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Defects in Translational Regulation contributing to Human Cognitive and Behavioral Disease

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Abstract

Recent data suggests that the levels of many synaptic proteins may be tightly controlled by the opposing processes of new translation and protein turnover in neurons. Alterations in this balance or in the levels of such dosage-sensitive proteins that result in altered stoichiometry of protein complexes at developing and remodeling synapses may underlie several human cognitive diseases including Fragile X Syndrome, autism spectrum disorders, Angelman syndrome and non-syndromic mental retardation. While a significant amount is known about the transduction of membrane signals to the translational apparatus through kinase cascades acting on general translation factors, much less is understood about how such signals may influence the activity of mRNA-specific regulators, their mechanisms of action and the specific sets of mRNAs they regulate. New approaches to the unbiased *in vivo* identification of maps of binding sites for these proteins on mRNA is expected to greatly increase our understanding of this crucial level of regulation in neuronal development and function.

Introduction

Normal human cognition is dependent on the proper wiring of the central nervous system during critical periods in development, as well as the maintenance and plasticity of this network in response to experience and insult throughout life. Communication between neurons allows the formation and fine-tuning of neuronal connections to coordinate cellular activity into circuits. A fundamental unit of communication in neuronal networks is the synapse. Synapses are comprised of a relatively well-defined set of proteins many of which function in multi-protein complexes. As such, they may be present in defined stoichiometric ratios arising in some cases from the coordinated synthesis, packaging and delivery of “units” of these multi-protein complexes to axons or dendrites where new or modified synapses are needed. One example of such a cellular strategy for achieving proper stoichiometry is the set of presynaptic scaffolding proteins including bassoon, piccolo, RIM and munc13 [1, 2]. These proteins are synthesized in the neuronal cell body and transported down the axon of the presynaptic cell in “piccolo transport vesicles” or PTVs. In response to signals enticing formation of a new synapse, one or more of these quanta of synaptic proteins is inserted into the presynaptic membrane [1].

Perhaps due to these stoichiometric constraints, alterations in the functional levels of several synaptic proteins are believed to underlie defects in cognition and behavior in human disease. Autism is one example; haplo-insufficiency of Shank3, neurexins or neuroligins, can cause the disease [3, 4]. Shank3 is a scaffolding protein in the postsynaptic density (or PSD, the assembly of postsynaptic proteins of excitatory glutamatergic synapses), and is

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believed to be a key organizer of the PSD as a component of defined multi-protein complexes [5, 6]. Similarly, haplo-insufficient mutations in neurexins and neuroligins, pre- and post-synaptic cell adhesion molecules mediating synapse formation and stabilization, have been linked to autism spectrum disorders (ASDs), Tourette's syndrome, schizophrenia and nonspecific learning disabilities [7].

Variation in protein expression levels can also arise in individuals due to *de novo* microdeletions and microduplications, giving rise to one or three alleles of a gene rather than the usual two (referred to as copy number variations; CNVs) [8]. CNVs have been shown to be much more common than expected; as many as one in eight births harbors a microdeletion and one in fifty, a microduplication [9]. Several large-scale studies in human copy number variation have examined the relationship of such events with cognitive diseases such as the ASDs and schizophrenia [10]. A fascinating conclusion from these studies is that 50% *increases* in levels of certain dosage-sensitive synaptic proteins is linked to cognitive diseases as well as the more commonly appreciated 50% decreases arising from loss-of-function mutations. Interestingly, many of the individual synaptic proteins whose dysregulation or mutation is related to the ASDs have now been linked to disease through both under- and overexpression. Again, Shank3 is a good example; duplication of the 22q13 region encompassing the Shank3 gene has been linked to severe impairment of social communication [4]. This and similar examples can explained by the gene balance hypothesis, which posits that deleterious phenotypes can arise from under- or overexpression of the same dosage-sensitive proteins because either can disrupt the stoichiometry of the same complex [11–13]. In sum, this evidence supports the concept that the *expression levels* of many synaptic proteins are critical to the formation and maintenance of proper synaptic function.

The expression level of many synaptic proteins may be tightly controlled by the balance between translation and turnover. The growing number of developmental cognitive diseases whose underlying cause is a defect in the regulation of either translation or turnover suggests that the equilibrium between these opposing processes is a sensitive point in establishing normal cognition and behavior. The first such disease to be characterized was Fragile X Syndrome, caused by a triplet repeat expansion which silences expression of the Fragile X Mental Retardation protein, FMRP, thought to repress neuronal activity-dependent translation [14]. Subsequently, some cases of autism were found to be caused by mutations in PTEN, TSC2 and NF1, three proteins with a shared function to repress the mammalian target of rapamycin (mTOR) pathway that is important for activity-dependent initiation of new translation [15]. On the side of protein turnover, both Angelman syndrome and X-linked syndromic mental retardation have been linked to defects or alterations in expression of the ubiquitin ligases UBE3A and HUWE1 respectively [16–18] which mark proteins for degradation by the proteasome. Taken together, a compelling argument can be made that elucidating the mechanisms regulating neuronal protein translation and turnover are likely to shed light on fundamental aspects of human cognition and neuronal function. This review is focused primarily on the function and regulation of neuronal translation, and the reader is directed to excellent reviews on synaptic activity-regulated protein turnover for a complementary view of this equilibrium [19, 20].

Mechanisms for translational regulation in neurons

While most cells have the ability to alter translation in response to environmental signals neurons have an additional need for specific mechanisms of translational control because of their architecture (Figure 1). This is in part due to a need for spatial control, exemplified by the ability of local groups of synapses to alter their "strength" in response to local input using mechanisms dependent on new protein synthesis in the processes rather than cell

body. There is an additional need for local synthesis to solve temporal control issues, as somatic polyribosomes can be a great distance from the site where new proteins are needed to bring about activity-dependent changes. In order to effect control over protein expression, specific mRNAs must be transported to the neuronal processes, requiring translational repression during localization. Following delivery to dendrites or axons these mRNAs may be maintained in a repressed form until synaptic stimulation triggers the local activation of translation to make specific proteins needed for changes in synaptic strength. Finally, mechanisms must exist to halt the translation of dosage-sensitive genes following a burst of synthesis.

Several fundamental pieces of information are needed to understand how activity-dependent translation controls the formation, maintenance and plasticity of synapses in a neuronal network. These include elucidation of the molecular pathways transducing synaptic activity into new protein synthesis, the regulatory factors that stimulate or repress this translation, and the set of activity-dependent plasticity proteins being synthesized.

Translational regulatory factors might be divided into three categories: (1) general translation factors, including the initiation and elongation factors regulated by phosphorylation, (2) sequence-specific RNA binding proteins (RNABPs) and (3) small noncoding RNAs such as miRNAs that regulate translation of specific sets of mRNAs. Two primary pathways for signal transduction from neuronal receptors to these regulatory factors, including the PI3K/Akt/mTOR and MEK/ERK kinase cascades have been extensively reviewed [21–25]. Activation of mTOR affects initiation by phosphorylation of the eIF4E binding proteins (4EBPs) causing their release from eIF4E which increases initiation; activation of the MEK/ERK cascade leads to phosphorylation of eIF4E with the same result. Inhibition of initiation can result from stimuli that cause phosphorylation of eIF2⟨ through the activation of eIF2⟨ kinases. In addition, inhibition of translation during elongation can be elicited through glutamate receptor activation which leads to eEF2 phosphorylation [26, 27]. Effects on general factors such as eIF4E, 4EBPs, eIF2⟨ and eEF2 might be expected to globally alter local translation, although in some cases specificity may be mediated by mechanisms involving competition of specific mRNAs for the translation apparatus [26, 28–31]. Nonetheless, it seems unlikely that general mechanisms of translational control can fully account for the complexity of protein synthesis-dependent synaptic plasticity.

Specific changes in gene expression may also be mediated by mRNA-specific RNABPs or Argonaute(Ago)/microRNA(miRNA) complexes that respond to signaling pathways to fine-tune translation of specific mRNAs or sets of functionally related mRNAs. The identification of relevant RNABPs and the sets of mRNAs that they regulate has been a major hurdle in connecting the compelling evidence for the role of local translation in human cognition and synaptic function with a molecular understanding of its function.

Evaluation of neuronal RNABPs that are implicated in translation regulation underlying neuronal development, activity-dependent synapse formation, stabilization and plasticity should consider several relevant questions: (1) Is there a human disease of cognition or behavior linked to dysfunction of the RNABP? (2) Do bidirectional RNABP level changes in model organisms cause relevant phenotypes such as defects in synaptic plasticity, spine structure, synapse number, or axonal/dendritic morphology? (3) Is the RNABP associated with polyribosomes, stalled initiation complexes, or transport/stress/P-body granules? (4) Is the expression or phosphorylation state of the RNABP activity- or experience-dependent? (5) What is known about how the RNABP regulates translation at the mechanistic level and is this regulation associated with an additional function in regulating stability or localization? This type of analysis suggests several mRNA binding proteins and non-coding

RNAs that may serve this function in neurons including FMRP, the CPEBs, pumilio1/2, ZBP1 (and mammalian homologs IGF2BP1–3), caprin1/2, HuB, C and D, Ago/miRNA complexes and the noncoding RNAs BC1 and BC200.

FMRP: an example of an mRNA-specific regulatory protein

Perhaps the most appealing approach to identifying the most relevant mRNA-specific translation factors is to mine the documentation of naturally occurring human mutations in neuronal RNABPs whose function is likely to include translational control and that are causally linked to cognitive and behavioral symptoms. Fragile X Syndrome, characterized by mental retardation, autistic symptoms, and childhood seizures is a model example. In Fragile X patients, a CGG triplet repeat expansion in the 5'UTR of the Fragile X Mental Retardation (*FMR1*) gene results in loss of expression of the encoded RNA binding protein FMRP [32, 33]. FMRP is recognized to be an RNABP by the presence of three canonical RNA binding domains, two KH-type and a C-terminal RGG box [34] and a role in regulating translation in neurons was suggested by its polyribosome association in brain [35–37]. Loss of polyribosome association due to a point mutation (I304N) in the KH2 RNA binding domain was reported in a severely affected Fragile X Syndrome patient [38, 39] and this mutation was subsequently confirmed to cause a Fragile X phenotype in a knock-in mouse model of the I304N mutation [40]. These observations suggest that loss of the *specific* function of FMRP in regulating translation in association with polyribosomes underlies Fragile X Syndrome, and has incited a great deal of interest in understanding the mechanisms by which FMRP controls translational and in identifying its mRNA targets.

Loss of function of FMRP has been linked to many defects in synaptic plasticity in a knockout (KO) mouse model of the disease (reviewed by [41–43]) including the finding of enhanced Group 1 metabotropic glutamate receptor-dependent long term depression (mGluR-LTD) in hippocampal CA1 neurons in the *Fmr1* KO mouse [44] which led to the "mGluR theory of Fragile X Syndrome", a compelling explanation for the neurologic and psychiatric aspects of the disease based on known properties of the mGluR signaling pathways [45]. mGluR-LTD is a form of plasticity that requires local postsynaptic protein synthesis in dendrites for its expression [46]. Remarkably, mGluR-LTD loses protein-synthesis dependence in the absence of FMRP [47, 48] suggesting that the plasticity-related proteins needed for LTD expression are already present in excess due to loss of translational repression by FMRP. This hypothesis has been extensively reviewed [14, 41, 49]. FMRP has been implicated in other forms of protein synthesis-dependent long-term plasticity as well, and taken together, the data suggest that FMRP plays a widespread role in regulating synaptic strength in response to activity in the central nervous system by regulating translation (reviewed in [41–43]).

Interest in the function of FMRP was heightened by reports of its rapid synthesis in synaptoneurosomes in response to mGluR activation [50], in hippocampal slices in which mGluR-LTD is induced [51], in mouse brain after behavioral stimulation [52], visual experience [53], or whisker stimulation [54]. The Drosophila homolog, dFMRP, was strongly up-regulated after spaced training which induces a protein synthesis-dependent form of long-term memory [55]. Klann and coworkers demonstrated that FMRP was also rapidly degraded by the proteasome during mGluR-LTD and turnover was necessary for this form of synaptic plasticity [51] suggesting that much of the activity-dependent alteration in FMRP levels might be mediated by a decrease in turnover rate. In light of the importance of proteasome-mediated turnover in plasticity and neurologic disease, further understanding of the fine control of FMRP levels is fundamental to understanding its role in synaptogenesis and plasticity.

An important issue for translational regulation in neurons is to what extent *pre*synaptic regulation of local translation will affect synapse formation and plasticity. A presynaptic function for FMRP has been suggested by localization studies that have found FMRP in axons and growth cones [35, 56, 57] as well as the characterization of axonal growth cone motility [57, 58], elongation [59] and pathfinding [60] defects in both fly and mouse models of Fragile X Syndrome. In addition, there is experimental support for a presynaptic role in synapse formation and the establishment of circuitry [61–64]. Recent studies on the role of the Aplysia FMRP homolog (ApFMRP) in sensory to motor neuron synaptic plasticity supports both a pre- and post-synaptic role for FMRP in regulating protein synthesis in response to synaptic stimulation [65]. A fruitful area for further research is to connect these observations with the set of presynaptic mRNAs whose translation is regulated by FMRP [66].

Historically, two independent studies suggested that FMRP acted to repress translation but were met with some skepticism as neither the FMRP nor the “mRNA” reporters represented endogenous interactions and specificity was lacking [67, 68]. However, several ensuing studies support a role for FMRP as a translational repressor though there is little consensus as to its mechanism of action [59, 69–74]. Two studies addressing mechanism arrived at different conclusions as to whether FMRP inhibits elongation [75] or initiation [76]. Very recent work used *in vivo* UV-crosslinking and a brain polyribosome-programmed *in vitro* translation assay designed to preserve endogenous interactions between FMRP and its mRNA targets in neurons. This study found that FMRP interacts along the length of the coding region of target mRNAs and stalls ribosome translocation to repress translation during elongation [66].

Challenges inherent in the identification of sets of regulated mRNAs

While identification of important neuronal regulators of translation (illustrated above by work on FMRP but also including CPEB, pumilio, ZBP, caprin, Hu, Ago/miRNA complexes and the noncoding RNAs BC1 and BC200) through genetic and biochemical approaches has been quite successful, identification of the mRNAs regulated by these binding proteins remains a major hurdle. Previous approaches have included *in vitro* RNA selection [77, 78] and co-IP followed by either microarray (RIP-Chip, also called “ribonomics”) [79] or by directed PCR for candidate mRNA ligands. *In vitro* RNA selection studies using fusion proteins with pools of random RNA ligands have been performed in the hope of characterizing high affinity RNA ligands for a given RNABP so that this information can be used to identify *in vivo* binding sites bioinformatically. While these experiments have been somewhat successful at reproducing *in vivo* binding motifs [80–82], their bioinformatic identification *in vivo* still presents a formidable challenge, especially in cases where secondary structure is involved [83, 84] or when high affinity binding requires multimers of a binding motif with variable spacing [85]. Because important cofactors may be missing, the RNA may not fold properly, and binding is influenced by lab buffers the experiment is more similar to a filter binding assay than to the milieu of a cell.

Co-IP of RNABP:RNA complexes is significantly more physiologic, except that once cells are lysed in a particular buffer, and cellular compartments are broken down and contents significantly diluted, the binding of RNABPs to RNA again takes on the character of an equilibrium binding assay, shown experimentally by Joan Steitz and colleagues [86]. In addition, the approach suffers from a failure to identify the sites of RNA binding, and from the need to use relatively gentle IP conditions so as not to lose the RNABP:RNA interaction which precludes the use of very high or low salt buffers, or ionic detergents. This results in a low stringency situation where the RNABP of interest may be IPed in a complex with other

RNABPs in addition to the problem of crossreactivity of the antibody with nonspecific proteins under low stringency IP conditions.

Attempts to identify the set of mRNAs whose translation is regulated by FMRP illustrate these issues. FMRP was originally defined as an RNABP by the presence of two KH domains and a C-terminal RGG box [34] and *in vitro* RNA selection revealed that FMRP binds a G-quadruplex RNA motif through its RGG box [83, 87], and a kissing complex RNA motif through the disease-associated KH2 domain [84]. G-quadruplex forming sequences have been reported in a number of mRNAs, and have been shown to interact with FMRP by *in vitro* binding assays, but their relevance to *in vivo* RNA binding remains unclear. Kissing complex RNA structures (kcRNA) cannot be predicted bioinformatically [84] although this motif has been identified structurally in other RNAs [88]. FMRP has been shown to interact with hundreds of neuronal mRNAs by RIP-Chip assays from mouse brain [89] and other attempts to identify its targets have been reviewed [41], however these studies have resulted in little consensus in FMRP target identification.

A significant advance in this area uses UV-crosslinking to introduce a covalent bond between the RNABP and RNA. We have recently combined UV crosslinking of RNABP-RNA complexes in intact cells or tissue with stringent immunoprecipitation (CLIP) to purify RNABPs away from nonspecific cellular RNABPs (Figure 2) [90–92]. Following linker addition the crosslinked RNA can be sequenced by high throughput techniques (HITS-CLIP, also referred to as CLIP-seq), allowing genome-wide assessment of bound RNAs. The advantages of this technique are that 1) UV crosslinking creates a bond between the RNABP and RNA only if the distance between them is within a bond length, in contrast to formaldehyde crosslinking which crosslinks molecules at some distance; 2) because intact tissue is crosslinked the physiologic/endogenous state of RNABP:RNA interactions is preserved; and 3) the covalent crosslink between RNABP and RNA allows the use of very stringent wash conditions to purify the RNABP of interest and remove free RNA. The sum of these, performed correctly, is that the sequenced RNAs represent a snapshot of what the RNABP was bound to *in vivo* at the time of crosslinking, with very little noise (false positives) in the picture. Because limiting RNAse digestion is used to reduce the size of the RNA “tags” this technique also reveals binding sites. Validation experiments can then be designed to examine altered metabolism (translation, splicing, turnover) of these RNAs *in vivo*, depending on where the RNA binding sites lie in the transcripts, ideally using animal models lacking the RNABP [93]. Recent studies using HITS-CLIP to identify mRNAs directly bound by FMRP on neuronal polyribosomes have confirmed that approximately 50% of those mRNAs identified by RIP-Chip are directly bound (and therefore likely to be directly regulated) by FMRP and have expanded this set of targets to more than 800 high confidence mRNA targets for FMRP directly bound *in vivo* [66]. Significantly, a large proportion of these mRNAs are components of either the pre-or postsynaptic proteomes of neurons.

Concluding remarks

Application of HITS-CLIP to other specific mRNA-binding proteins implicated in the control of activity-dependent translation in brain, such as CPEB, ZBP, Hu, caprin or pumilio should greatly expand our understanding of the mRNA targets they regulate. Indeed, application to Ago/miRNA complexes in P13 mouse brain has yielded a compelling map of *in vivo* Ago binding sites on mRNA [92]. Using HITS-CLIP analysis to quantify changes in binding due to activity, or in subcellular fractions such as polyribosomes or purifiable granules, or during development is expected to lead to dramatic advances in our understanding of how these proteins act individually and in concert to control the synthesis of neuronal proteins underlying development and plasticity.

Highlights

1. Activity-dependent translation is required for proper synaptic development and plasticity
2. Specific mRNA-binding proteins including FMRP may regulate such translation in neurons
3. New methods are being used to identify physiologically relevant mRNA targets of RNABPs

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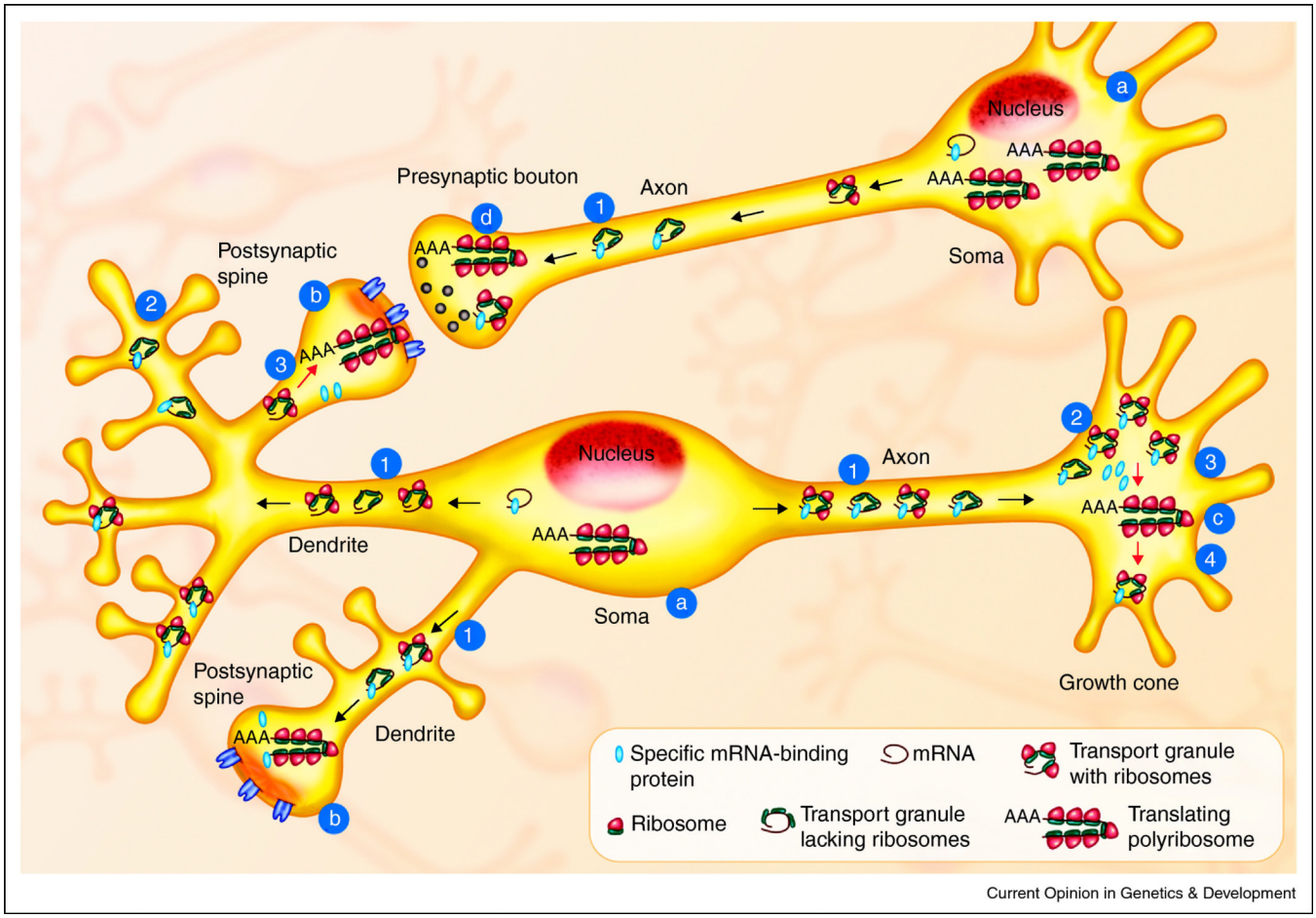

Figure 1. Points of Translational Regulation in Neurons

Neuronal networks (light blue) have a greater need for spatial and temporal control of translation than many other cell types because of their architecture and need to rapidly alter protein synthesis in response to signals. While the soma was originally believed to be the site of all protein synthesis in the neuron (**a**) it is now clear that actively translating polyribosomes are present in and near the dendritic spines (the sites of postsynaptic excitatory input, **b**), in growth cones during development and regeneration after injury (**c**) and likely on the presynaptic side of synapses as well (**d**). Localized protein synthesis permits rapid changes in the local proteome at sites distant from the soma but requires delivery of the mRNA templates (black spirals) and synthetic machinery to these sites in the form of transport granules with or without ribosomes (40S and 60S subunits are yellow and blue dots, respectively). The prevailing theory is that specific mRNA-binding proteins (red dots) repress translation during transport (**1**) and maintain the mRNA in a repressed state until new protein synthesis is needed (**2**). Mechanisms exist to activate the synthesis of specific proteins in the dendrites and growth cones (**3**) and finally, specific mechanisms halt their translation as well (**4**).

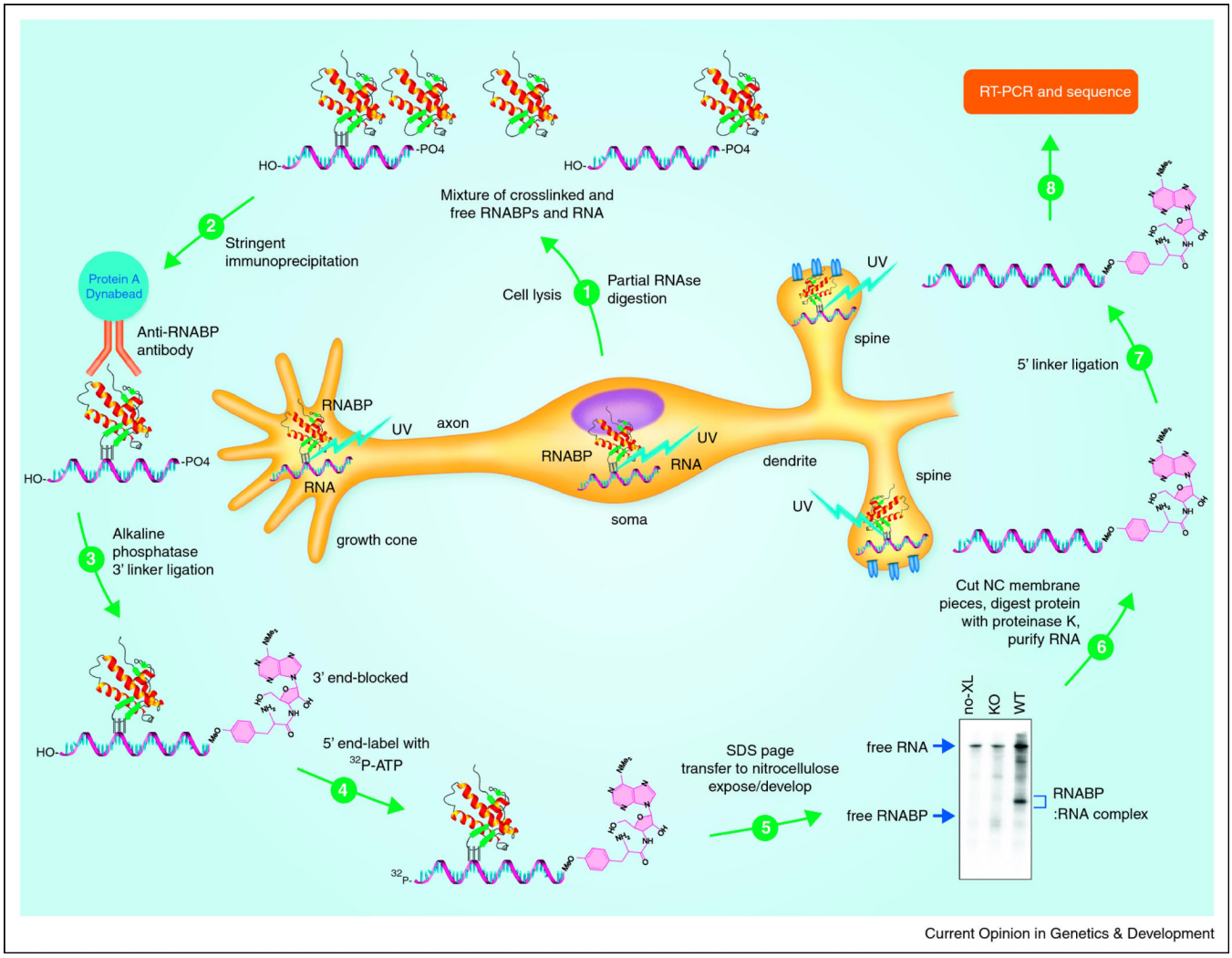

Figure 2. Schematic of the HITS-CLIP protocol to identify the *in vivo* RNA ligands of a neuronal RNABP

UV crosslinking at 254 nm (lightning bolts) captures endogenous interactions between RNABPs (colored ribbon structures) interaction with RNA (grey helices) occurring at the time of crosslinking of the tissue. A stylized neuron is shown to illustrate that interactions in the growth cones, dendrites, neuronal soma and other cellular compartments are captured by this technique which creates a covalent bond (triple black lines) between the RNABP and the RNA. **1**. Following crosslinking of cells or tissue, cells are lysed and treated with limiting RNAse digestion to reduce the modal size of the RNA "tags" to around 60 nucleotides to permit identification of RNA binding sites with good resolution, and improve purification in subsequent steps. This creates a mixture of RNA fragments, cellular RNABPs and RNABPs crosslinked to their RNA binding sites. The art of CLIP is in the purification of the RNABP of interest from this mixture. **2**. The first step in this purification is typically a stringent immunoprecipitation in a buffer that dissociates endogenous RNP complexes. **3**. The IP beads are then stringently washed, treated with alkaline phosphatase, and a blocked 3' RNA linker added. **4**. The RNA tags are labeled with T4 polynucleotide kinase and ^{32}P-γATP. **5**. A second important purification step is SDS-PAGE gel electrophoresis to separate complexes by size. After transfer to nitrocellulose crosslinked RNABP:^{32}P-RNA complexes can be visualized and success of the experiment evaluated by comparison of a sample (WT)

with a control lacking the RNABP of interest (KO) or additional antibodies, both irrelevant (negative control) and directed against other RNABP epitopes on the same RNABP (positive controls). An otherwise identical sample that has not been crosslinked serves as a negative control for contaminating free ^{32}P-RNA. **6.** The pieces of nitrocellulose containing complexes of interest, migrating approx. 20 kDa larger than the free RNABP (accounting for the 60 nt RNA tag) are excised, RNABP digested away with proteinase K, and the RNA purified. **7**. After ligation of an RNA linker to the 5'end of RNA tags, **8.** the tags can be amplified by RT-PCR and products sent for high throughput sequencing (HITS). Resulting sequences can be aligned with the genome or any database of interest, including noncoding RNAs, to identify novel, physiologically relevant maps of RNA binding for a given RNABP. References for a more detailed method for HITSCLIP are given in the text.