizing RNABP functions (Licatalosi and Darnell 2010; Darnell 2013). These strategies may also allow for the elucidation of neural development in the field of RNABPs. This review highlights the roles of neural tissue-enriched RNABPs (nRNABPs) obtained from using evolving RNA methodologies during neural development (Table 1).

Victory of Genetic approach ~ RNABP research in neural development~

nRNABPs are identified in several independent experiments, for example in mutant screening and clinical research, both of which are approaches that take advantage of subjects with neural dysfunctions. One representative nRNABP is the embryonic lethal abnormal visual system (Elav) family of proteins, which were found in studies involving *Drosophila* genetics and autoimmune antigens from human diseases. In *Drosophila*, Elav was found in mutant screening studies of eye development and was proven necessary for neuronal development and survival (Robinow et al. 1988). The mammalian homologues, the Elav-like family of proteins (also known as HuR, HuB-D), were found as an antigen of autoimmune antibodies from patients suffering from paraneoplastic neurologic disorders ((Szabo et al. 1991; Darnell and Posner 2003). In spite of the physiological importance of nRNABPs, many questions still remain to be addressed with regards to how they bind and regulate RNA target transcripts and how their targets connect to the physiological functions.

The first successes were raised by *Drosophila* genetics from two nRNABPs, dfxr and dMusashi (dMsi) (Table 1). dfxr is the *Drosophila* homologue of the FMRP protein, which is a protein product of the causative gene of fragile X-syndrome mental retardation. A study carried out by Zhang et al. (2001) demonstrated for the first time that both dfxr loss-of-function *Drosophila* mutants and dfxr over-expressing flies displayed neurological phenotypes, including enlarged synaptic buttons, rough eye and neuromuscular junction defects, which are similar to the symptoms observed in fragile X-syndrome patients. A candidate approach was used to search phenotypes that are similar to those seen in the *dfxr* over-expression phenotype, to explain which target of dfxr contributes to the regulation of synaptic structure. *Futsch*, a mammalian MAP1B homologue that shows synaptic structure defects, was picked as a candidate. The structure and function of the synapse is restored in *dfxr and futsch* double mutants, which indicates a genetic interaction between *dfxr* and *Futsch*. Furthermore, in vitro biochemical assays reveal that dfxr binds to *futsch* mRNA and Futsch proteins show excess expression in the *dfxr* mutant. Finally, the group proposed that dfxr acts as a translational repressor of *futsch* to regulate microtubule-dependent synaptic growth and functions.

In addition, *dMsi* mutants exhibit a defect of asymmetric cell division in the sensory organ precursor cells (SOP) of the adult *Drosophila* external sensory organ (Nakamura et al. 1994). The molecular mechanism for dMsi was also used in a phenotype-based candidate approach to identify a functionally relevant target. Among mutants seen with defects in asymmetric cell divisions of SOP, the zinc finger-type transcriptional repressor protein from the *ttk69* gene whose expression pattern is necessary for determining non-neuronal cell fate in *Drosophila* SOP, indicates an opposite function to *dMsi*. To prove that there is a genetic interaction between *dMsi* and *ttk69 genes*, Okabe et al. (2001) used hypomorphic mutants lacking one copy of the *ttk69* gene and a mutant with an insertion of the P element in the *ttk69* 3'UTR, which contains dMsi1-binding sites that were identified by in vitro RNA selection methods. The dMsi defect is restored in the *ttk69* mutant, which lacks one copy of the *ttk69* gene. In another *ttk69* mutant, which lacks ten of the fifteen dMsi putative-binding sites in the *ttk69* 3'UTR, the dMsi defect is mimicked and manifests a double-bristle phenotype. Finally, a study that combined an in vitro reporter assay confirmed that dMsi represses ttk69 protein synthesis and thereby controls asymmetric cell division in the SOP. The dMsi and dFxr studies (Okabe et al. 2001; Zhang et al. 2001) provide biochemical evidence using traditional in vitro assays and a clear genetic interaction between the RNABPs and their targets. These two factors were both used in a "phenotype-based candidate approach" to explain which targets are required for RNABP-dependent physiological functions.

To observe and understand the physiological functions of nRNABPs in mammals, similar studies were performed using genetic and in vivo approaches, such as generating knock-out mice, conditional knockdown and over-expression studies (Table 1). The biological functions were observed in the brain of several nRNABP-null mutant mice. Mouse Musashi family, *Drosophila dmsi* homologue, is enriched in the developing neural stem/progenitor cells (NSPCs) in brain (Sakakibara et al. 1996), suggesting a link to neural progenitor function similar to *Drosophila dMsi.* In vitro neurosphere assays revealed that neural stem cell self-renewal activity does not appear to be affected by a single *Msi1* knockout in the brain-derived neural stem cell. However, a single *Msi1* knockout combined with the knock-down of another family member, *Msi2*, which is co-expressed in NSPCs (Sakakibara et al. 2001), dramatically reduced neural stem cell maintenance

Table 1 Functions of neural RNA-binding protein

Organisms	RNABPs	Expression pattern (cell type)	Developmental phenotype (mutant)	CLIP	Biological relevant RNA targets	Molecular function	Ref.
Drosophila	Elav	Post-mitotic neuron	Embryonic lethal eye defect	No		Alt splicing	Robinow et al. 1988 Lisbin et al. 2001 Soller and White 2003
	dFxr		Enlarged synaptic button eye defect neuromuscullar junction defect	No	*Futsch* (synaptic growth and functions)	Translational repression	Zhang et al. 2001
	dMsi	Neural stem/progenitor cell	Double sensory bristles	No	*ttk69* (asymmetric cell division)	Translational repression	Okabe et al. 2001
Mouse	Msil	Neural stem/progenitor cell	Hydrocephalus	No		Translational repression	Sakakibara et al. 2002 Imai et al. 2001 Kawahara et al. 2008
	nElavls	Neural progenitor cell post-mitotic neuron	Ataxia Defect of neural progenitor proliferation (Elavl4)	Elavl2 No Elavl3/4 Yes		Translational activation Alt splicing	Szabo et al. 1991 Darnell and Posner 2003 Akamatsu et al. 1999 Akamatsu et al. 2005 Antic et al. 1999 Yano et al. 2005
	FMRP	Neural stem cell post-mitotic neuron	Cell fate transition synaptic dysfunction			Translational repression	Saffary and Xie 2011 Darnell et al. 2011
	Nova 1	Specific neuron	Motor deficit (for spinal and brainstem neuron death)		*Agrin* (NMJ formation)	Alt splicing	Buckanovich et al. 1996 Jensen et al. 2000 Licatalosi et al. 2008 Ule et al. 2005 Ruggiu et al. 2009
	Nova2	Post-mitotic neuron	Cortical layer malformation		*Agrin* (NMJ formation) *Dab I* (neuronal migration)	Alt splicing	Ruggiu et al. 2009 Yano et al. 2010 Licatalosi et al. 2008 Ule et al. 2005
	Ptbpl	Neural stem cell	Hydrocephalus	Yes (in HeLa)		Alt splicing	Shibasaki et al. 2013 Boutz et al. 2007 Makeyev et al. 2007 Xue et al. 2009 Zheng et al. 2012
	Ptbp2	Neural stem/progenitor cell post-mitotic neuron and glia	Postnatal lethality	Yes		Alt splicing	Polydorides et al. 2000 Licatalosi et al. 2012 Boutz et al. 2007 Makeyev et al. 2007

Table 1 (continued)

Organisms	RNABPs	Expression pattern (cell type)	Developmental phenotype (mutant)	CLIP	Biological relevant RNA targets	Molecular function	Ref.
							Li et al. 2014
							Zheng et al. 2012
	Rbfox1	Neuron	Epileptic and hyperexcitable highly irregular firing of purkinje	Yes (in hESC)		Alt splicing	Jin et. al. 2003
							Gehman et al. 2011
	Rbfox2	Ubiquitous	Prone to hydrocephalus, abnormal cerebellar morphology, highly irregular firing of purkinje	0 (in hESC)		Alt splicing	Yeo et al. 2009
							Gehman et al. 2012

and proliferation. It was concluded that the Msi family acts as neural stem regulators (Sakakibara et al. 2002). Also, several in vitro assays have provided a model for Msi1-dependent RNA regulation, such that Msi1 acts as a translational repressor that competes with the translational initiation complexes, eIF4G, by the interaction of Msi1 with the poly-A binding protein (Imai et al. 2001; Kawahara et al. 2008).

Next, in the *Drosophila* Elav homologue, mouse Elav-like (Elavl1-4) family members are specifically expressed in neurons except for Elavl1. Many studies provide evidence of nElavl (2–4) promoting neuronal differentiation by gain-of-function studies (Akamatsu et al. 1999; Antic et al. 1999; Yano et al. 2005), suggesting a link to their expression pattern and functional similarity to *Drosophila* Elav. Several groups report that nElavl proteins bind to mRNAs encoding cell-cycle regulators and neuronal transcripts (Joseph et al. 1998; Chung et al. 1997; Antic et al. 1999) and regulate the stability and translation of the respective mRNA. In contrast, molecular function of *Drosophila* Elav has been focused on the alternative splicing (Lisbin et al. 2001; Soller and White 2003). The first loss-of-function study of the nElavl family genes was *Elavl4*. *Elavl4* null mutant mice display abnormal hind-limb reflexes, poor rotarod performance and reduced proliferative activity in neural progenitor cells. Given these multiple phenotypes, Elavl4 proteins might be involved in multiple steps of neuronal developmental events (Akamatsu et al. 2005). However, analysis of the *Elavl4* null brain failed to detect any differences in the expression patterns and levels of putative targets, possibly due to the compensatory expression of other Elavl proteins (Akamatsu et al. 2005).

Another neuron-specific RNABP family was first defined as a vertebrate tissue-specific splicing factor, Nova (neuro-onconeural ventral antigens), also identified as autoimmune targets in patient sera with Paraneoplastic Opsoclonus Myoclonus Ataxia (POMA) (Buckanovich et al. 1996). *Nova1*-null mutants exhibit motor deficits associated with the cell death of spinal and brainstem neurons. This *Nova1*-null mutant mouse study identified two clear in vivo splicing targets, $GABA_A$ receptor and glycine receptor alpha2, suggesting a link that these targets might be related to a failure of the inhibition of the motor system, similar to that seen in patients with POMA (Jensen et al. 2000). Another Nova family member, Nova2, regulates cortical and Purkinje neuron migration but not neural progenitor proliferation (Yano et al. 2010). Nova2-null mice manifest a neuronal migration defect in Purkinje neurons; this phenotype was also observed in RNA-binding fox homologue2, *Rbfox2* CNS-specific null mice, albeit to a lesser extent. In addition, the postnatal deletion of RbFox2 in Purkinje neurons also displays deficits in neuronal excitability (Gehman et al. 2012). Another member of this family, Rbfox1, was first identified as an Ataxin-2 interacting protein. This protein mutated in spinocerebeller ataxia type II (Martin et al. 2007) and was later shown to be involved in the control

of mature neuronal excitation in the *Rbfox1* null mouse brain (Gehman et al. 2011).

The pyrimidine tract-binding protein2, PTBP2, which was found as an interacting protein of Nova1, antagonizes Nova1 splicing functions (Polydorides et al. 2000) and regulates interkinetic nuclear migration of NSPCs associated with neurogenesis (Licatalosi et al. 2012). In studies of another member of the PTBP family, *PTBP1*-null mice exhibit a more severe phenotype of neural stem cell (Shibasaki et al. 2013). Therefore, both PTBP1/2 play important roles in neural stem cell to neuron switching, which is related to previous in vitro neurogenesis studies of PTBP1 and PTBP2, whereby a switching model was proposed in neuronal differentiation (Boutz et al. 2007; Makeyev et al. 2007). These two members of Ptbp1/2 also regulate the post-synaptic density protein 95 (Dlg4), which is important for synaptic maturation and plasticity, through its exon18 skip-mediating premature translational termination (Zheng et al. 2012). In addition, a recent analysis of PTBP2 conditional knock-out mice suggests that Ptbp2 is involved in multiple steps of neurogenesis, particularly in the final neuronal maturation stage and during neuronal differentiation through the neuronal RNA splicing program (Li et al. 2014).

FMRP, whose main function is related to the synapse, also plays a role in early neural development. Extensive in vivo short hairpin RNA (shRNA) knock-down studies have revealed that FMRP suppresses the transition from radial glia cells (cortical NSPCs) to inter-progenitor cells during neocortical development by an actin-dependent mechanism (Saffary and Xie 2011). It is becoming clear that each nRNABP participates in several steps of neural development and in most cases the phenotypes of nRNABP-null mice are complex (Fig. 1 and Table 1). This may be due to the involvement of nRNABPs in many pathways and in greater RNA regulatory complexity; thus, more detailed analyses are required for brain developmental studies, for example the use of conditional knock-out technology and in vivo manipulation assays. There are also issues that need to be addressed, such as which targets of RNABP play a role in a specific physiological event and the identification of biological relevant in vivo RNA targets of RNABP.

Genome-wide view of in vivo "true" RNA targets of nRNABPs

The importance of RNABPs in gene regulation is supported by genetic studies in *Drosophila*, mouse and human disease (Cooper et al. 2009; Darnell 2013). However, despite their biological importance, understanding how RNABPs regulate and orchestrate genes remains a mystery. Advances in microarray technologies could succeed in identifying potential RNABP targets in combination with genetically modified animals such as knock-out mouse brains. Advanced exon-junction microarrays revealed alternative splicing defects that are linked to synaptic function in the *Nova2*-null brain (Ule et al. 2005). Subsequent exon array analyses with genetic tools for *Rbfox* and *Ptbp* revealed that these RNABPs are associated with an alternative splicing program linked to neuronal functions and neural development (Gehman et al. 2012; Li et al. 2014). Such analyses remain important in assessing and validating whether these changed transcripts are direct RNA targets of RNABPs.

Over a few decades, in vitro RNA selection experiments have defined the binding consensus motif of numerous RNABPs. For example, the best characterized, neuron-specific RNABP, Nova1/2, binds to a YCAY sequence, indicating very low specificity; such a match occurs by chance [~1 site per 64 nucleotide (nt) on the genome; Buckanovich and Darnell 1997)]. In general, these RNABP-binding motifs may show lower complexity than those of DNA transcription factors. Given that approximately 80 % of the human DNA genome is transcribed, it would be very difficult to predict the in vivo RNA target sites by tracing RNABPs harboring consensus sequences. This also relates to the greater complexity of protein-RNA dynamics in the genome and the difficulty of an in vivo RNABP–RNA interaction study. There are also substantial technical limitations in understanding the connections between the molecular mechanisms and physiology. Several attempts have been made to find the in vivo RNA targets of RNABPs, by multiple approaches using biochemistry and bioinformatics. A breakthrough in mapping protein–RNA interactions at the transcriptome-wide level is CLIP (UV cross-linked immunoprecipitation methods) technology, a technique that uses UV-B irradiation to form a covalent bond between a protein and an RNA when they come into direct contact (~1 Angstrom contact each other) in living tissue and cells. This event can be applied to snapshot protein–RNA interaction in a correct cellular context. Once protein and RNA complexes are cross-linked, they are rigorously purified and specific-bound RNA libraries are obtained via several biochemical processes (Ule et al. 2003).

The recent adaptation of high-throughput sequencing technologies provides a new way to generate RNA–protein interaction maps at the transcriptome-wide level, known as HITS-CLIP (high-throughput sequencing CLIP) and their various derivations, CLIP-seq, iCLIP and PAR-CLIP (Licatalosi and Darnell 2010). In vivo biochemical maps of protein–RNA interaction by CLIPs assay could allow us to understand the regulation of RNA processing by RNABPs at the genome-wide level. On the other hand, traditional biochemical studies have also succeeded in defining how RNABPs work. For example, Jin et al. (2003) performed detailed functional analyses of the Rbfox-1 and revealed that this protein regulates tissue-specific splicing events in both exon inclusion and skipping and is dependent on the Rbfox-1 binding site,

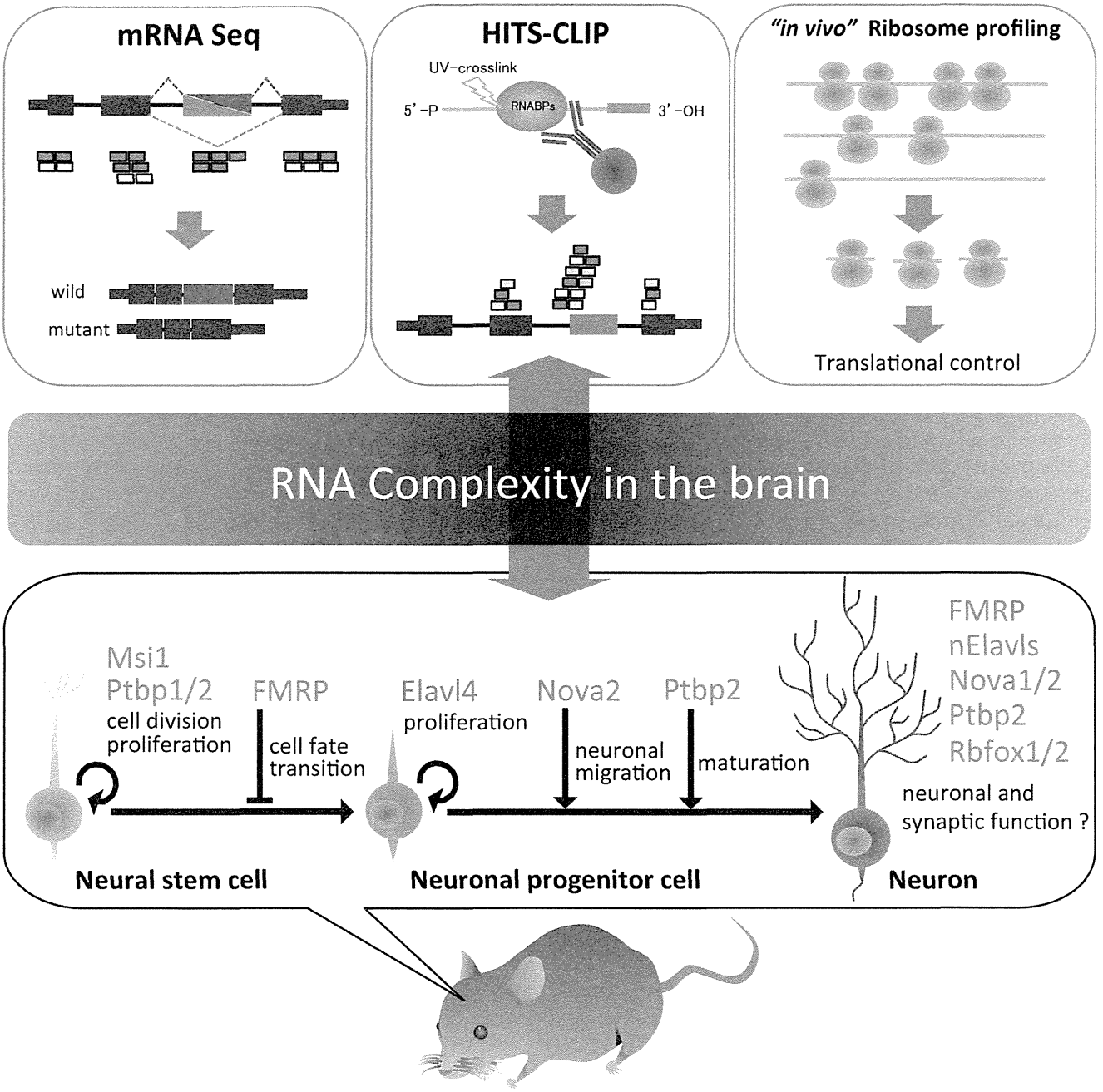

Fig. 1 Transcriptome-wide analysis of neural RNA-binding proteins (nRNABPs) will provide a new platform for bridging the gap between RNABPs and their physiological functions in the brain.

GCAUG. However, it is insufficient to generalize the molecular functions of Rbfox-1 and nRNABPs by a validation obtained from a few candidates, or to predict the direction or mechanisms underlying splicing regulation. HITS-CLIP, however, is able to determine the sequences that any RNABP directly binds to, "in an unbiased way" and "in situ" in the brain. This new platform technology also enables the functional insights, regulatory rules and unexpected new functions to be revealed when combined with microarray, bioinformatics and genetic systems for validation. The first HITS-CLIP paper by Licatalosi et al. (2008) validated a Nova-dependent alternative splicing regulatory rule. The group specified that Nova acts on exon inclusion when Nova binds to downstream intron of alternative exons and exon exclusion when Nova binds to upstream intron of alternative exons and discovered an unexpected role for Nova in regulating alternative poly-A usage selection in the brain. This suggests that RNABP regulates multiple RNA maturation processes of many RNA targets, leading a generation of biological diversity. Subsequent CLIP studies for other RNABPs, such as Elavl3 and

PTBP2 by HITS-CLIP, Fox2 and PTBP1 by CLIP-seq and hnRNPC and TDP43 by iCLIP, have demonstrated similar position-dependent splicing actions of RNABPs on exon skipping and inclusion patterns in a variety of cell and tissue types (Yeo et al. 2009; Ince-Dunn et al. 2012; Licatalosi et al. 2012; Xue et al. 2009; Konig et al. 2010; Tollervey et al. 2011; for other RNABPs, see Darnell 2013). HITS-CLIP is also adapted for translational mechanisms other than RNA processing studies. HITS-CLIP experiments of FMRP, a translational repressor, revealed that FMRP interacts with the RNA of the protein-coding regions of transcripts and is linked to autism spectrum disorders. The underlying mechanism is that FMRP represses translation of its RNA targets by stalling ribosomal translocation (Darnell et al. 2011). Furthermore, recent advances in integrative modeling, Bayesian networks using microarrays, CLIP datasets and the evolutionary conservation of Nova-binding sites have helped to define and understand the function of RNA splicing networks with a high level of sensitivity (Zhang et al. 2010). These new platforms will allow a new approach to understanding tissue-specific functions and human disease and provides a powerful tool for understanding the evolution and dynamics of the mammalian brain.

RNA-binding protein studies with Genetics and "in vivo" biochemistry in neural development

Advanced technologies, such as the aforementioned HITS-CLIP, can also be adapted for neural development studies (Fig. 1). A comprehensive map of protein–RNA interactions identified robust RNA targets of protein-coding genes. These datasets could predict biological functions with a bioinformatic assay. Nova regulates the RNA of biologically coherent sets of targets involved in synaptic transmission, as shown by exon-junction microarray (Ule et al. 2005) and HITS-CLIP (Licatalosi et al. 2008). Indeed, *Nova2*-null mice display loss of inhibitory synaptic plasticity, and long-term potentiation of spontaneous inhibitory post-synaptic currents but not excitatory post-synaptic currents (Huang et al. 2005). HITS-CLIP, in combination with genetic studies, exon arrays and bioinformatics, succeeded in giving an answer to a specific biological question, such as 'what is the role of Nova2 in neural development?' *Nova2*-null mice exhibit both cortical and Purkinje neuron migration defects, similar to those seen in Reelin-related molecule-null mutants. The use of high-throughput technologies with *Nova2*-null mice succeeded in identifying a pair of Nova2-dependent exons, termed *Dab1.7bc*, in the *Dab1* transcript, which are developmentally regulated during a crucial timeframe of neuronal migration in the embryonic brain (Yano et al. 2010). By in utero electroporation assay, wild-type Dab1 expression in Nova2 knock-out mice brain could partially mitigate the Nova2 neuronal migration defect phenotype. This is the first study to show the involvement of an alternative splicing in the development of the laminar structure of the mammalian brain (Yano et al. 2010). In addition, the double knock-out of *Nova1* and *Nova2* exhibits paralysis and failed synaptic formation between motor neurons and muscle. A suspected target of Nova-dependent splicing is the alternatively spliced variant of *agrin*, Z+ agrin, which is essential for development of neuromuscular junction (NMJ). The mating of Nova double-mutant mice with transgenic mice constitutively expressing Z + agrin in motor neurons rescues NMJ formation but paralysis still remains, indicating that specific RNA targets of Nova contribute to specific biological events in neuronal function (Ruggiu et al. 2009). An alternative use for HITS-CLIP in biological discovery is in PTBP2 analysis. Licatalosi et al. (2012) identified a bona fide protein–RNA interaction map of the neural-specific PTBP family, PTBP2, using a combination of advanced technologies, HITS-CLIP and exon-junction array analysis. Gene ontology and KEGG pathway analyses using a list of the PTBP2-dependent alternative splicing targets enriched the particular cellular functions, including cell proliferation and actin cytoskeleton regulation. In fact, immunohistochemical analysis showed that Ptbp2 is expressed with proliferating NSPCs in the embryonic brain but not in non-neural tissue. Traditional BrdU labeling assays using *Ptbp2* mice revealed that Ptbp2 controls NSPCs functions. Such biologically relevant genes, among the newly indentified targets by an unbiased way of new platform technologies, could promise a prediction of new physiological roles in neural development (Licatalosi et al. 2012). On the other hand, the analysis of RNABP–null mutants using histology and physiological assays, like those described above, reveals complicated phenotypes (Table 1). For example, Fox2 knock-down in embryonic stem cells exhibit cell mortality. It could be difficult to explain these effects because they originate from several pathways and multiple Fox2-dependent gene regulation events and may not be due to a single target in this instance (Yeo et al. 2009). More detailed biological assays could predict the targets responsible by the use of datasets for these RNABP-comprehensive targets.

Continuous technical advances and future remarks

Here, we described the roles of RNABP function in neural development with recent advanced technologies and a physiological link between the regulation of RNABPs and their targets. At present, global analysis at the transcriptome level provides a new starting point for understanding the functional connections between RNA regulation and neural development. CLIP technologies, which are described above, are also evolving to use ternary complexes in studies between RISC proteins, miRNA and their mRNA targets, termed AGO-HITS-CLIP (Chi et al. 2009). In addition, recent modifications of AGO-HITS-CLIP, called cross-linking,

ligation and sequencing of hybrids (CLASH method), have been developed to enable the identification of endogenous mRNA targets of a specific miRNA with canonical- and non-canonical-binding motif by direct monitoring of inter-RNA interactions with RISC complexes (Helwak et al. 2013). Furthermore, CLIP assays also lead to an enhanced resolution; HITS-CLIP and iCLIP succeed in identifying protein–RNA interactions at the 1 nucleotide resolution (Zhang and Darnell 2011; Konig et al. 2011) and quantitative level. A study using AGO-HITS-CLIP with *miR-155* knock-out mice, named differential CLIP (dCLIP), succeeded in identifying transcriptome-wide *miR-155* binding targets, including non-canonical *miR-155* seed-binding sites (Loeb et al. 2012). In addition, to snapshot translational control, Ingolia et al. (2011) developed an original translational assay, "ribosome profiling", which uses purification of intact ribosome to monitor translation at the transcriptome level in mammalian cells (Ingolia et al. 2011). Alternatively, the Heintz laboratory group developed the BAC-TRAP assay, which uses affinity purification of polysomal mRNAs from genetically defined cell populations with enhanced green fluorescent protein-tagged ribosomal protein L10a in the brain (Doyle et al. 2008; Heiman et al. 2008). All the new technologies commonly suggest that the accurate comprehensive mapping of RNA will allow us to reach our goal of generalizing mechanistic roles of RNABP regulation to physiological roles in future RNABP research in the developing brain (Fig. 1).

Two recent independent comprehensive studies, using RNA-seq, exon microarray and CLIP assays with the muscleblind-like (MBNL) family of RNABPs, revealed its functional roles in RNA regulation and provided new implications in several phenotypes, synaptic plasticity and REM sleep regulation consistent with an abnormality observed in muscle dystrophy (Charizanis et al. 2012; Wang et al. 2012). However, molecular mechanisms like sleep disturbance through RNA regulation remains an open question and might be involved in multiple MBNL direct RNA targets. In the future, it would be interesting to see how we bridge the gap between new technical advances in developmental biology and in vivo biochemistry and understand RNA regulatory networks and developmental pathways on a global scale (for reviews of new RNA methodologies see Licatalosi and Darnell 2010; Ingolia 2014) (Fig. 1).

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