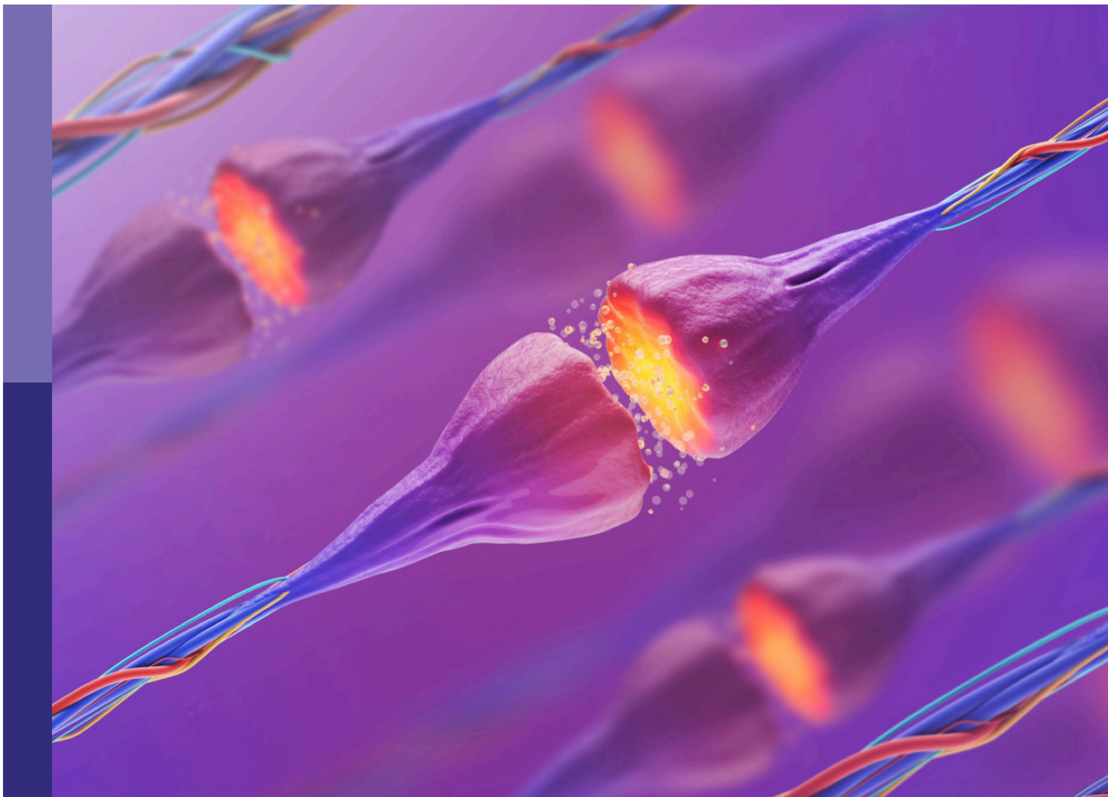
frontiers | Research Topics

Come as you R(NA): post-transcriptional regulation will do the rest

Edited by
Oriane Mauger, Michael A. Kiebler
and Clémence Bernard

Published in
Frontiers in Molecular Neuroscience



ISSN 1664-8714
ISBN 978-2-8325-6610-7
DOI 10.3389/978-2-8325-6610-7

Generative AI statement
Any alternative text (Alt text) provided alongside figures in the articles in this ebook has been generated by Frontiers with the support of artificial intelligence and reasonable efforts have been made to ensure accuracy, including review by the authors wherever possible. If you identify any issues, please contact us.

About Frontiers

Frontiers is more than just an open access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers journal series

The Frontiers journal series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the *Frontiers journal series* operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

Come as you R(NA): post-transcriptional regulation will do the rest

Topic editors
Oriane Mauger — Max Planck Institute of Psychiatry, Germany
Michael A. Kiebler — Ludwig Maximilian University of Munich, Germany
Clémence Bernard — University of Exeter, United Kingdom

Citation
Mauger, O., Kiebler, M. A., Bernard, C., eds. (2025). *Come as you R(NA): post-transcriptional regulation will do the rest*. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-8325-6610-7

Table of contents

frontiers | Frontiers in Molecular Neuroscience

TYPE Editorial
PUBLISHED 01 July 2025
DOI 10.3389/fnmol.2025.1644067

Check for updates

OPEN ACCESS

EDITED AND REVIEWED BY
Clive R. Bramham,
University of Bergen, Norway

*CORRESPONDENCE
Oriane Mauger
✉ oriane_mauger@psych.mpg.de
Michael A. Kiebler
✉ michael.kiebler@med.uni-muenchen.de
Clémence Bernard
✉ c.bernard@exeter.ac.uk

RECEIVED 09 June 2025
ACCEPTED 11 June 2025
PUBLISHED 01 July 2025

CITATION
Mauger O, Kiebler MA and Bernard C (2025) Editorial: Come as you R(NA): post-transcriptional regulation will do the rest. *Front. Mol. Neurosci.* 18:1644067. doi: 10.3389/fnmol.2025.1644067

Editorial: Come as you R(NA): post-transcriptional regulation will do the rest

Oriane Mauger[1]*, Michael A. Kiebler[2]* and Clémence Bernard[3]*

[1]Max Planck Institute of Psychiatry, Munich, Germany, [2]Biomedical Center, Department of Cell Biology and Anatomy, Medical Faculty, LMU, Planegg, Germany, [3]Department of Clinical and Biomedical Sciences, Faculty of Health and Life Sciences, University of Exeter, Exeter, United Kingdom

KEYWORDS

RNA processing, neuron development and plasticity, translation regulation, neurological diseases, RNA-binding proteins

Editorial on the Research Topic
Come as you R(NA): post-transcriptional regulation will do the rest

If RNA were simply a messenger between genes and proteins, cells would not function. RNA is a regulatory hub, a feature particularly leveraged in the central nervous system, where post-transcriptional processes (PTPs) control RNA stability, localization, translation and protein isoforms, mediating precise spatio-temporal control of gene expression (Alfonso-Gonzalez and Hilgers, 2024; Flamand et al., 2023; Ule and Blencowe, 2019). The extensive repertoire of PTPs, their widespread programs, the logic of their regulation and their physiological relevance have recently taken their full meaning. Indeed, PTPs tightly parallel the intricacy of the brain's spectacular diversity of cells with complex morphologies that need to integrate many extrinsic and intrinsic signals (Bauer et al., 2022, 2023; Darnell, 2013; Furlanis and Scheiffele, 2018; Holt et al., 2019). This editorial introduces the articles collected in this Research Topic to highlight the recent progress in the field of post-transcriptional control of gene expression in the central nervous system in health and disease (Figure 1).

Diversity of post-transcriptional processes

While the diversity of PTPs has been known for several decades, new mechanisms are continuously revealed to have pivotal roles in shaping gene expression for brain development and function. For instance, epitranscriptomics represents a rapidly expanding area of research, with chemical modifications of mRNAs now emerging as being crucial for neurodevelopment and cognitive functions (Tegowski and Meyer). Dogmas in the PTP field are being revisited: for a long time, only one open-reading frame (ORF) per mRNA was thought to be active, ultimately giving rise to one protein isoform. Recent evidence has however revealed that multiple ORFs within the same mRNA can produce different protein isoforms. Many of these newly identified ORFs are often located in the improperly called "untranslated regions" of mRNA and code for microproteins that are likely relevant for neuronal cell functions (Duffy et al.). Beyond intracellular mechanisms, the intercellular transfer of secreted factors that influence PTPs (de la Cruz-Gambra and Baleriola) and of transcripts themselves (Taylor and Nikolaou) have started to be uncovered. For instance, factors secreted by astrocytes were found to regulate local translation of mRNAs located in neighboring neurons in culture (de la Cruz-Gambra and Baleriola). While PTPs are mainly investigated for coding RNAs, non-coding (nc) RNAs are also under post-transcriptional control. Many ncRNAs have been observed in neuronal processes including at synapses (Taylor and Nikolaou) with an increasing number shown to have coding capacity, revising our textbook vision of gene expression (Duffy et al.; Taylor and Nikolaou).

Interplay between post-transcriptional mechanisms and other gene expression steps

The interplay between PTPs and other gene expression mechanisms is becoming increasingly evident and appears to control the availability, levels and isoforms of PTP factors. For instance, alternative splicing can control the production of transcription factor isoforms with distinct impact on neurodevelopmental transcription programs (Nazim). The expression levels of post-transcriptional factors can also be controlled by post-translational modifications. Ubiquitination—a key step of proteostasis—can target RNA-binding proteins (RBPs) such as splicing factors, and subsequently affect the splicing regime in the brain (Elu et al.).

This interplay is also clearly evidenced by the various functions exerted by PTP factors at different stages of the RNA life cycle. Many RBPs, such as RNA helicases and heterogeneous nuclear ribonucleoproteins (hnRNPs), exert distinct roles in different subcellular compartments (Lederbauer et al.; Tilliole et al.). The splicing factor poly-pyrimidine tract binding protein PTBP2 has also been shown to be transported in neuronal processes where it controls local translation (Salehi et al.). Finally, the interplay between PTPs and other gene expression processes can result from a local synergy, where RNA processing factors can be recruited at regulatory transcription regions such as promoters and enhancers. This crosstalk seems to play a pivotal role in dictating the developing neuronal transcriptome (Ozbulut and Hilgers).

Specificity of post-transcriptional processes

Recent research has revealed a high specificity of PTPs, from subcellular localization to cell type and species differences. In neurons, specific PTPs are observed at the subcellular level, with neuronal processes and synapses exhibiting diverse molecular landscapes (Taylor and Nikolaou). More broadly, the many neuronal subtypes observed in the brain exhibit distinct transcripts and protein repertoires, to which different PTPs contribute. During development, cellular differentiation and specification are associated with dedicated PTPs (Ozbulut and Hilgers). Finally, another level of specificity is observed between species, raising the tantalizing hypothesis that PTPs also contribute to species divergence and precise features of individual species across evolution (Dando et al.).

Post-transcriptional processes in disease

Several PTPs have been shown to be dysregulated in a range of neurological diseases. Pathological variants of RBPs such as RNA helicases and hnRNPs have been associated with neurodevelopmental disorders, including developmental delay, intellectual disability and brain anomalies (Lederbauer et al.; Tilliole et al.). Defects in RBPs have been linked to degenerative disorders, ranging from spinal muscular atrophy (Salehi et al.) to the frontotemporal lobar degeneration—amyotrophic lateral sclerosis spectrum and Alzheimer's disease (Tilliole et al.). Defective regulation of protein homeostasis has also been reported in several neurological disorders (Elu et al.).

A better understanding of PTPs in both health and disease opens the door to novel therapeutic means (Elu et al.; Salehi et al.). RNA-based tools such as splice-switching oligonucleotides have shown great promise to treat spinal muscular atrophy and amyotrophic lateral sclerosis (Zhang). RNA-targeting CRISPR-Cas9 technologies (Tegowski and Meyer) are also being developed, which will offer innovative options for therapeutical interventions.

Technical challenges and looking forward

The recent progress described in the articles of this Research Topic is continuously accelerated by major technological advances, such as third generation sequencing and spatial transcriptomics (Taylor and Nikolaou). This is particularly exemplified by our recent ability to identify RNA modifications, and direct sequencing will provide more opportunities to study their effect at the single-molecule level (Tegowski and Meyer). More technical developments, such as single-synapse characterization and live imaging of translation, will bring unprecedented resolution to our understanding of the roles of PTPs in spatio-temporal control of gene expression. Artificial intelligence and machine learning will certainly revolutionize prediction of cis- and trans-regulatory elements. This will facilitate the implementation of emerging antisense oligonucleotide strategies to manipulate PTPs and investigate their functional relevance for neuronal circuits and cognition *in vivo*.

We hope that this Research Topic provides valuable material on the latest advances in PTP research and stimulates new avenues for our long-term goal to elucidate the foundational connections between these processes and brain function. The coming years will undoubtedly lead to a more precise understanding of the various levels of PTP regulation and their consequences, with impact on both basic science and translational investigations.

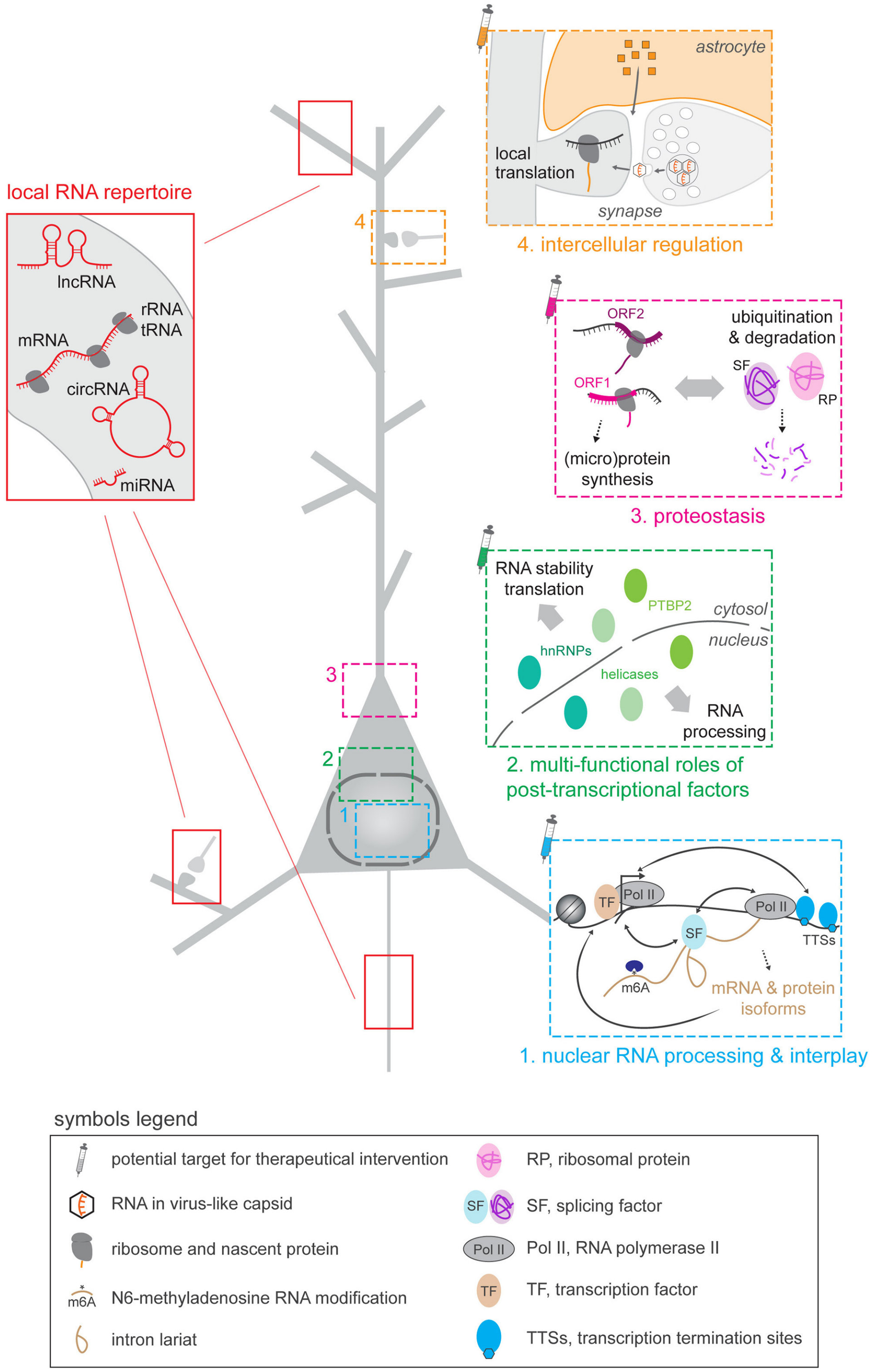


FIGURE 1
Post-transcriptional processes (PTPs) exert crucial spatio-temporal control of gene expression in the brain under basal conditions and in response to stimulation, a conserved feature observed across a wide range of species. These PTPs act upon a wide array of RNA categories in the various compartments formed by the complex morphology of neurons (**red box**). Within the nucleus **(1, blue box)**, various PTPs interact with each other and with other gene expression mechanisms, including transcription. The interplay of PTPs is further influenced by the dual localization of many PTP factors, which carry different functions in the nucleus and the cytosol to regulate RNA metabolism **(2, green box)**. Protein abundance is governed by tightly regulated protein synthesis, generating multiple protein and micro-protein forms, coupled with precise protein degradation **(3, pink box)**. The complex architecture of neurons requires local control of gene expression notably at synapses, involving the intercellular transfer of molecules such as astrocyte-secreted factors and RNA transported via virus-like capsids **(4, orange box)**. Many PTPs therefore represent potential targets for therapeutic interventions in a range of brain disorders.

Author contributions

OM: Writing – review & editing, Writing – original draft. MK: Writing – review & editing, Writing – original draft. CB: Writing – review & editing, Writing – original draft.

Funding

The author(s) declare that financial support was received for the research and/or publication of this article. This work was supported by the Max Planck Institute of Psychiatry (OM), by the DFG (Ki 502/9-1 and 11-1) (MK) and by the University of Exeter (CB).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The authors declare that no Gen AI was used in the creation of this manuscript.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

Alfonso-Gonzalez, C., and Hilgers, V. (2024). (Alternative) transcription start sites as regulators of RNA processing. *Trends Cell. Biol.* 34, 1018–1028. doi: 10.1016/j.tcb.2024.02.010

Bauer, K. E., Bargenda, N., Schieweck, R., Illig, C., Segura, I., Harner, M., et al. (2022). RNA supply drives physiological granule assembly in neurons. *Nat. Commun.* 13:2781. doi: 10.1038/s41467-022-30067-3

Bauer, K. E., de Queiroz, B. R., Kiebler, M. A., and Besse, F. (2023). RNA granules in neuronal plasticity and disease. *Trends Neurosci.* 46, 525–538. doi: 10.1016/j.tins.2023.04.004

Darnell, R. B. (2013). RNA protein interaction in neurons. *Annu. Rev. Neurosci.* 36, 243–270. doi: 10.1146/annurev-neuro-062912-114322

Flamand, M. N., Tegowski, M., and Meyer, K. D. (2023). The proteins of mRNA modification: writers, readers, and erasers. *Annu. Rev. Biochem.* 92, 145–173. doi: 10.1146/annurev-biochem-052521-035330

Furlanis, E., and Scheiffele, P. (2018). Regulation of neuronal differentiation, function, and plasticity by alternative splicing. *Annu. Rev. Cell Dev. Biol.* 34, 451–469. doi: 10.1146/annurev-cellbio-100617-062826

Holt, C. E., Martin, K. C., and Schuman, E. M. (2019). Local translation in neurons: visualization and function. *Nat. Struct. Mol. Biol.* 26, 557–566. doi: 10.1038/s41594-019-0263-5

Ule, J., and Blencowe, B. J. (2019). Alternative splicing regulatory networks: functions, mechanisms, and evolution. *Mol. Cell.* 76, 329–345. doi: 10.1016/j.molcel.2019.09.017

frontiers | Frontiers in Molecular Neuroscience

TYPE Perspective
PUBLISHED 22 April 2024
DOI 10.3389/fnmol.2024.1393973

OPEN ACCESS

EDITED BY
Clémence Bernard,
University of Exeter, United Kingdom

REVIEWED BY
Gary Patrick Brennan,
University College Dublin, Ireland
Dan Ohtan Wang,
New York University Abu Dhabi,
United Arab Emirates

*CORRESPONDENCE
Kate D. Meyer
kate.meyer@duke.edu

RECEIVED 29 February 2024
ACCEPTED 12 April 2024
PUBLISHED 22 April 2024

CITATION
Tegowski M and Meyer KD (2024) Studying m⁶A in the brain: a perspective on current methods, challenges, and future directions. *Front. Mol. Neurosci.* 17:1393973.
doi: 10.3389/fnmol.2024.1393973

Studying m^{6}A in the brain: a perspective on current methods, challenges, and future directions

Matthew Tegowski[1] and Kate D. Meyer[1,2]*

[1]Department of Biochemistry, Duke University School of Medicine, Durham, NC, United States,
[2]Department of Neurobiology, Duke University School of Medicine, Durham, NC, United States

A major mechanism of post-transcriptional RNA regulation in cells is the addition of chemical modifications to RNA nucleosides, which contributes to nearly every aspect of the RNA life cycle. N^6-methyladenosine (m^{6}A) is a highly prevalent modification in cellular mRNAs and non-coding RNAs, and it plays important roles in the control of gene expression and cellular function. Within the brain, proper regulation of m^{6}A is critical for neurodevelopment, learning and memory, and the response to injury, and m^{6}A dysregulation has been implicated in a variety of neurological disorders. Thus, understanding m^{6}A and how it is regulated in the brain is important for uncovering its roles in brain function and potentially identifying novel therapeutic pathways for human disease. Much of our knowledge of m^{6}A has been driven by technical advances in the ability to map and quantify m^{6}A sites. Here, we review current technologies for characterizing m^{6}A and highlight emerging methods. We discuss the advantages and limitations of current tools as well as major challenges going forward, and we provide our perspective on how continued developments in this area can propel our understanding of m^{6}A in the brain and its role in brain disease.

KEYWORDS
RNA, m^{6}A, epitranscriptome, methods, brain

Introduction

RNAs contain over 170 distinct chemical modifications which play important roles in regulating RNA processing and function. Although most of these modifications occur in non-coding RNAs such as ribosomal RNA and tRNA, recent studies have revealed a diverse and dynamic "epitranscriptome" within cellular mRNAs as well. N^6-methyladenosine (m^{6}A) is the most abundant internal mRNA modification and is found in thousands of cellular mRNAs, in addition to a large number of non-coding RNAs. m^{6}A plays important roles in several RNA processing events, including splicing, nuclear export, stability, and translation, making it a critical regulator of gene expression in cells (Murakami and Jaffrey, 2022; Flamand et al., 2023). Indeed, m^{6}A contributes to a wide variety of physiological processes, including development, innate immunity, gametogenesis, and the cellular stress response. Additionally, and consistent with its importance for cellular function, m^{6}A dysregulation has been implicated in a variety of human diseases, including several cancers (Yang et al., 2020; He and He, 2023). Thus, understanding m^{6}A distribution, regulation, and function is critical for advancing our knowledge of human health and disease.

Within the brain, m^{6}A levels are particularly abundant compared to other tissues (Meyer et al., 2012; Liu et al., 2020), and proper regulation of m^{6}A is critical for processes such as neural stem cell function, brain development, learning and memory, response to stress, and neuronal signaling (Flamand and Meyer, 2019; Livneh et al., 2020).

Our current knowledge of m^{6}A has been accelerated by technological advances which have enabled the identification of m^{6}A sites transcriptome-wide. Additionally, emerging technologies for targeted m^{6}A manipulation in select RNAs are promising tools that can enable functional studies of m^{6}A in the brain and other tissues. Here, we review m^{6}A detection and manipulation strategies and discuss major challenges that need to be overcome. We also provide our perspective on future directions and areas that are likely to drive the field forward.

m^{6}A function and regulation in the brain

m^{6}A is deposited in the nucleus co-transcriptionally by a large methyltransferase complex which includes METTL3 as the catalytic subunit and several additional accessory proteins including METTL14, WTAP, HAKAI, VIRMA, ZC3H13, and RBM15/15B (Shi et al., 2019; Zaccara et al., 2019; Flamand et al., 2023). Methylation occurs preferentially within the DRACH (D = A, G, U; R = A, G; H = A, C, U) consensus sequence, and recent studies have revealed that sequence specificity and gene architecture are the major determinants of methylation within cellular mRNAs (Garcia-Campos et al., 2019; Yang et al., 2022; He et al., 2023; Uzonyi et al., 2023). In addition, m^{6}A can be removed by two eraser proteins, FTO and ALKBH5, which can contribute to dynamic regulation of m^{6}A and gene expression under certain contexts (Shi et al., 2019; Flamand et al., 2023).

m^{6}A has been shown to influence nearly every aspect of the RNA life cycle, including splicing, export, stability, localization, and translation (Shi et al., 2019; Zaccara et al., 2019; Flamand et al., 2023). However, the most well-established function of m^{6}A in mRNAs is to recruit RNA degradation machinery through the binding of YTHDF proteins (Shi et al., 2019; Zaccara et al., 2019; Kontur et al., 2020; Zaccara and Jaffrey, 2020; Flamand et al., 2023). This m^{6}A-dependent control of mRNA stability is critical for proper brain development, as this mechanism helps regulate the abundance of mRNAs that participate in neuronal stem cell function and cell cycle regulation (Yoon et al., 2017; Wang et al., 2018). m^{6}A has also been shown to regulate mRNA metabolism in the brain in other ways, including promoting translation and nuclear export (Shi et al., 2019; Zaccara et al., 2019; Flamand et al., 2023). These functions are mediated by a variety of m^{6}A reader proteins. For instance, YTHDF1 promotes methylated mRNA translation in neurons to control synaptic activity and learning and memory (Shi et al., 2018; Zou et al., 2023), and YTHDF2 promotes the differentiation of neural progenitors by degrading methylated transcripts (Li et al., 2018). The fragile X messenger ribonucleoprotein (FMRP) has been shown to preferentially bind methylated transcripts and facilitate their nuclear export (Edens et al., 2019). Additionally, our group identified RBM45 as a brain-enriched m^{6}A reader protein that can impact splicing and regulate neuronal differentiation (Choi et al., 2022).

In addition to cortical development and neurogenesis, m^{6}A also has important roles in regulating the function of mature neurons. Neurons are highly polarized cells, with complex dendritic processes that can make thousands of synaptic connections with other neurons. Proper synaptic function and plasticity requires the trafficking and local translation of mRNAs to synapses in an activity-dependent manner (Doyle and Kiebler, 2011; Holt et al., 2019; Roy et al., 2020). RNA localization is mediated by a variety of *cis*-acting elements, such as sequence and structure, which are bound by RNA-binding effector proteins (Doyle and Kiebler, 2011). m^{6}A profiling of synaptic RNAs showed that several methylated transcripts are localized at synapses, suggesting that m^{6}A could serve as an additional *cis*-acting element to control RNA localization in neurons (Merkurjev et al., 2018). Indeed, subsequent work from our group showed that hundreds of transcripts, including many that encode proteins important for synaptic maintenance and plasticity, are localized to distal processes in neurons in an m^{6}A-dependent manner (Flamand and Meyer, 2022). We further showed that this is mediated through YTHDF proteins. However, why some methylated transcripts are degraded by YTHDF proteins while others are transported to distal processes is unknown, and it likely depends on other context-dependent factors, such as additional sequence and structural elements and interactions with other RBPs.

Interestingly, a recent study showed that mRNA stability is a major determinant of mRNA localization in neurons, with more stable transcripts being enriched in neurites (Loedige et al., 2023). The authors reported that neurite-enriched RNAs have lower levels of m^{6}A, and they found that disrupting m^{6}A or other factors that control RNA stability promotes neurite enrichment of neuronal transcripts. These studies examined m^{6}A-mediated localization in primary cortical neurons, in contrast to hippocampal neurons used in our work, so it is possible that m^{6}A may have unique roles in different neuronal subtypes. However, even within hippocampal neurons, we identified many transcripts with increased neurite localization following *Mettl3* depletion in addition to the hundreds of transcripts that showed decreased neurite localization (Flamand and Meyer, 2022). Thus, the effects of m^{6}A on RNA localization may be transcript-specific. Further studies will be necessary for defining the cell type-and transcript-dependent effects of m^{6}A on RNA localization in the brain.

In addition to RNA localization, recent work has demonstrated that m^{6}A promotes local, activity-dependent translation of mRNAs in hippocampal neurons. This process is mediated by YTHDF1, which is required in hippocampal neurons for proper learning and memory (Shi et al., 2018). Supporting these data, deletion of *Mettl3* in the mouse hippocampus also leads to impaired learning and memory (Zhang et al., 2018). Furthermore, a mechanism by which YTHDF1 can promote activity-dependent translation in the hippocampus has been uncovered. Basal interactions between FMRP and YTHDF1 sequester YTHDF1. However, FMRP is phosphorylated upon neuronal activity, resulting the release of YTHDF1, allowing it to promote the translation of methylated transcripts (Zou et al., 2023). Altogether, m^{6}A has been shown to regulate neuronal development and function by regulating RNA stability, localization, and translation.

Current methods and recent advances in m^{6}A mapping

The first method for transcriptome-wide m^{6}A mapping was developed in 2012 and involved using m^{6}A antibodies to immunoprecipitate methylated RNAs followed by next-generation sequencing to identify the methylated targets (Dominissini et al., 2012; Meyer et al., 2012). This method, called MeRIP-seq or m^{6}A-seq, has been widely used to globally profile m^{6}A across a variety of tissues, cell types, and conditions, and it continues to be the predominant method used in most studies. Improvements to the technique have enabled single-nucleotide resolution m^{6}A mapping (miCLIP and m^{6}A-CLIP) (Linder et al., 2015; Ke et al., 2017), provided profiles of m^{6}A within individual RNA isoforms (m^{6}A-LAIC-seq) (Molinie et al., 2016), and reduced the RNA input requirements through more efficient library preparation (Zeng et al., 2018; Dierks et al., 2021).

Although widely used, antibody-based m^{6}A mapping methods have their drawbacks. This includes cross-reactivity of m^{6}A antibodies with m^{6}A$_m$, a chemically similar modification that is part of the 5′ cap structure. In addition, m^{6}A site calling can be stochastic due to variability in antibody immunoprecipitation efficiency, and most studies lack sufficient replicate numbers to make accurate site calls (McIntyre et al., 2020). Furthermore, most global m^{6}A mapping strategies lack the ability to quantify m^{6}A stoichiometry. This has made studies of m^{6}A dynamics difficult and has contributed to discrepancies in the literature regarding how m^{6}A responds to cellular stress and other states.

Recently, two methods for simultaneous m^{6}A mapping and quantification have overcome this problem. GLORI uses nitrous acid to deaminate unmodified A to I while leaving m^{6}A unchanged. This results in unmodified A being read as G in sequencing reads, with m^{6}A remaining as A (Liu et al., 2023). eTAM-seq similarly relies on exclusive deamination of unmodified A, but it does so through an evolved TadA8.20 enzyme which selectively targets unmodified A (Xiao et al., 2023). Both methods offer a simple approach for identifying m^{6}A with nucleotide specificity, and they have the added advantage of being able to measure m^{6}A stoichiometry transcriptome-wide. Further improvements to GLORI and eTAM-seq to limit RNA degradation will facilitate more widespread use of these methods and will help pave the way for their potential use in single-cell m^{6}A mapping (below). Additionally, several other antibody-independent m^{6}A profiling methods have been developed in recent years (reviewed in Owens et al., 2021). These approaches employ a variety of different strategies, including the use of methionine analogs to label m^{6}A sites (m^{6}A-label-seq) (Shu et al., 2020), chemical labeling of FTO-directed m^{6}A demethylation intermediates (m^{6}A-SEAL) (Wang et al., 2020), and treating RNA with modification-sensitive endoribonucleases (MAZTER-seq and m^{6}A-REF-seq) (Garcia-Campos et al., 2019; Chen et al., 2022). Strategies for site-specific m^{6}A quantification in RNAs of interest have also been developed, which serve as useful tools for investigating m^{6}A within individual transcripts and/or validating the results of global m^{6}A mapping for a subset of RNAs (Liu et al., 2013; Xiao et al., 2018; Castellanos-Rubio et al., 2019).

In addition to antibody-based, enzyme-assisted, and biochemical methods for m^{6}A mapping, nanopore sequencing has emerged as a technology with great promise for profiling m^{6}A and other RNA modifications. This direct RNA sequencing method involves driving RNAs through a protein nanopore and measuring the variations in ionic current that occur as different nucleotides pass through the pore (Garalde et al., 2018) (Figure 1). Chemical modifications in RNAs can alter the current intensity or dwell time of the RNA as it moves through the pore, and these unique signatures can then be used to detect the presence of modifications (Jain et al., 2022). Several studies have demonstrated the ability of nanopore technology to call m^{6}A sites (Zhong et al., 2023). A key advantage of this approach is that native, full-length RNA molecules can be sequenced, therefore enabling a deeper understanding of m^{6}A distribution within distinct transcript isoforms, the presence of m^{6}A clusters in single RNA molecules, and potential co-occurrence of m^{6}A with other modifications (Leger et al., 2021; Huang et al., 2024; Mateos et al., 2024).

Mapping m^{6}A in single cells

The brain is a complex mixture of diverse cell types. However, all m^{6}A profiling studies done in the brain thus far have used bulk tissue samples, which represent the cumulative m^{6}A signal across different cell types and provide no information on the distribution or abundance of m^{6}A within individual cells. By mapping methylated transcripts in single cells, the methylomes of all cell types can be elucidated, which would provide unprecedented insights into how m^{6}A contributes to brain function and disease through influencing gene expression in distinct cell types.

Several approaches have recently been developed to achieve single-cell m^{6}A profiling. Some studies have used m^{6}A antibodies to perform a low-input MeRIP-seq from single cells (Li et al., 2023; Yao et al., 2023). These methods can identify methylated transcripts from individual cells, but they have some drawbacks. First, the high signal-to-noise ratio resulting from antibody enrichment complicates peak calling, especially when using low input samples. Second, these methods are generally not highly scalable and have profiled m^{6}A in a few dozen cells at most (Li et al., 2023; Yao et al., 2023). The recent development of single-nucleus m6A-CUT&Tag (sn-m6A-CT) addresses these issues by coupling antibody-based methylated RNA enrichment with Tn5 transposase-mediated tagmentation (Hamashima et al., 2023). While this method still relies on m^{6}A antibodies, it improves signal-to-noise relative to strategies based on immunoprecipitation alone and can be used in any cell type or tissue of interest. Furthermore, it is amenable to droplet-based library preparation methods, making it a truly high-throughput technique.

Antibody-independent strategies for single-cell m^{6}A profiling have also been developed. In 2022, our group introduced single-cell DART-seq (scDART-seq), which installs a unique mutation signature adjacent to m^{6}A sites (Meyer, 2019; Tegowski et al., 2022). This is achieved by expressing a fusion protein consisting of the m^{6}A-binding YTH domain tethered to the cytidine deaminase APOBEC1 (Meyer, 2019). The YTH domain recruits the fusion protein to sites of methylation while APOBEC1 edits nearby cytidines to uridines, enabling m^{6}A sites to be identified as C-to-T mutations in the sequencing data. This method is compatible with any scRNA-seq preparation method and does not require additional RNA processing steps, making it a highly scalable strategy which is straightforward to implement (Figure 1). However, one limitation of scDART-seq is that it requires expressing the APOBEC1-YTH protein in cells of interest. This is easy to do in many cultured cell types but can be more challenging for certain tissues. Furthermore, expression of APOBEC1-YTH could also influence cell biology if expression is prolonged (Tegowski et al., 2022).

Emerging technologies for studying m^{6}A at the single-molecule level

Most methods for m^{6}A mapping rely on short read sequencing. Although these techniques can reveal m^{6}A sites, they are unable to describe how these sites are distributed on individual RNA molecules.

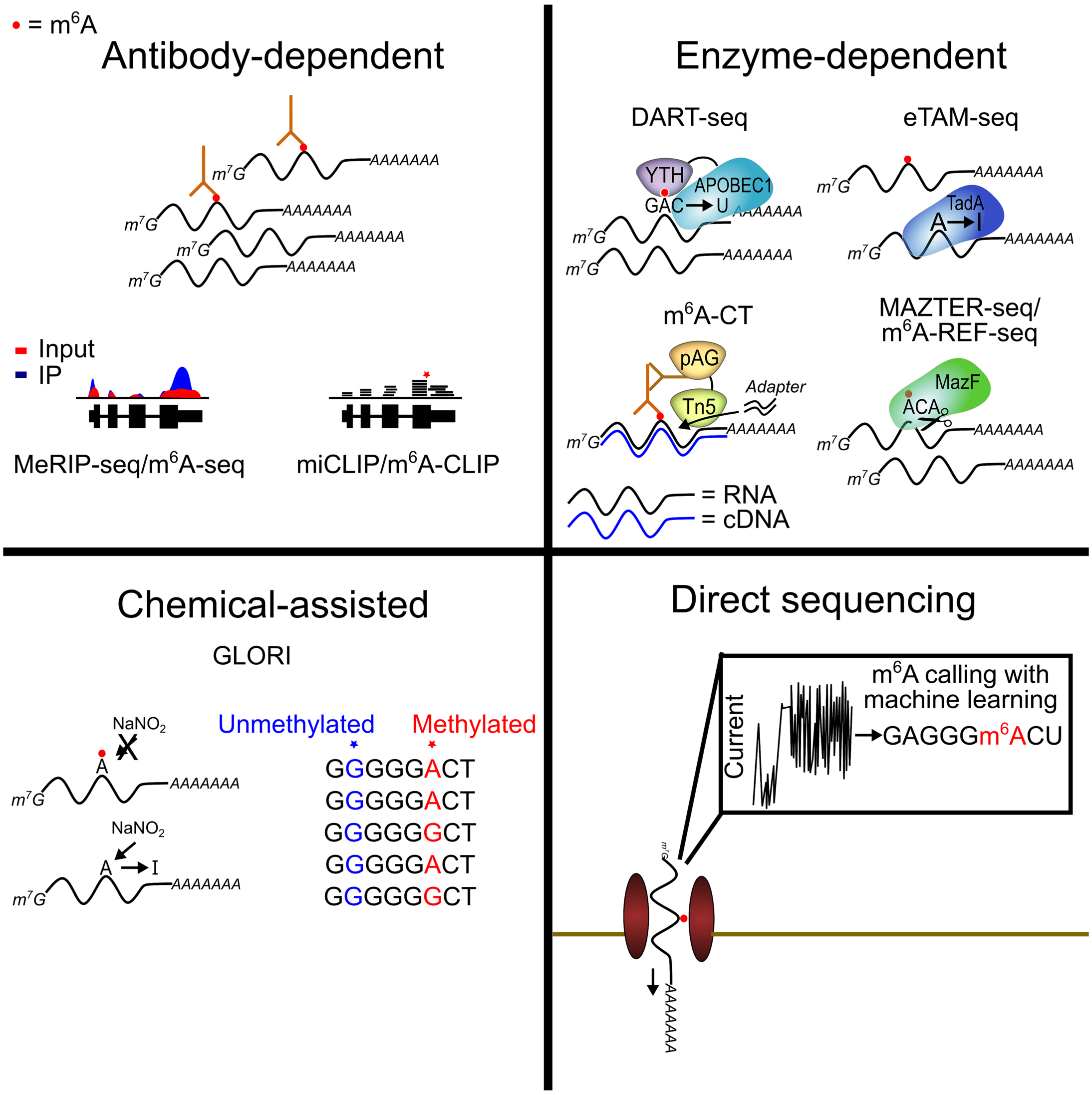


FIGURE 1
Current transcriptome-wide m⁶A profiling methods. Recent advances have provided new techniques for m⁶A profiling across the transcriptome. Some of these methods allow for highly quantitative detection of m⁶A sites at single-nucleotide resolution (GLORI and eTAM-seq). Others have facilitated the use of low-input samples, including single cells (DART-seq and m⁶A-CT).

For example, many RNAs have multiple m⁶A sites, but whether these sites co-occur on the same RNA molecules is unknown. In addition, the distribution of m⁶A within distinct transcript isoforms is often difficult to assess when only a short fragment of the parent RNA is sequenced. Exploring methylation at the single-molecule level can help address these important questions.

As discussed above, nanopore sequencing has emerged as a technology with great promise for profiling m⁶A and other RNA modifications. This strategy provides information on full-length RNA molecules, which enables greater insight into the presence of modifications in splice variants or other RNA isoforms. Additionally, since RNA molecules are sequenced directly, potential biases introduced during cDNA synthesis and PCR amplification steps are avoided. However, nanopore-based RNA modification sequencing has some limitations. First, identification of modification sites requires the use of machine learning algorithms trained on datasets to enable *de novo* modification site calls, or the use of modification-free control samples to enable modification detection by comparative analysis (Hendra et al., 2022; Jain et al., 2022). For m⁶A, several computational tools have been developed for identifying methylated sites from nanopore data, with substantial variations in called sites and estimated accuracy (Zhong et al., 2023). More fundamentally, training m⁶A calling algorithms requires a known "ground-truth," which can be difficult to know with certainty in all model systems.

In addition to DRS technologies such as nanopore sequencing, other methods exist that enable m⁶A identification in individual RNA molecules. For instance, DART-seq has been used with PacBio sequencing, which has enabled the identification of m⁶A sites along the full length of individual mRNAs (Meyer, 2019). In theory, other methods that induce m⁶A-associated mutations, such as eTAM-seq and GLORI, could also be combined with long-read sequencing to explore m⁶A on single molecules. However, these approaches have not been widely used, and given the rapid developments in nanopore technology, DRS will likely emerge as the method of choice for single-molecule m^{6}A mapping.

In addition to sequencing-based approaches, other methods have been developed that enable analysis of individual methylated RNA molecules in cells. m^{6}AISH-PLA uses proximity ligation between an m^{6}A-recognizing antibody and a sequence-specific oligo targeted to sequences flanking the m^{6}A site of interest. After ligation, rolling circle amplification (RCA) amplifies an engineered sequence recognized by a fluorescent detection probe (Ren et al., 2021). This method allows for the visualization of single methylated molecules *in situ*, facilitating novel investigations into m^{6}A-mediated RNA localization and trafficking. One drawback to this method is that it cannot simultaneously visualize unmethylated transcripts, which could lead to misinterpretations if both methylated and unmethylated RNAs are similarly trafficked. However, an adaptation of the DART-seq technology, termed DART-FISH, can detect methylated and unmethylated transcripts (Sheehan et al., 2023). By expressing the APOBEC1-YTH enzyme in cells, transcripts with m^{6}A-dependent C-to-U mutations can be discriminated from unmodified transcripts using padlock probe hybridization followed by RCA and hybridization of detection probes. By using distinct padlock probes for the C and U variants adjacent to an m^{6}A site of interest, the unmethylated and methylated copies of an individual transcript can be visualized simultaneously. Since m^{6}A has been implicated in subcellular RNA localization, approaches such as these which enable *in situ* visualization of m^{6}A-modified transcripts can be powerful approaches for understanding the role of m^{6}A in RNA trafficking or partitioning to subcellular compartments such as stress granules (Anders et al., 2018; Fu and Zhuang, 2020; Khong et al., 2022; Ries et al., 2023).

Strategies for targeted m^{6}A manipulation and m^{6}A-dependent gene expression control

Several groups have developed tools for targeted addition or removal of m^{6}A in cellular RNAs of interest. These methods involve fusing m^{6}A methyltransferase or demethylase enzymes to catalytically inactive Cas proteins coupled with guide RNA (gRNA)-mediated targeting of specific transcripts. For instance, Wilson et al. fused METTL3/14 to dCas13 to achieve site-specific methylation of several cellular mRNAs, including *GAPDH*, *FOXM1*, and *SOX2*. In addition, they showed that targeted methylation of *ACTB* led to transcript degradation and that methylation of the *BRD8* and *ZNF638* transcripts impacted splicing, consistent with previous reports of m^{6}A function in these mRNAs (Wilson et al., 2020). Li et al. showed that ALKBH5 tethered to dCas13b can remove m^{6}A from oncogenic transcripts *EGFR* and *MYC* in the presence of transcript-targeting gRNAs, leading to decreased protein expression and reduced cell proliferation (Li et al., 2020). Tethering of dCas9 to m^{6}A methyltransferases and demethylases has also been used to achieve targeted m^{6}A writing and erasing, respectively (Liu et al., 2019). Collectively, these tools have utility not only for basic research into m^{6}A function but also as a potential therapeutic strategy to overcome the effects of hyper or hypomethylation during disease. Current challenges include minimizing off-targeting effects to ensure transcript specificity and optimization of methylation and demethylation efficiency. However, the use of CRISPR/Cas-based technologies for targeting RNA has accelerated at a rapid pace, and as these and other methods continue to expand, we anticipate that the tools for manipulating m^{6}A and other RNA modifications will also improve. Indeed, these methods have already been expanded to include light-activated m^{6}A modification systems which add temporal specificity (Lan et al., 2021; Shi et al., 2022).

The tools above use targeted manipulation of m^{6}A levels in specific RNAs to control the expression of genes of interest. This holds promise as a potential therapeutic strategy, since m^{6}A dysregulation can lead to abnormal expression of specific genes to promote the pathogenesis of cancer and other diseases (Yang et al., 2020; Jiang et al., 2021; Delaunay et al., 2024). However, an alternative approach is to couple the presence of m^{6}A with the expression of desired proteins. Recently, our group developed a genetically encoded m^{6}A sensor system (GEMS), which couples mRNA methylation with expression of a protein of interest (Marayati et al., 2024). This is achieved by expressing a reporter mRNA together with APOBEC1-YTH in cells. The reporter mRNA contains an m^{6}A sensor sequence that, when methylated, recruits APOBEC1-YTH to convert nearby cytidines to uridines, in turn generating one or more stop codons that block translation of a degradation tag after the coding sequence of the protein of interest. The result is stable protein production only when the mRNA is methylated. We used this system to achieve m^{6}A-coupled expression of tumor suppressor proteins in cancer cells, which led to decreased cell proliferation and migration (Marayati et al., 2024). Although m^{6}A-coupled protein expression technologies such as this still require further optimization, the ability to sense m^{6}A in living cells offers an attractive platform both for methylation-sensitive protein expression as well as for studies of m^{6}A dynamics in the brain and other tissues.

Discussion

Much of our understanding of m^{6}A in the brain has been driven by recent advances in m^{6}A mapping technologies. These tools have not only enabled the identification of methylated RNAs within the brain and other tissues but have also provided a deeper understanding of m^{6}A dynamics and function. Although antibody-based methods have been the predominant method of choice for transcriptome-wide m^{6}A mapping, newer approaches have emerged in the last few years which overcome many of the limitations of antibody-based approaches. For instance, GLORI and eTAM-seq offer not only nucleotide-resolution m^{6}A mapping, but they also enable quantification of m^{6}A stoichiometry. The ability to measure changes in m^{6}A abundance is an important advance, since methods for reliable, sensitive quantification of m^{6}A stoichiometry transcriptome-wide have been largely elusive, which has contributed to discrepancies regarding the dynamic nature of m^{6}A. Although GLORI and eTAM-seq have great potential for becoming the new gold standard of m^{6}A mapping and quantification, further refinements of these methods to improve sensitivity and reduce RNA degradation will be needed for their widespread adoption.

Direct RNA sequencing with nanopore technology also holds great promise for enabling m^{6}A identification at the single-molecule level and within different transcript isoforms. Additionally, nanopore sequencing can potentially be used to identify multiple modifications within a single RNA molecule, which is an area that we currently have little knowledge about. However, achieving these goals will require improved throughput and accuracy, as well as establishment of consistent data analysis pipelines and appropriate training datasets. Nevertheless, rapid progress is being made in nanopore-based modification mapping, so we anticipate that this technology will become increasingly widespread in the coming years.

The ability to map m6A in single cells is an important step forward for deepening our understanding of m6A regulation and function. The recent development of scDART-seq (Tegowski et al., 2022), scm6A-seq (Yao et al., 2023), picoMeRIP-seq (Li et al., 2023), and single-nucleus m6A-CUT&Tag (sn-m6A-CT) (Hamashima et al., 2023) have been critical advances and have revealed new insights into m6A distribution and regulation within individual cells of a population. Applying single-cell m6A mapping methods to the brain will undoubtedly uncover new information about m6A dynamics and regulation within distinct brain cell types. In particular, our understanding of m6A function in non-neuronal cells is limited, so such studies will greatly facilitate future discoveries in this area.

Going forward, it will be important to further develop single-cell m6A mapping technologies to enable their widespread use across cell or tissue types of interest. Additionally, methods such as GLORI or eTAM-seq may be promising antibody-independent strategies for single-cell m6A mapping, but their sensitivity for low-input RNA must be further developed, and their propensity to induce RNA degradation must be addressed. Nevertheless, this is an exciting time for m6A mapping technology in single cells, with a few tools already available and further developments undoubtedly on the horizon. Having the ability to combine m6A mapping with other single-cell "omics" technologies will be very powerful for furthering our understanding of the interplay between m6A and other gene regulatory processes such as chromatin remodeling, transcription regulation, and RNA processing events.

In addition to technologies for mapping and quantifying m6A, there are emerging tools for targeted manipulation of m6A which can achieve selective methylation or demethylation of RNAs of interest. The ability to selectively add or remove m6A from RNAs is a useful tool for investigating m6A function. However, one consideration is that m6A sites cluster in RNAs, and recent studies have indicated that cellular RNAs contain many more m6A sites than previously thought (Tegowski et al., 2022; Liu et al., 2023). Thus, the effects of adding or removing a single m6A site may be compensated for through methylation of other nearby adenosines within a given region of methylation. This is also an important consideration when developing m6A targeting tools for therapeutic applications, as multiple m6A sites may exist at nearby positions in a transcript of interest. However, methylating or demethylating single sites has been shown to impact RNA expression in cells (Liu et al., 2019; Li et al., 2020; Wilson et al., 2020), suggesting that compensation by nearby m6A sites does not happen for all RNAs. It is also possible that the individual m6A sites that make up methylation "clusters" occur on different RNA molecules, which would make compensation by nearby adenosines less likely. Most m6A profiling strategies do not report the individual RNA molecules in which m6A sites reside, underscoring the need to develop better tools for single-molecule m6A mapping.

Going forward, it will be important for the field to address issues related to sensitivity and reproducibility of methods for studying m6A. Newer technologies such as GLORI and eTAM-seq that enable high-resolution m6A mapping as well as quantification can potentially enable better insights into m6A dynamics, since many m6A sites may be regulated by changes in abundance as opposed to strict gain or loss of methylation. Additionally, the recent development of tools for sensing m6A provide new opportunities for studying m6A dynamics in living cells, in contrast to other methods that require RNA isolation. Our understanding of how m6A is regulated within the brain during both healthy and disease states will undoubtedly be accelerated by the ability to map and quantify m6A within the brain and in specific cell types. Thus, further development of single-cell m6A profiling approaches will be important. Finally, nanopore sequencing or other methods that provide single-molecule information have the potential to provide deeper insights into roles of m6A in distinct transcript isoforms, as well as the possibility of multiple different modifications co-occurring on the same RNAs. We anticipate that continued development of these methods in the coming years will make them more widely used for studies of m6A in the brain.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

MT: Writing – original draft, Writing – review & editing. KM: Writing – original draft, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This work was supported by the National Institutes of Health (1DP1DA046584, 1R01MH118366, and 1RM1HG011563 to KM).

Acknowledgments

We thank Dr. Clémence Bernard, Dr. Michael Kiebler, and Dr. Oriane Mauger for giving us the opportunity to write this perspective review.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Anders, M., Chelysheva, I., Goebel, I., Trenkner, T., Zhou, J., Mao, Y., et al. (2018). Dynamic m6A methylation facilitates mRNA triaging to stress granules. *Life Sci. Alliance* 1:e201800113. doi: 10.26508/lsa.201800113

Castellanos-Rubio, A., Santin, I., Olazagoitia-Garmendia, A., Romero-Garmendia, I., Jauregi-Miguel, A., Legarda, M., et al. (2019). A novel RT-QPCR-based assay for the relative quantification of residue specific m6A RNA methylation. *Sci. Rep.* 9, 1–7. doi: 10.1038/s41598-019-40018-6

Chen, H.-X., Zhang, Z., Ma, D.-Z., Chen, L.-Q., and Luo, G.-Z. (2022). Mapping single-nucleotide m6A by m6A-REF-seq. *Methods* 203, 392–398. doi: 10.1016/j.ymeth.2021.06.013

Choi, S. H., Flamand, M. N., Liu, B., Zhu, H., Hu, M., Wang, M., et al. (2022). RBM45 is an m6A-binding protein that affects neuronal differentiation and the splicing of a subset of mRNAs. *Cell Rep.* 40:111293. doi: 10.1016/j.celrep.2022.111293

Delaunay, S., Helm, M., and Frye, M. (2024). RNA modifications in physiology and disease: towards clinical applications. *Nat. Rev. Genet.* 25, 104–122. doi: 10.1038/s41576-023-00645-2

Dierks, D., Garcia-Campos, M. A., Uzonyi, A., Safra, M., Edelheit, S., Rossi, A., et al. (2021). Multiplexed profiling facilitates robust m6A quantification at site, gene and sample resolution. *Nat. Methods* 18, 1060–1067. doi: 10.1038/s41592-021-01242-z

Dominissini, D., Moshitch-Moshkovitz, S., Schwartz, S., Salmon-Divon, M., Ungar, L., Osenberg, S., et al. (2012). Topology of the human and mouse m6A RNA methylomes revealed by m6A-seq. *Nature* 485, 201–206. doi: 10.1038/nature11112

Doyle, M., and Kiebler, M. A. (2011). Mechanisms of dendritic mRNA transport and its role in synaptic tagging. *EMBO J.* 30, 3540–3552. doi: 10.1038/emboj.2011.278

Edens, B. M., Vissers, C., Su, J., Arumugam, S., Xu, Z., Shi, H., et al. (2019). FMRP modulates neural differentiation through m6A-dependent mRNA nuclear export. *Cell Rep.* 28, 845–854.e5. doi: 10.1016/J.CELREP.2019.06.072

Flamand, M. N., and Meyer, K. D. (2019). The epitranscriptome and synaptic plasticity. *Curr. Opin. Neurobiol.* 59, 41–48. doi: 10.1016/J.CONB.2019.04.007

Flamand, M. N., and Meyer, K. D. (2022). m6A and YTHDF proteins contribute to the localization of select neuronal mRNAs. *Nucleic Acids Res.* 50, 4464–4483. doi: 10.1093/nar/gkac251

Flamand, M. N., Tegowski, M., and Meyer, K. D. (2023). The proteins of mRNA modification: writers, readers, and erasers. *Annu. Rev. Biochem.* 92, 145–173. doi: 10.1146/annurev-biochem-052521-035330

Fu, Y., and Zhuang, X. (2020). m6A-binding YTHDF proteins promote stress granule formation. *Nat. Chem. Biol.* 16, 955–963. doi: 10.1038/s41589-020-0524-y

Garalde, D. R., Snell, E. A., Jachimowicz, D., Sipos, B., Lloyd, J. H., Bruce, M., et al. (2018). Highly parallel direct RNA sequencing on an array of nanopores. *Nat. Methods* 15, 201–206. doi: 10.1038/nmeth.4577

Garcia-Campos, M. A., Edelheit, S., Toth, U., Safra, M., Shachar, R., Viukov, S., et al. (2019). Deciphering the "m6A code" via antibody-independent quantitative profiling. *Cell* 178, 731–747.e16. doi: 10.1016/J.CELL.2019.06.013

Hamashima, K., Wong, K. W., Sam, T. W., Teo, J. H. J., Taneja, R., Le, M. T. N., et al. (2023). Single-nucleus multiomic mapping of m6A methylomes and transcriptomes in native populations of cells with sn-m6A-CT. *Mol. Cell* 83, 3205–3216.e5. doi: 10.1016/j.molcel.2023.08.010

He, P. C., and He, C. (2023). mRNA accessibility within mRNPs as a determinant of gene expression. *Trends Biochem. Sci.* 49, 199–207. doi: 10.1016/j.tibs.2023.11.003

He, P. C., Wei, J., Dou, X., Harada, B. T., Zhang, Z., Ge, R., et al. (2023). Exon architecture controls mRNA m6A suppression and gene expression. *Science* 379, 677–682. doi: 10.1126/science.abj9090

Hendra, C., Pratanwanich, P. N., Wan, Y. K., Goh, W. S. S., Thiery, A., and Göke, J. (2022). Detection of m6A from direct RNA sequencing using a multiple instance learning framework. *Nat. Methods* 19, 1590–1598. doi: 10.1038/s41592-022-01666-1

Holt, C. E., Martin, K. C., and Schuman, E. M. (2019). Local translation in neurons: visualization and function. *Nat. Struct. Mol. Biol.* 26, 557–566. doi: 10.1038/s41594-019-0263-5

Huang, S., Wylder, A. C., and Pan, T. (2024). Simultaneous nanopore profiling of mRNA m6A and pseudouridine reveals translation coordination. *Nat. Biotechnol.* 42, 1–5. doi: 10.1038/s41587-024-02135-0

Jain, M., Abu-Shumays, R., Olsen, H. E., and Akeson, M. (2022). Advances in nanopore direct RNA sequencing. *Nat. Methods* 19, 1160–1164. doi: 10.1038/s41592-022-01633-w

Jiang, X., Liu, B., Nie, Z., Duan, L., Xiong, Q., Jin, Z., et al. (2021). The role of m6A modification in the biological functions and diseases. *Signal Transduct. Target. Ther.* 6, 1–16. doi: 10.1038/s41392-020-00450-x

Ke, S., Pandya-Jones, A., Saito, Y., Fak, J. J., Vågbø, C. B., Geula, S., et al. (2017). m6A mRNA modifications are deposited in nascent pre-mRNA and are not required for splicing but do specify cytoplasmic turnover. *Genes Dev.* 31, 990–1006. doi: 10.1101/gad.301036.117

Khong, A., Matheny, T., Huynh, T. N., Babl, V., and Parker, R. (2022). Limited effects of m6A modification on mRNA partitioning into stress granules. *Nat. Commun.* 13:3735. doi: 10.1038/s41467-022-31358-5

Kontur, C., Jeong, M., Cifuentes, D., and Giraldez, A. J. (2020). Ythdf m6A readers function redundantly during zebrafish development. *Cell Rep.* 33:108598. doi: 10.1016/j.celrep.2020.108598

Lan, L., Sun, Y.-J., Jin, X.-Y., Xie, L.-J., Liu, L., and Cheng, L. (2021). A light-controllable chemical modulation of m6A RNA methylation. *Angew. Chem. Int. Ed.* 60, 18116–18121. doi: 10.1002/anie.202103854

Leger, A., Amaral, P. P., Pandolfini, L., Capitanchik, C., Capraro, F., Miano, V., et al. (2021). RNA modifications detection by comparative Nanopore direct RNA sequencing. *Nat. Commun.* 12:7198. doi: 10.1038/s41467-021-27393-3

Li, J., Chen, Z., Chen, F., Xie, G., Ling, Y., Peng, Y., et al. (2020). Targeted mRNA demethylation using an engineered dCas13b-ALKBH5 fusion protein. *Nucleic Acids Res.* 48, 5684–5694. doi: 10.1093/nar/gkaa269

Li, Y., Wang, Y., Vera-Rodriguez, M., Lindeman, L. C., Skuggen, L. E., Rasmussen, E. M. K., et al. (2023). Single-cell m6A mapping *in vivo* using picoMeRIP–seq. *Nat. Biotechnol.* 41, 1–6. doi: 10.1038/s41587-023-01831-7

Li, M., Zhao, X., Wang, W., Shi, H., Pan, Q., Lu, Z., et al. (2018). Ythdf2-mediated m6A mRNA clearance modulates neural development in mice. *Genome Biol.* 19, 1–16. doi: 10.1186/S13059-018-1436-Y/TABLES/1

Linder, B., Grozhik, A. V., Olarerin-George, A. O., Meydan, C., Mason, C. E., and Jaffrey, S. R. (2015). Single-nucleotide-resolution mapping of m6A and m6Am throughout the transcriptome. *Nat. Methods* 12, 767–772. doi: 10.1038/nmeth.3453

Liu, J., Li, K., Cai, J., Zhang, M., Zhang, X., Xiong, X., et al. (2020). Landscape and regulation of m6A and m6Am Methylome across human and mouse tissues. *Mol. Cell* 77, 426–440.e6. doi: 10.1016/J.MOLCEL.2019.09.032

Liu, N., Parisien, M., Dai, Q., Zheng, G., He, C., and Pan, T. (2013). Probing N6-methyladenosine RNA modification status at single nucleotide resolution in mRNA and long noncoding RNA. *RNA* 19, 1848–1856. doi: 10.1261/rna.041178.113

Liu, C., Sun, H., Yi, Y., Shen, W., Li, K., Xiao, Y., et al. (2023). Absolute quantification of single-base m6A methylation in the mammalian transcriptome using GLORI. *Nat. Biotechnol.* 41, 355–366. doi: 10.1038/s41587-022-01487-9

Liu, X.-M., Zhou, J., Mao, Y., Ji, Q., and Qian, S.-B. (2019). Programmable RNA N6-methyladenosine editing by CRISPR-Cas9 conjugates. *Nat. Chem. Biol.* 15, 865–871. doi: 10.1038/s41589-019-0327-1

Livneh, I., Moshitch-Moshkovitz, S., Amariglio, N., Rechavi, G., and Dominissini, D. (2020). The m6A epitranscriptome: transcriptome plasticity in brain development and function. *Nat. Rev. Neurosci.* 21, 36–51. doi: 10.1038/s41583-019-0244-z

Loedige, I., Baranovskii, A., Mendonsa, S., Dantsuji, S., Popitsch, N., Breimann, L., et al. (2023). mRNA stability and m6A are major determinants of subcellular mRNA localization in neurons. *Mol. Cell* 83, 2709–2725.e10. doi: 10.1016/j.molcel.2023.06.021

Marayati, B. F., Thompson, M. G., Holley, C. L., Horner, S. M., and Meyer, K. D. (2024). Programmable protein expression using a genetically encoded m6A sensor. *Nat. Biotechnol.* 42, 1–12. doi: 10.1038/s41587-023-01978-3

Mateos, P. A., Sethi, A. J., Ravindran, A., Srivastava, A., Kanchi, M., Mahmud, S., et al. (2024). Simultaneous identification of m6A and m5C reveals coordinated RNA modification at single-molecule resolution. *bioRxiv*. doi: 10.1101/2022.03.14.484124

McIntyre, A. B. R., Gokhale, N. S., Cerchietti, L., Jaffrey, S. R., Horner, S. M., and Mason, C. E. (2020). Limits in the detection of m6A changes using MeRIP/m6A-seq. *Sci. Rep.* 10:6590. doi: 10.1038/s41598-020-63355-3

Merkurjev, D., Hong, W.-T., Iida, K., Oomoto, I., Goldie, B. J., Yamaguti, H., et al. (2018). Synaptic N6-methyladenosine (m6A) epitranscriptome reveals functional partitioning of localized transcripts. *Nat. Neurosci.* 21, 1004–1014. doi: 10.1038/s41593-018-0173-6

Meyer, K. D. (2019). DART-seq: an antibody-free method for global m6A detection. *Nat. Methods* 16, 1275–1280. doi: 10.1038/s41592-019-0570-0

Meyer, K. D., Saletore, Y., Zumbo, P., Elemento, O., Mason, C. E., and Jaffrey, S. R. (2012). Comprehensive analysis of mRNA methylation reveals enrichment in 3′ UTRs and near stop codons. *Cell* 149, 1635–1646. doi: 10.1016/J.CELL.2012.05.003

Molinie, B., Wang, J., Lim, K. S., Hillebrand, R., Lu, Z. X., Van Wittenberghe, N., et al. (2016). M6 A-LAIC-seq reveals the census and complexity of the m6 a epitranscriptome. *Nat. Methods* 13, 692–698. doi: 10.1038/nmeth.3898

Murakami, S., and Jaffrey, S. R. (2022). Hidden codes in mRNA: control of gene expression by m6A. *Mol. Cell* 82, 2236–2251. doi: 10.1016/J.MOLCEL.2022.05.029

Owens, M. C., Zhang, C., and Liu, K. F. (2021). Recent technical advances in the study of nucleic acid modifications. *Mol. Cell* 81, 4116–4136. doi: 10.1016/j.molcel.2021.07.036

Ren, X., Deng, R., Zhang, K., Sun, Y., Li, Y., and Li, J. (2021). Single-cell imaging of m6A modified RNA using m6A-specific *in situ* hybridization mediated proximity ligation assay (m6AISH-PLA). *Angew. Chem. Int. Ed.* 60, 22646–22651. doi: 10.1002/anie.202109118

Ries, R. J., Pickering, B. F., Poh, H. X., Namkoong, S., and Jaffrey, S. R. (2023). m6A governs length-dependent enrichment of mRNAs in stress granules. *Nat. Struct. Mol. Biol.* 30, 1525–1535. doi: 10.1038/s41594-023-01089-2

Roy, R., Shiina, N., and Wang, D. O. (2020). More dynamic, more quantitative, unexpectedly intricate: advanced understanding on synaptic RNA localization in learning and memory. *Neurobiol. Learn. Mem.* 168:107149. doi: 10.1016/j.nlm.2019.107149

Sheehan, C. J., Marayati, B. F., Bhatia, J., and Meyer, K. D. (2023). *In situ* visualization of m6A sites in cellular mRNAs. *Nucleic Acids Res.* 51:e101. doi: 10.1093/nar/gkad787

Shi, H., Wei, J., and He, C. (2019). Where, when, and how: context-dependent functions of RNA methylation writers, readers, and erasers. *Mol. Cell* 74, 640–650. doi: 10.1016/j.molcel.2019.04.025

Shi, H., Xu, Y., Tian, N., Yang, M., and Liang, F.-S. (2022). Inducible and reversible RNA N6-methyladenosine editing. *Nat Commun.* 13:1958. doi: 10.1038/s41467-022-29665-y

Shi, H., Zhang, X., Weng, Y.-L., Lu, Z., Liu, Y., Lu, Z., et al. (2018). m6A facilitates hippocampus-dependent learning and memory through YTHDF1. *Nature* 563, 249–253. doi: 10.1038/s41586-018-0666-1

Shu, X., Cao, J., Cheng, M., Xiang, S., Gao, M., Li, T., et al. (2020). A metabolic labeling method detects m6A transcriptome-wide at single base resolution. *Nat. Chem. Biol.* 16, 887–895. doi: 10.1038/s41589-020-0526-9

Tegowski, M., Flamand, M. N., and Meyer, K. D. (2022). scDART-seq reveals distinct m6A signatures and mRNA methylation heterogeneity in single cells. *Mol. Cell* 82, 868–878.e10. doi: 10.1016/J.MOLCEL.2021.12.038

Uzonyi, A., Dierks, D., Nir, R., Kwon, O. S., Toth, U., Barbosa, I., et al. (2023). Exclusion of m6A from splice-site proximal regions by the exon junction complex dictates m6A topologies and mRNA stability. *Mol. Cell* 83, 237–251.e7. doi: 10.1016/j.molcel.2022.12.026

Wang, Y., Li, Y., Yue, M., Wang, J., Kumar, S., Wechsler-Reya, R. J., et al. (2018). N6-methyladenosine RNA modification regulates embryonic neural stem cell self-renewal through histone modifications. *Nat. Neurosci.* 21, 195–206. doi: 10.1038/s41593-017-0057-1

Wang, Y., Xiao, Y., Dong, S., Yu, Q., and Jia, G. (2020). Antibody-free enzyme-assisted chemical approach for detection of N6-methyladenosine. *Nat. Chem. Biol.* 16, 896–903. doi: 10.1038/s41589-020-0525-x

Wilson, C., Chen, P. J., Miao, Z., and Liu, D. R. (2020). Programmable m6A modification of cellular RNAs with a Cas13-directed methyltransferase. *Nat. Biotechnol.* 38, 1431–1440. doi: 10.1038/s41587-020-0572-6

Xiao, Y.-L., Liu, S., Ge, R., Wu, Y., He, C., Chen, M., et al. (2023). Transcriptome-wide profiling and quantification of N6-methyladenosine by enzyme-assisted adenosine deamination. *Nat. Biotechnol.* 41, 993–1003. doi: 10.1038/s41587-022-01587-6

Xiao, Y., Wang, Y., Tang, Q., Wei, L., Zhang, X., and Jia, G. (2018). An elongation-and ligation-based qPCR amplification method for the radiolabeling-free detection of locus-specific N6-Methyladenosine modification. *Angew. Chem. Int. Ed.* 57, 15995–16000. doi: 10.1002/anie.201807942

Yang, C., Hu, Y., Zhou, B., Bao, Y., Li, Z., Gong, C., et al. (2020). The role of m6A modification in physiology and disease. *Cell Death Dis.* 11, 960–916. doi: 10.1038/s41419-020-03143-z

Yang, X., Triboulet, R., Liu, Q., Sendinc, E., and Gregory, R. I. (2022). Exon junction complex shapes the m6A epitranscriptome. *Nat. Commun.* 13:7904. doi: 10.1038/s41467-022-35643-1

Yao, H., Gao, C.-C., Zhang, D., Xu, J., Song, G., Fan, X., et al. (2023). scm6A-seq reveals single-cell landscapes of the dynamic m6A during oocyte maturation and early embryonic development. *Nat. Commun.* 14:315. doi: 10.1038/s41467-023-35958-7

Yoon, K.-J., Ringeling, F. R., Vissers, C., Jacob, F., Pokrass, M., Jimenez-Cyrus, D., et al. (2017). Temporal control of mammalian cortical neurogenesis by m6A methylation. *Cell* 171, 877–889.e17. doi: 10.1016/J.CELL.2017.09.003

Zaccara, S., and Jaffrey, S. R. (2020). A unified model for the function of YTHDF proteins in regulating m6A-modified mRNA. *Cell* 181, 1582–1595.e18. doi: 10.1016/J.CELL.2020.05.012

Zaccara, S., Ries, R. J., and Jaffrey, S. R. (2019). Reading, writing and erasing mRNA methylation. *Nat. Rev. Mol. Cell Biol.* 20, 608–624. doi: 10.1038/s41580-019-0168-5

Zeng, Y., Wang, S., Gao, S., Soares, F., Ahmed, M., Guo, H., et al. (2018). Refined RIP-seq protocol for epitranscriptome analysis with low input materials. *PLoS Biol.* 16:e2006092. doi: 10.1371/journal.pbio.2006092

Zhang, Z., Wang, M., Xie, D., Huang, Z., Zhang, L., Yang, Y., et al. (2018). METTL3-mediated N6-methyladenosine mRNA modification enhances long-term memory consolidation. *Cell Res.* 28, 1050–1061. doi: 10.1038/s41422-018-0092-9

Zhong, Z.-D., Xie, Y.-Y., Chen, H.-X., Lan, Y.-L., Liu, X.-H., Ji, J.-Y., et al. (2023). Systematic comparison of tools used for m6A mapping from nanopore direct RNA sequencing. *Nat. Commun.* 14:1906. doi: 10.1038/s41467-023-37596-5

Zou, Z., Wei, J., Chen, Y., Kang, Y., Shi, H., Yang, F., et al. (2023). FMRP phosphorylation modulates neuronal translation through YTHDF1. *Mol. Cell* 83, 4304–4317.e8. doi: 10.1016/j.molcel.2023.10.028

frontiers | Frontiers in Molecular Neuroscience

TYPE Perspective
PUBLISHED 14 May 2024
DOI 10.3389/fnmol.2024.1386219

Check for updates

OPEN ACCESS

EDITED BY
Oriane Mauger,
Max Planck Institute of Psychiatry, Germany

REVIEWED BY
Alexandros Poulopoulos,
University of Maryland, United States
Hiruy Meharena,
University of California, San Diego,
United States

*CORRESPONDENCE
Erin E. Duffy
erin_duffylacy@hms.harvard.edu
Brian T. Kalish
brian.kalish@sickkids.ca

RECEIVED 14 February 2024
ACCEPTED 30 April 2024
PUBLISHED 14 May 2024

CITATION
Duffy EE, Assad EG, Kalish BT and Greenberg ME (2024) Small but mighty: the rise of microprotein biology in neuroscience. *Front. Mol. Neurosci.* 17:1386219.
doi: 10.3389/fnmol.2024.1386219

Small but mighty: the rise of microprotein biology in neuroscience

Erin E. Duffy[1]*, Elena G. Assad[1], Brian T. Kalish[2,3,4]* and Michael E. Greenberg[1]

[1]Department of Neurobiology, Harvard Medical School, Boston, MA, United States, [2]Program in Neuroscience and Mental Health, SickKids Research Institute, Toronto, ON, Canada, [3]Department of Molecular Genetics, University of Toronto, Toronto, ON, Canada, [4]Division of Neonatology, Department of Paediatrics, Hospital for Sick Children, Toronto, ON, Canada

The mammalian central nervous system coordinates a network of signaling pathways and cellular interactions, which enable a myriad of complex cognitive and physiological functions. While traditional efforts to understand the molecular basis of brain function have focused on well-characterized proteins, recent advances in high-throughput translatome profiling have revealed a staggering number of proteins translated from non-canonical open reading frames (ncORFs) such as 5′ and 3′ untranslated regions of annotated proteins, out-of-frame internal ORFs, and previously annotated non-coding RNAs. Of note, microproteins <100 amino acids (AA) that are translated from such ncORFs have often been neglected due to computational and biochemical challenges. Thousands of putative microproteins have been identified in cell lines and tissues including the brain, with some serving critical biological functions. In this perspective, we highlight the recent discovery of microproteins in the brain and describe several hypotheses that have emerged concerning microprotein function in the developing and mature nervous system.

KEYWORDS

microprotein, RNA translation, mitochondrial, DNA repair, mammalian, brain

Introduction

> "And though she be but little, she is fierce."
> - William Shakespeare

Regulated translation of RNA into protein represents a pivotal mechanism in the control of gene expression, enabling the cell to modulate the quantity, diversity, and functionality of proteins. In the mammalian nervous system, this protein diversity allows for the establishment of specific cell types, the organization of neural circuits, and the execution of complex behaviors. Historically, one mRNA was thought to encode a single protein product, but transcriptome-wide identification of translated open reading frames (ORFs) has revealed thousands of proteins that are translated from alternative ORFs, thereby exponentially increasing proteomic diversity by encoding multiple proteins from a single mRNA. These non-canonical ORFs (ncORFs) are distinct from the coding sequence included in the reference annotation, which we will refer to as the canonical ORF. A subset of these ncORFs are microproteins, defined as proteins 100 amino acids (AA) or less in length that are translated from an independent small open reading frame (sORF, also referred to as a smORF), which have emerged as versatile regulators of cellular function. In the literature, microproteins have been interchangeably referred to as "micropeptides" and "miniproteins", both denoting proteins that arise from sORFs. In this perspective, we will use the term "microprotein" to distinguish these proteins from proteolytic cleavage products of larger proteins.

While relatively few studies have performed rigorous functional characterization of microproteins, these small proteins have immense potential in the brain. Small secreted peptides such as Brain-Derived Neurotrophic Factor (BDNF), Nerve Growth Factor (NGF) and Neuropeptide Y (NPY) have well-established roles in neural plasticity, learning, and memory (Chao, 2003). While these neuropeptides are cleavage products from larger proteins, the *de novo* translation of sORFs may similarly serve critical cell signaling functions in the brain. Moreover, microproteins with specific functions in other tissues and cell lines, such as mitochondrial respiration, stress granule formation and DNA repair, may possess unique roles within the brain during health and disease. This perspective will highlight methods for microprotein discovery and functional characterization in the mammalian nervous system.

Microprotein discovery in mammals

Microproteins have been historically under-studied in protein research, primarily due to the technical limitations of traditional bioinformatic and mass spectrometry analyses (Figure 1A). In bioinformatics, efforts to annotate the genome based on predicted protein-coding potential, such as those pioneered by the FANTOM consortium, introduced a cutoff of 100 AA to protein prediction to reduce the risk of false discovery of sORFs within predicted long non-coding RNAs (lncRNAs) (Okazaki et al., 2002; Dinger et al., 2008). Consequently, many potentially translated and/or functional microproteins that fell below this threshold were overlooked in the final genome annotation. Similarly, traditional mass spectrometry-based approaches have posed significant obstacles to microprotein detection due to multiple factors such as purification column size cutoffs, low microprotein abundance relative to annotated proteins, limited trypsin cleavage sites, and similarity to existing protein domains based on AA sequence (Saghatelian and Couso, 2015).

The development and widespread utilization of high throughput RNA sequencing methods to study mRNA translation subsequently enabled the discovery and cataloging of sORFs and their encoded microproteins. In particular, ribosome profiling (Ribo-seq, also known as ribosome footprinting) enabled the sequencing of ribosome-protected RNA fragments and the subsequent identification of actively translated open reading frames (Ingolia et al., 2009). This approach circumvented many technical challenges associated with proteomic discovery of microproteins and revealed >1,000 non-canonical translation events in the 5′ untranslated regions (5′UTRs) of genes in budding yeast. With the advent of Ribo-seq technologies came an explosion of studies that revealed widespread non-canonical translation across numerous eukaryotic species including zebrafish (Bazzini et al., 2014) and mouse (Harnett et al., 2022; Martinez et al., 2023), as well as human tissues including heart (van Heesch et al., 2019), kidney (Loayza-Puch et al., 2016), skeletal muscle (Wein et al., 2014), cortex (Duffy et al., 2022), and thalamus (Chothani et al., 2022). These studies also inspired targeted searches for microprotein expression using bioinformatic and mass spectrometry approaches. For example, Mackowiak et al. (2015) bioinformatically identified thousands of sORFs based on their high conservation between human, mouse, drosophila and *C. elegans*. Furthermore, modified mass spectrometry approaches that enrich small proteins and use custom protein databases generated from RNA-seq have accelerated microprotein identification (Saghatelian and Couso, 2015).

Collectively, these studies have shown that much of the transcriptome that was previously annotated as "non-coding" can encode small proteins (Figure 1B). Microproteins have been identified in 5′UTRs, where they are termed upstream open reading frames (uORFs). Classically, uORFs are thought to negatively regulate the downstream translation of canonical ORFs. For example, two uORFs in the 5′UTR of the stress response gene *Atf4* repress downstream ATF4 protein expression, and this repression is relieved by the integrated stress response (Harding et al., 2000). However, more recent high-throughput methods have shown that translational repression of downstream ORFs is uncommon for uORFs (Ingolia et al., 2009; van Heesch et al., 2019; Duffy et al., 2022), and some uORFs may exert cis- or trans-effects (Chen et al., 2020; Barragan-Iglesias et al., 2021) that depend on the sequence of the encoded microprotein rather than the act of their translation. Although downstream ORFs (dORFs) encoded by polycistronic sequences in 3′UTRs represent a relatively small proportion of all sORFs (e.g., 3.4% of sORFs in Duffy et al., 2022), these sORFs can also encode microproteins. While the mechanisms for dORF translation remain unclear, the presence of a dORF in translation reporter assays can enhance the translation of the upstream reporter ORF, suggesting a mechanistic coupling between the translation of both ORFs (Wu et al., 2020). Microproteins can also be encoded from out-of-frame sORFs with larger annotated ORFs. For example, altFUS is a highly conserved internal out-of-frame ORF translated in brain tissue, where altFUS, but not FUS, is responsible for the inhibition of autophagy in neurons (Brunet et al., 2021). Finally, many RNAs that are annotated as non-coding indeed encode functional microproteins. For example, the TUNAR lncRNA [also known as *Megamind* in zebrafish (Ulitsky et al., 2011)] encodes an evolutionarily conserved 48 AA transmembrane protein that modulates intracellular calcium dynamics through its interaction with the calcium transporter SERCA2 in the nervous system (Senís et al., 2021). These studies have revealed the translation of thousands of sORFs from annotated non-coding RNAs, thereby expanding the diversity of the known proteome.

General properties of microproteins

Microproteins share distinct properties compared to longer annotated proteins. They are enriched for translation from non-AUG start codons (Ingolia et al., 2009; van Heesch et al., 2019; Duffy et al., 2022), and are more recently evolved on average compared to known proteins (Ruiz-Orera et al., 2014; Duffy et al., 2022; Vakirlis et al., 2022), making them challenging to detect based on sequence conservation or start codon usage alone. They also tend to exhibit lower protein expression compared to longer annotated proteins, making them more challenging to detect by mass spectrometry as discussed above. As a result, a relatively small fraction of

10.3389/fnmol.2024.1386219

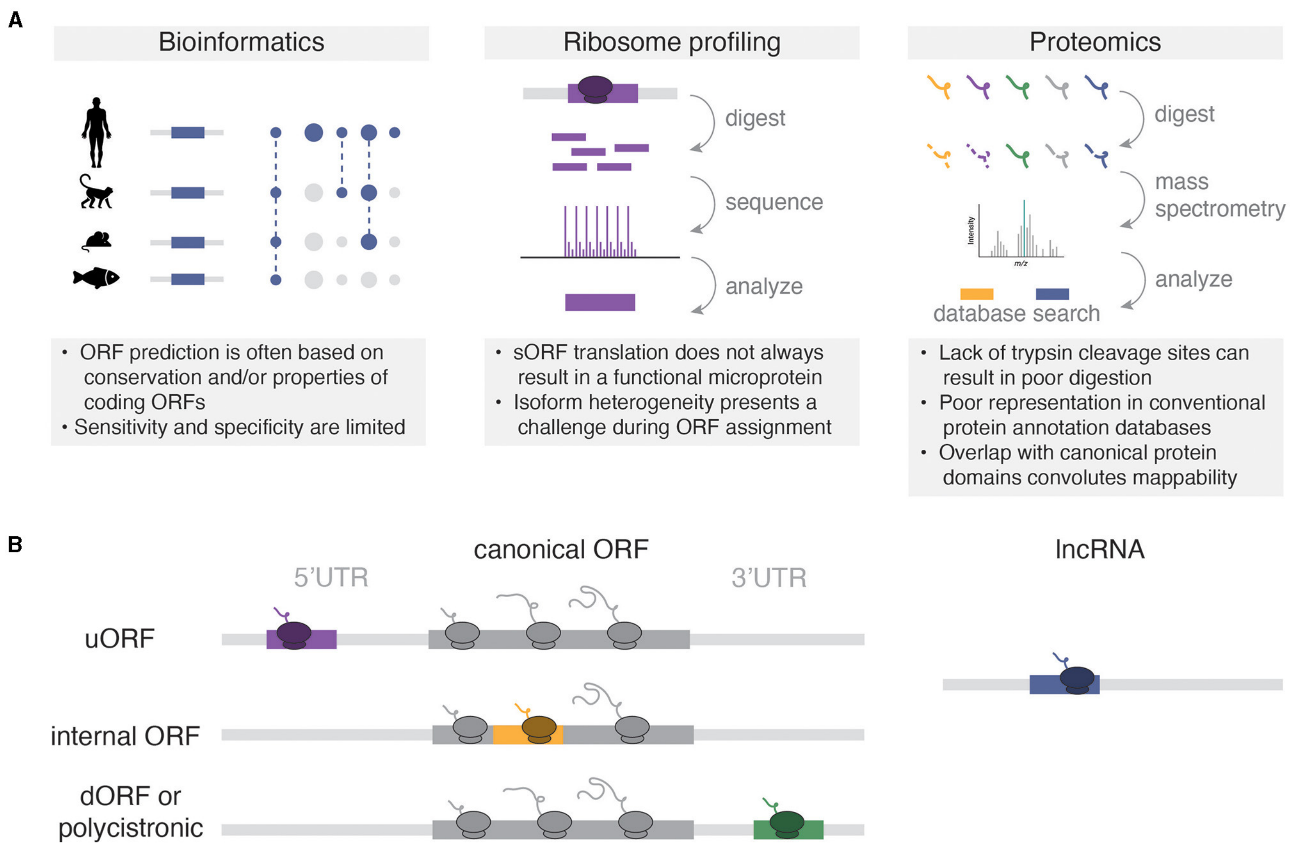


FIGURE 1
(A) Methods for microprotein discovery and general caveats of each approach. **(B)** Types of microRNAs relative to canonical coding sequences. Not pictured are variations of overlapping sORFs (e.g., uORFs that overlap the start codon of the canonical ORF), or rarer sORFs from non-coding RNAs like circular RNAs, pseudogenes, and microRNA precursors.

microproteins observed as translated by Ribo-seq has subsequently been detected by mass spectrometry, sparking a debate over whether newly evolved, lowly translated or unstable microproteins have the capacity for function. These characteristics align with the classic view that evolutionarily conserved or highly abundant sORFs are more likely to carry out important functions in the cell; however, newly evolved microproteins may represent evolutionary experiments, in which a given sORF becomes translated without necessarily being conserved in subsequent evolution. While newly evolved microproteins may not have yet acquired function, it is possible for them to introduce species-specific functions to the proteome, indeed, >100 human-specific microproteins detected as translated in the human brain (Duffy et al., 2022) exhibit a significant growth phenotype when knocked out in human cell lines (Chen et al., 2020). Furthermore, several groups have found examples of newly evolved proteins that acquire function in a given species, highlighting the importance of studying these evolutionarily young proteins in addition to those that are conserved (Ruiz-Orera and Albà, 2019). In the context of neurobiology, evolutionarily new microproteins have the potential to explain some of the unique properties of the human brain relative to other species. While these hypotheses remain to be tested for human brain microproteins, they motivate the study of poorly conserved microproteins in addition to those that are highly conserved.

As protein structure is often tied to function, microproteins that adopt stable structures may also be prioritized for functional characterization. For example, microproteins that mimic the domains of larger proteins, such as Id (Benezra et al., 1990) and LITTLE ZIPPER (Wenkel et al., 2007) can act as competitive inhibitors of larger protein complexes. However, while some microproteins can adopt simple structures such as alpha helices and transmembrane domains, as a class of proteins they are enriched for intrinsically disordered regions relative to the known proteome (Duffy et al., 2022). These unique properties can confer interesting potential functions to microproteins compared to previously annotated proteins. Intrinsically disordered microproteins may be able to interact with other biomolecules either in a promiscuous or substrate-specific manner that is similar to that of intrinsically disordered regions of larger proteins, potentially allowing them to drive or disrupt macromolecular structures such as biomolecular condensates (Chakrabarti and Chakravarty, 2022). These properties make microproteins both potentially interesting and challenging to functionally characterize.

Microprotein functional characterization

It is important to note that the studies of microproteins in mammals are built upon excellent foundational work in non-mammalian systems (Saghatelian and Couso, 2015; Hemm et al., 2020; Kushwaha et al., 2022), and the work in non-mammalian species can inform future experiments on microproteins in the brain. While only a handful of microproteins have been functionally characterized in the nervous system to date, many microproteins in other tissues have important functions that may also be relevant in the brain. For the purposes of this perspective, we will discuss microproteins that have been functionally characterized in other tissues and reported to be expressed in the mammalian brain based on existing ribosome profiling and proteomic data (Figure 2, Wang et al., 2021; Chothani et al., 2022; Duffy et al., 2022).

Many functionally characterized microproteins have been shown to be important in mitochondrial energy homeostasis (Stein et al., 2018; Chu et al., 2019; Zhang et al., 2020; Brunet et al., 2021; Liang et al., 2022), which is critical in neurons to produce the ATP required for various neuronal processes including neurotransmitter synthesis and metabolism, maintaining ion gradients, neutralizing oxidative stress, and supporting signaling pathways. The well-characterized microprotein Humanin (HN, Hashimoto et al., 2001) can exhibit neuroprotective effects in part by binding to the cytosolic proteins Bcl2-associated X protein (BAX) and Bid to inhibit their translocation to the mitochondrial membrane. This in turn impedes Bax pore formation in the mitochondrial outer membrane and suppresses mitochondrial-dependent apoptosis (Zhu et al., 2022). In addition, several microproteins with mitochondrial function have been assayed in the mammalian brain. MP31 which is encoded by the uORF of the *PTEN* transcript, limits mitochondrial lactate-pyruvate conversion by competing with mitochondrial lactate dehydrogenase for nicotinamide adenine dinucleotide (NAD+, Huang et al., 2021). The lncRNA-encoded microprotein STMP1 is expressed in microglia and is thought to regulate mitochondrial function and protect retinal ganglion cells from oxidative damage by inhibiting the Nlrp3 inflammasome pathway (Zheng et al., 2023).

Microproteins have also been shown to play important roles in the nucleus in the context of transcription and DNA repair. The function of DNA damage repair in neurons is to preserve genomic stability and maintain the functional and structural integrity of the neuronal circuit. As neurons are post-mitotic, they rely on non-homologous end joining (NHEJ) rather than homologous repair, which requires mitotic DNA replication. While microproteins involved in nuclear function have not been characterized in neurons to date, the DDUP microprotein encoded by the DNA damage-induced lncRNA CTBP1-DT protects cells from DNA damage, likely through binding to the DNA repair factor RAD18 (Ren et al., 2023). Furthermore, the microprotein CYREN (also known as MRI-2) binds to Ku to regulate NHEJ and double-stranded break repair (Slavoff et al., 2014; Arnoult et al., 2017). Other microproteins function as subunits of RNA polymerase II (POLR2L, Woychik and Young, 1990) and regulate the binding of transcription factors to chromatin. One such protein is the microprotein EMBOW, which facilitates WDR5 protein complex assembly and regulates the DNA binding specificity of the complex (Chen et al., 2023). As WDR5 also regulates neurodevelopment and dendritic polarity (Ka et al., 2022), it is plausible that microproteins such as EMBOW participate in the regulation of transcription during nervous system development.

Several microproteins are themselves transmembrane proteins or interact with proteins on cellular membranes and facilitate cell signaling. For example, the microprotein phospholemman (PLM) is a single-pass transmembrane protein that regulates the activity of the Na,K-ATPase (NK) complex to maintain Na+ and K+ gradients across cell membranes (Crambert et al., 2002). The microprotein CGRP, which is expressed from a uORF of the calcitonin (*Calca*) gene, promotes pain sensitization in mouse dorsal root ganglia through GPCR signaling (Barragan-Iglesias et al., 2021). Several SERCA-inhibiting microproteins regulate calcium signaling in the heart (Anderson et al., 2016), and one of these microproteins, SLN, is also translated in the human brain (Duffy et al., 2022), suggesting a potentially interesting role in neuronal calcium signaling. The microprotein MAVI1, encoded by the gene *Smim30*, is a transmembrane protein localized to the endoplasmic reticulum where it interacts with the mitochondrial protein MAVS to block innate immune responses (Shi et al., 2023). The expression of MAVI1 in the human brain suggests potential additional functions of MAVI1 beyond antiviral innate immune responses.

Finally, there are limited but interesting examples of microproteins that regulate RNA metabolism and translational control. The 25 AA ribosomal subunit RPL41 is a highly conserved microprotein from yeast to mammals (Klaudiny et al., 1992). RPL41 expression has recently been suggested to be a useful biomarker for Alzheimer's disease (Cruz-Rivera et al., 2018). The microprotein NoBody (NBDY) regulates mRNA decapping and stability through its interaction with processing bodies, cytoplasmic ribonucleoprotein (RNP) granules that are made up of translationally repressed mRNAs and proteins related to mRNA decay (D'Lima et al., 2017), where P-bodies are hypothesized to regulate local RNA translation at synapses (Zeitelhofer et al., 2008). Investigating the role that microproteins play in RNA translation and metabolism in neurons represents a fascinating future direction in microprotein research.

Discussion

Challenges of studying microproteins

The precise spatiotemporal expression of proteins is fundamental to synapse plasticity and circuit remodeling. Much of the work to date on the role of translation in the nervous system has focused on the canonical proteome, but advances in proteomics and genomics in the last decade have revealed an expansive landscape of ncORFs, including sORFs that encode microproteins. Moving forward, the noncanonical proteome is a potentially rich source of underexplored neurobiology, but several challenges have limited mechanistic studies. Herein, we define critical scientific priorities, technical challenges, and potential

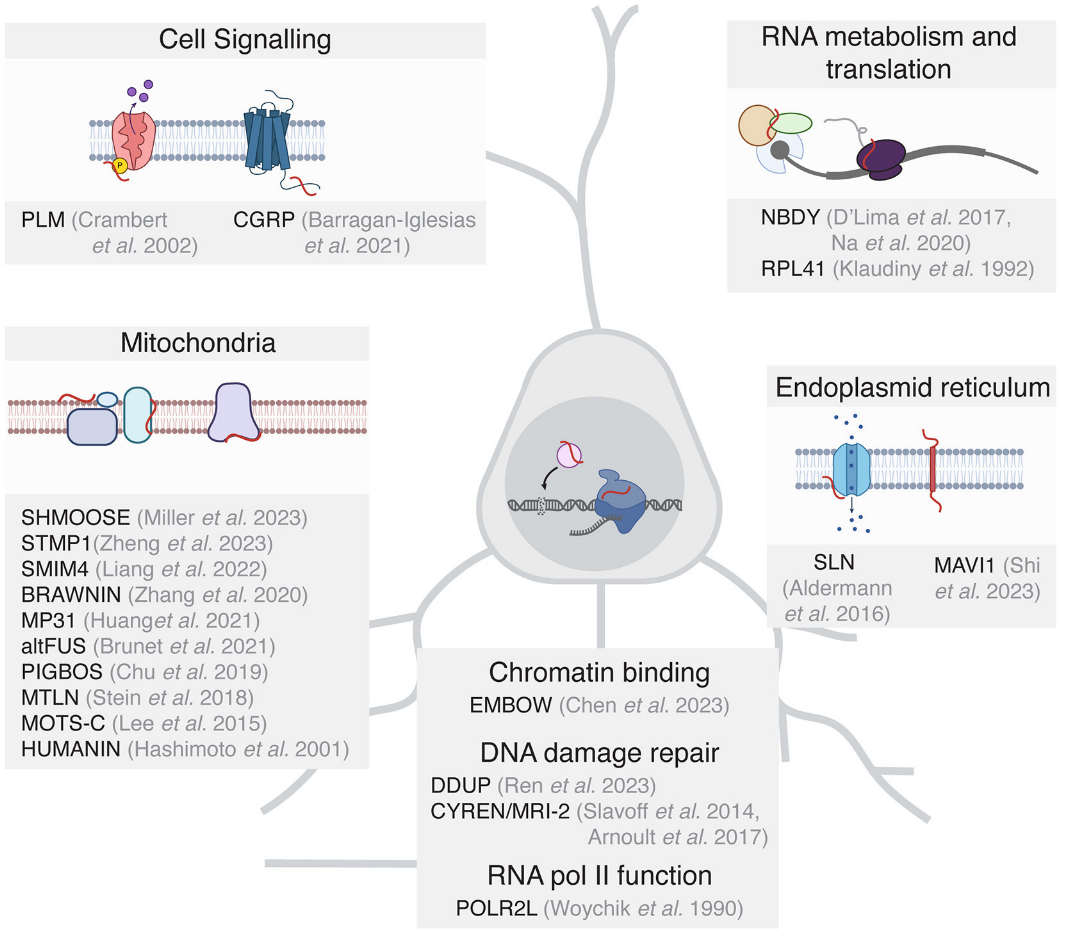


FIGURE 2
Functionally characterized microproteins grouped by functional potential in the mammalian brain. For clarity, only microproteins are included where the sORF is detected as translated in the mammalian brain.

opportunities for investigation that lie at the intersection of microprotein biology and neuroscience.

The foremost challenge is identifying a high-confidence set of brain microproteins, which can then be exploited for functional interrogation. There is currently a lack of standardization in the experimental methods, data quality control, and analysis of sORFs and microproteins, which has led to significant variability in the identification of translated sORFs. Given the need to adopt rigorous, uniform standards for microprotein validation, several groups have proposed consensus definitions to improve the reliability and consistency of ncORF and protein coding identification (Mudge et al., 2022; Chothani et al., 2023; Prensner et al., 2023). These definitions include the independent identification of a sORF across multiple studies, detection by multiple experimental methods (e.g., Ribo-seq plus mass spectrometry, epitope tagging and western blot, or detection by endogenous antibodies), and/or the presence within the microprotein of disease-associated mutations (Table 1).

Another challenge for microprotein neurobiology is the difficulty in prioritizing candidate sORFs for functional investigation. Approaches to filter and prioritize sORFs, based on their physicochemical properties, sequence conservation, predicted structure (using AlphaFold) and subcellular localization are likely to accelerate biological insight. However, these approaches have significant limitations when applied to microproteins.

TABLE 1 Suggested criteria for prioritizing sORFs for functional characterization.

Criteria	Comments
Detection by more than one experimental method (e.g., Ribo-seq plus mass spectrometry, epitope tagging and western blot, or detection by endogenous antibodies)	Proteins that are expressed at high enough levels to be detected by mass spectrometry or western blot are more likely to execute important functions
Evolutionary conservation	While not required for function, selective pressure increases the probability of function
Homology with protein of known function	Microproteins that mimic known proteins can act as positive or negative regulators of cellular functions
Presence of disease-associated mutations	This includes microproteins whose expression is misregulated in disease states

Importantly, not all criteria must be simultaneously satisfied.

AlphaFold, for instance, has not been trained on microproteins and thus may provide misleading predictions for putative microproteins and their potential protein-protein interactions (Jumper et al., 2021). Empirical data will be necessary to train more comprehensive machine-learning models for noncanonical proteins. Another potential avenue to elucidate functionally relevant microproteins in the brain is to identify candidates that are associated with neurologic disease vulnerability. Specifically, sORFs with enrichment of disease-associated genomic variants may be more likely to have biologically relevant functions. For example, single nucleotide polymorphisms (SNPs) in patients with Alzheimer's disease have been identified in the mitochondrial microproteins HN and SCHMOOSE (Niikura, 2022; Miller et al., 2023). However, such analyses are complicated by the proximity or overlap of sORFs with canonical ORFs and therefore require the development of new computational tools to incorporate non-canonical ORFs into genome annotations and variant calling algorithms. Alternatively, microproteins that show differential expression in different neurodevelopmental or disease states offer interesting candidates for functional characterization. For example, thousands of microproteins detected in the human brain show differential RNA expression and translatability in the fetal vs. adult brain (Duffy et al., 2022).

To circumvent the laborious process of functionally characterizing individual microproteins, several groups have pioneered high-throughput, unbiased testing of microprotein function. For example, Chen et al. (2020) used CRISPR-Cas9 strategies to investigate the function of thousands of microproteins in mammalian cells by mutating the start codon of individual sORFs and identified hundreds of microproteins that are important for cell growth and fitness. Hofman et al. (2024) used a similar approach to identify microproteins translated from uORFs and lncRNAs that are required for medullablastoma cell survival. Conversely, a recently described translation-activating RNA technology may be a useful technique to promote the targeted upregulation of specific sORFs (Cao et al., 2023). While these approaches facilitate the nomination of biologically important microproteins from the thousands of potential sORF candidates, they have, to date, been limited to biological assays of cell growth and survival. Future screens will need to employ more neurobiologically relevant assays, including neural differentiation, electrophysiology, bioenergetics, and synapse complexity and composition.

Beyond the need to confidently identify, prioritize, and predict functionality of brain ncORFs and microproteins, the field will require new computational and experimental tools to interrogate microprotein function at single-cell resolution in the brain. Microprotein expression in the brain may be cell type-specific, developmentally regulated, or expressed in response to specific stimuli or disease states, all of which will be challenging to study using current methods and may require a combination of *in vitro* models and an examination of primary tissue. Recently described approaches for single-cell ribosome profiling (Ozadam et al., 2023) and *in situ* spatial translatome mapping (Zeng et al., 2023) raise the promise of studying translation more precisely in heterogeneous tissues such as the brain. For example, microglia may employ a unique repertoire of microproteins, as immune cells often leverage microproteins in the context of antigen recognition and presentation (Malekos and Carpenter, 2022). Therefore, ribosome profiling of specific glial populations, combined with proteomics approaches to identify small immunopeptides presented on the cell surface, are likely to uncover unique microproteins that contribute to the neuro-immune landscape.

Future directions and conclusions

Moving forward, the brain poses unique challenges to microprotein research that will require the development and consensus of rigorous experimental and computational approaches to define and characterize microproteins across development and disease. Despite these challenges, microproteins remain an exciting avenue for future research aimed at understanding the importance of non-canonical translation for cognitive development and brain function.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

ED: Writing—original draft, Writing—review & editing. EA: Writing—review & editing. BK: Writing—original draft, Writing—review & editing. MG: Writing—review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This research was supported by the Allen Discovery Center program, a Paul G. Allen Frontiers Group advised program of the Paul G. Allen Family Foundation. ED was a Damon Runyon-National Mah Jongg League, Inc. Breakthrough Scientist supported by the Damon Runyon Cancer Research Foundation and was supported by the Warren Alpert Distinguished Scholar Award. MG was supported by funding from NINDS R01 NS115965. This study received funding from the National Mah Jongg League, Inc. through the Damon Runyon Cancer Research Foundation. The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article, or the decision to submit it for publication.

Acknowledgments

We thank members of the Greenberg laboratory for helpful discussions on the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

Anderson, D. M., Makarewich, C. A., Anderson, K. M., Shelton, J. M., Bezprozvannaya, S., Bassel-Duby, R., et al. (2016). Widespread control of calcium signaling by a family of SERCA-inhibiting micropeptides. *Sci. Signal* 9:ra119. doi: 10.1126/scisignal.aaj1460

Arnoult, N., Correia, A., Ma, J., Merlo, A., Garcia-Gomez, S., Maric, M., et al. (2017). Regulation of DNA repair pathway choice in S and G2 phases by the NHEJ inhibitor CYREN. *Nature* 549, 548–552. doi: 10.1038/nature24023

Barragan-Iglesias, P., Kunder, N., Wanghzou, A., Black, B., Ray, P. R., Lou, T.-F., et al. (2021). A peptide encoded within a 5′ untranslated region promotes pain sensitization in mice. *Pain* 162, 1864–1875. doi: 10.1097/j.pain.0000000000002191

Bazzini, A. A., Johnstone, T. G., Christiano, R., Mackowiak, S. D., Obermayer, B., Fleming, E. S., et al. (2014). Identification of small ORFs in vertebrates using ribosome footprinting and evolutionary conservation. *EMBO J.* 33, 981–993. doi: 10.1002/embj.201488411

Benezra, R., Davis, R. L., Lockshon, D., Turner, D. L., and Weintraub, H. (1990). The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. *Cell* 61, 49–59. doi: 10.1016/0092-8674(90)90214-y

Brunet, M. A., Jacques, J.-F., Nassari, S., Tyzack, G. E., McGoldrick, P., Zinman, L., et al. (2021). The FUS gene is dual-coding with both proteins contributing to FUS-mediated toxicity. *EMBO Rep.* 22:e50640. doi: 10.15252/embr.202050640

Cao, Y., Liu, H., Lu, S. S., Jones, K. A., Govind, A. P., Jeyifous, O., et al. (2023). RNA-based translation activators for targeted gene upregulation. *Nat. Commun.* 14:6827. doi: 10.1038/s41467-023-42252-z

Chakrabarti, P., and Chakravarty, D. (2022). Intrinsically disordered proteins/regions and insight into their biomolecular interactions. *Biophys. Chem.* 283:106769. doi: 10.1016/j.bpc.2022.106769

Chao, M. V. (2003). Neurotrophins and their receptors: a convergence point for many signalling pathways. *Nat. Rev. Neurosci.* 4, 299–309. doi: 10.1038/nrn1078

Chen, J., Brunner, A.-D., Cogan, J. Z., Nuñez, J. K., Fields, A. P., Adamson, B., et al. (2020). Pervasive functional translation of noncanonical human open reading frames. *Science* 367, 1140–1146. doi: 10.1126/science.aay0262

Chen, Y., Su, H., Zhao, J., Na, Z., Jiang, K., Bacchiocchi, A., et al. (2023). Unannotated microprotein EMBOW regulates the interactome and chromatin and mitotic functions of WDR5. *Cell Rep.* 42:113145. doi: 10.1016/j.celrep.2023.113145

Chothani, S., Ho, L., Schafer, S., and Rackham, O. (2023). Discovering microproteins: making the most of ribosome profiling data. *RNA Biol.* 20, 943–954. doi: 10.1080/15476286.2023.2279845

Chothani, S. P., Adami, E., Widjaja, A. A., Langley, S. R., Viswanathan, S., Pua, C. J., et al. (2022). A high-resolution map of human RNA translation. *Mol. Cell* 82, 2885–2899.e8. doi: 10.1016/j.molcel.2022.06.023

Chu, Q., Martinez, T. F., Novak, S. W., Donaldson, C. J., Tan, D., Vaughan, J. M., et al. (2019). Regulation of the ER stress response by a mitochondrial microprotein. *Nat. Commun.* 10:4883. doi: 10.1038/s41467-019-12816-z

Crambert, G., Fuzesi, M., Garty, H., Karlish, S., and Geering, K. (2002). Phospholemman (FXYD1) associates with Na,K-ATPase and regulates its transport properties. *Proc. Natl. Acad. Sci. USA.* 99, 11476–11481. doi: 10.1073/pnas.182267299

Cruz-Rivera, Y. E., Perez-Morales, J., Santiago, Y. M., Gonzalez, V. M., Morales, L., Cabrera-Rios, M., et al. (2018). A selection of important genes and their correlated behavior in Alzheimer's disease. *J. Alzheimers. Dis.* 65, 193–205. doi: 10.3233/JAD-170799

Dinger, M. E., Pang, K. C., Mercer, T. R., and Mattick, J. S. (2008). Differentiating protein-coding and noncoding RNA: challenges and ambiguities. *PLoS Comput. Biol.* 4:e1000176. doi: 10.1371/journal.pcbi.1000176

D'Lima, N. G., Ma, J., Winkler, L., Chu, Q., Loh, K. H., Corpuz, E. O., et al. (2017). A human microprotein that interacts with the mRNA decapping complex. *Nat. Chem. Biol.* 13, 174–180. doi: 10.1038/nchembio.2249

Duffy, E. E., Finander, B., Choi, G., Carter, A. C., Pritisanac, I., Alam, A., et al. (2022). Developmental dynamics of RNA translation in the human brain. *Nat. Neurosci.* 25, 1353–1365. doi: 10.1038/s41593-022-01164-9

Harding, H. P., Novoa, I., Zhang, Y., Zeng, H., Wek, R., Schapira, M., et al. (2000). Regulated translation initiation controls stress-induced gene expression in mammalian cells. *Mol. Cell* 6, 1099–1108. doi: 10.1016/s1097-2765(00)00108-8

Harnett, D., Ambrozkiewicz, M. C., Zinnall, U., Rusanova, A., Borisova, E., Drescher, A. N., et al. (2022). A critical period of translational control during brain development at codon resolution. *Nat. Struct. Mol. Biol.* 29, 1277–1290. doi: 10.1038/s41594-022-00882-9

Hashimoto, Y., Ito, Y., Niikura, T., Shao, Z., Hata, M., Oyama, F., et al. (2001). Mechanisms of neuroprotection by a novel rescue factor humanin from Swedish mutant amyloid precursor protein. *Biochem. Biophys. Res. Commun.* 283, 460–468. doi: 10.1006/bbrc.2001.4765

Hemm, M. R., Weaver, J., and Storz, G. (2020). Escherichia coli small proteome. *EcoSal Plus* 2019:9. doi: 10.1128/ecosalplus.ESP-0031-2019

Hofman, D. A., Ruiz-Orera, J., Yannuzzi, I., Murugesan, R., Brown, A., Clauser, K. R., et al. (2024). Translation of non-canonical open reading frames as a cancer cell survival mechanism in childhood medulloblastoma. *Mol Cell* 84, 261–276.e18. doi: 10.1016/j.molcel.2023.12.003

Huang, N., Li, F., Zhang, M., Zhou, H., Chen, Z., Ma, X., et al. (2021). An upstream open reading frame in phosphatase and tensin homolog encodes a circuit breaker of lactate metabolism. *Cell Metab* 33, 128–144.e9. doi: 10.1016/j.cmet.2020.12.008

Ingolia, N. T., Ghaemmaghami, S., Newman, J. R. S., and Weissman, J. S. (2009). Genome-wide analysis *in vivo* of translation with nucleotide resolution using ribosome profiling. *Science.* 324, 218–223. doi: 10.1126/science.1168978

Jumper, J., Evans, R., Pritzel, A., Green, T., Figurnov, M., Ronneberger, O., et al. (2021). Highly accurate protein structure prediction with AlphaFold. *Nature* 596, 583–589. doi: 10.1038/s41586-021-03819-2

Ka, M., Kim, H.-G., and Kim, W.-Y. (2022). WDR5-HOTTIP histone modifying complex regulates neural migration and dendrite polarity of pyramidal neurons via reelin signaling. *Mol. Neurobiol.* 59, 5104–5120. doi: 10.1007/s12035-022-02905-4

Klaudiny, J., von der Kammer, H., and Scheit, K. H. (1992). Characterization by cDNA cloning of the mRNA of a highly basic human protein homologous to the yeast ribosomal protein YL41. *Biochem. Biophys. Res. Commun.* 187, 901–906. doi: 10.1016/0006-291x(92)91282-u

Kushwaha, A. K., Dwivedi, S., Mukherjee, A., Lingwan, M., Dar, M. A., Bhagavatula, L., et al. (2022). Plant microProteins: small but powerful modulators of plant development. *iScience* 25, 105400. doi: 10.1016/j.isci.2022.105400

Liang, C., Zhang, S., Robinson, D., Ploeg, M. V., Wilson, R., Nah, J., et al. (2022). Mitochondrial microproteins link metabolic cues to respiratory chain biogenesis. *Cell Rep.* 40, 111204. doi: 10.1016/j.celrep.2022.111204

Loayza-Puch, F., Rooijers, K., Buil, L. C. M., Zijlstra, J., Oude Vrielink, J. F., Lopes, R., et al. (2016). Tumour-specific proline vulnerability uncovered by differential ribosome codon reading. *Nature* 530, 490–494. doi: 10.1038/nature16982

Mackowiak, S. D., Zauber, H., Bielow, C., Thiel, D., Kutz, K., Calviello, L., et al. (2015). Extensive identification and analysis of conserved small ORFs in animals. *Genome Biol.* 16, 179. doi: 10.1186/s13059-015-0742-x

Malekos, E., and Carpenter, S. (2022). Short open reading frame genes in innate immunity: from discovery to characterization. *Trends Immunol.* 43, 741–756. doi: 10.1016/j.it.2022.07.005

Martinez, T. F., Lyons-Abbott, S., Bookout, A. L., De Souza, E. V., Donaldson, C., Vaughan, J. M., et al. (2023). Profiling mouse brown and white adipocytes to identify metabolically relevant small ORFs and functional microproteins. *Cell Metab.* 35, 166–183.e11. doi: 10.1016/j.cmet.2022.12.004

Miller, B., Kim, S.-J., Mehta, H. H., Cao, K., Kumagai, H., Thumaty, N., et al. (2023). Mitochondrial DNA variation in Alzheimer's disease reveals a unique microprotein called SHMOOSE. *Mol. Psychiatry* 28, 1813–1826. doi: 10.1038/s41380-022-01769-3

Mudge, J. M., Ruiz-Orera, J., Prensner, J. R., Brunet, M. A., Calvet, F., Jungreis, I., et al. (2022). Standardized annotation of translated open reading frames. *Nat. Biotechnol.* 40, 994–999. doi: 10.1038/s41587-022-01369-0

Niikura, T. (2022). Humanin and Alzheimer's disease: the beginning of a new field. *Biochim. Biophys. Acta Gen Subj.* 1866:130024. doi: 10.1016/j.bbagen.2021.130024

Okazaki, Y., Furuno, M., Kasukawa, T., Adachi, J., Bono, H., Kondo, S., et al. (2002). Analysis of the mouse transcriptome based on functional annotation of 60,770 full-length cDNAs. *Nature* 420, 563–573. doi: 10.1038/nature01266

Ozadam, H., Tonn, T., Han, C. M., Segura, A., Hoskins, I., Rao, S., et al. (2023). Single-cell quantification of ribosome occupancy in early mouse development. *Nature* 618, 1057–1064. doi: 10.1038/s41586-023-06228-9

Prensner, J. R., Abelin, J. G., Kok, L. W., Clauser, K. R., Mudge, J. M., Ruiz-Orera, J., et al. (2023). What can Ribo-seq, immunopeptidomics, and proteomics tell us about the noncanonical proteome? *Mol. Cell. Proteomics* 22, 100631. doi: 10.1016/j.mcpro.2023.100631

Ren, L., Qing, X., Wei, J., Mo, H., Liu, Y., Zhi, Y., et al. (2023). The DDUP protein encoded by the DNA damage-induced CTBP1-DT lncRNA confers cisplatin resistance in ovarian cancer. *Cell Death Dis.* 14, 568. doi: 10.1038/s41419-023-06084-5

Ruiz-Orera, J., and Albà, M. M. (2019). Translation of small open reading frames: roles in regulation and evolutionary innovation. *Trends Genet.* 35, 186–198. doi: 10.1016/j.tig.2018.12.003

Ruiz-Orera, J., Messeguer, X., Subirana, J. A., and Alba, M. M. (2014). Long non-coding RNAs as a source of new peptides. *Elife* 3:e03523. doi: 10.7554/eLife.03523

Saghatelian, A., and Couso, J. P. (2015). Discovery and characterization of smORF-encoded bioactive polypeptides. *Nat. Chem. Biol.* 11, 909–916. doi: 10.1038/nchembio.1964

Senís, E., Esgleas, M., Najas, S., Jiménez-Sábado, V., Bertani, C., Giménez-Alejandre, M., et al. (2021). TUNAR lncRNA encodes a microprotein that regulates neural differentiation and neurite formation by modulating calcium dynamics. *Front. Cell Dev. Biol.* 9, 747667. doi: 10.3389/fcell.2021.747667

Shi, T.-T., Huang, Y., Li, Y., Dai, X.-L., He, Y.-H., Ding, J.-C., et al. (2023). MAVI1, an endoplasmic reticulum-localized microprotein, suppresses antiviral innate immune response by targeting MAVS on mitochondrion. *Sci. Adv.* 9:eadg7053. doi: 10.1126/sciadv.adg7053

Slavoff, S. A., Heo, J., Budnik, B. A., Hanakahi, L. A., and Saghatelian, A. (2014). A human short open reading frame (sORF)-encoded polypeptide that stimulates DNA end joining. *J. Biol. Chem.* 289, 10950–10957. doi: 10.1074/jbc.C113.533968

Stein, C. S., Jadiya, P., Zhang, X., McLendon, J. M., Abouassaly, G. M., Witmer, N. H., et al. (2018). Mitoregulin: a lncRNA-encoded microprotein that supports mitochondrial supercomplexes and respiratory efficiency. *Cell Rep.* 23, 3710–3720.e8. doi: 10.1016/j.celrep.2018.06.002

Ulitsky, I., Shkumatava, A., Jan, C. H., Sive, H., and Bartel, D. P. (2011). Conserved function of lincRNAs in vertebrate embryonic development despite rapid sequence evolution. *Cell* 147, 1537–1550. doi: 10.1016/j.cell.2011.11.055

Vakirlis, N., Vance, Z., Duggan, K. M., and McLysaght, A. (2022). De novo birth of functional microproteins in the human lineage. *Cell Rep.* 41:111808. doi: 10.1016/j.celrep.2022.111808

van Heesch, S., Witte, F., Schneider-Lunitz, V., Schulz, J. F., Adami, E., Faber, A. B., et al. (2019). The translational landscape of the human heart. *Cell* 178, 242–260.e29. doi: 10.1016/j.cell.2019.05.010

Wang, H., Wang, Y., Yang, J., Zhao, Q., Tang, N., Chen, C., et al. (2021). Tissue- and stage-specific landscape of the mouse translatome. *Nucleic Acids Res.* 49, 6165–6180. doi: 10.1093/nar/gkab482

Wein, N., Vulin, A., Falzarano, M. S., Szigyarto, C. A.-K., Maiti, B., Findlay, A., et al. (2014). Translation from a DMD exon 5 IRES results in a functional dystrophin isoform that attenuates dystrophinopathy in humans and mice. *Nat. Med.* 20, 992–1000. doi: 10.1038/nm.3628

Wenkel, S., Emery, J., Hou, B.-H., Evans, M. M. S., and Barton, M. K. (2007). A feedback regulatory module formed by LITTLE ZIPPER and HD-ZIPIII genes. *Plant Cell* 19, 3379–3390. doi: 10.1105/tpc.107.055772

Woychik, N. A., and Young, R. A. (1990). RNA polymerase II subunit RPB10 is essential for yeast cell viability. *J. Biol. Chem.* 265, 17816–17819.

Wu, Q., Wright, M., Gogol, M. M., Bradford, W. D., Zhang, N., and Bazzini, A. A. (2020). Translation of small downstream ORFs enhances translation of canonical main open reading frames. *EMBO J.* 39:e104763. doi: 10.15252/embj.2020104763

Zeitelhofer, M., Macchi, P., and Dahm, R. (2008). Perplexing bodies: the putative roles of P-bodies in neurons. *RNA Biol.* 5, 244–248. doi: 10.4161/rna.6948

Zeng, H., Huang, J., Ren, J., Wang, C. K., Tang, Z., Zhou, H., et al. (2023). Spatially resolved single-cell translatomics at molecular resolution. *Science* 380:eadd3067. doi: 10.1126/science.add3067

Zhang, S., Reljić, B., Liang, C., Kerouanton, B., Francisco, J. C., Peh, J. H., et al. (2020). Mitochondrial peptide BRAWNIN is essential for vertebrate respiratory complex III assembly. *Nat. Commun.* 11:1312. doi: 10.1038/s41467-020-14999-2

Zheng, X., Wang, M., Liu, S., Chen, H., Li, Y., Yuan, F., et al. (2023). A lncRNA-encoded mitochondrial micropeptide exacerbates microglia-mediated neuroinflammation in retinal ischemia/reperfusion injury. *Cell Death Dis.* 14:126. doi: 10.1038/s41419-023-05617-2

Zhu, S., Hu, X., Bennett, S., Xu, J., and Mai, Y. (2022). The molecular structure and role of humanin in neural and skeletal diseases, and in tissue regeneration. *Front. Cell Dev. Biol.* 10:823354. doi: 10.3389/fcell.2022.823354

frontiers | Frontiers in Molecular Neuroscience

TYPE Review
PUBLISHED 17 July 2024
DOI 10.3389/fnmol.2024.1411639

Check for updates

OPEN ACCESS

EDITED BY
Clémence Bernard,
University of Exeter, United Kingdom

REVIEWED BY
Mladen-Roko Rasin,
The State University of New Jersey,
United States
Nicholas Clifton,
University of Exeter, United Kingdom

*CORRESPONDENCE
Juliette D. Godin
godin@igbmc.fr

RECEIVED 03 April 2024
ACCEPTED 17 June 2024
PUBLISHED 17 July 2024

CITATION
Tilliole P, Fix S and Godin JD (2024) hnRNPs: roles in neurodevelopment and implication for brain disorders.
Front. Mol. Neurosci. 17:1411639.
doi: 10.3389/fnmol.2024.1411639

hnRNPs: roles in neurodevelopment and implication for brain disorders

Pierre Tilliole[1,2,3,4], Simon Fix[1,2,3,4] and Juliette D. Godin[1,2,3,4]*

[1]Institut de Génétique et de Biologie Moléculaire et Cellulaire, IGBMC, Illkirch, France, [2]Centre National de la Recherche Scientifique, CNRS, UMR7104, Illkirch, France, [3]Institut National de la Santé et de la Recherche Médicale, INSERM, U1258, Illkirch, France, [4]Université de Strasbourg, Strasbourg, France

Heterogeneous nuclear ribonucleoproteins (hnRNPs) constitute a family of multifunctional RNA-binding proteins able to process nuclear pre-mRNAs into mature mRNAs and regulate gene expression in multiple ways. They comprise at least 20 different members in mammals, named from A (*HNRNP A1*) to U (*HNRNP U*). Many of these proteins are components of the spliceosome complex and can modulate alternative splicing in a tissue-specific manner. Notably, while genes encoding hnRNPs exhibit ubiquitous expression, increasing evidence associate these proteins to various neurodevelopmental and neurodegenerative disorders, such as intellectual disability, epilepsy, microcephaly, amyotrophic lateral sclerosis, or dementias, highlighting their crucial role in the central nervous system. This review explores the evolution of the hnRNPs family, highlighting the emergence of numerous new members within this family, and sheds light on their implications for brain development.

KEYWORDS
hnRNP proteins, alternative splicing, brain development, neurodevelopmental disorders, neurodegenerative disorders

1 Introduction

The exact number of protein-coding genes within the human genome remains a subject of intensive discussion, with estimated number that dropped from 30,000 to 40,000 since the initial publication of the human genome (Lander et al., 2001; Venter et al., 2001) to less than 20,000 today (Morales et al., 2022; Nurk et al., 2022). If each gene encoded a single protein, the estimated size of the proteome would be approximately 20,000. However, around 95% of the human multi-exon genes are able to produce multiple protein sequences (Pan et al., 2008; Wang et al., 2008), resulting in a number of distinct human proteins exceeding 70,000 (Aebersold et al., 2018). This extended protein diversity is the result of alternative splicing, a process that generates, in a tissue specific manner, several mRNA transcripts from the same gene. Notably, the brain is one of the organs with the highest number of splicing events (Mazin et al., 2021), making it particularly sensitive to defects in this process (Grabowski and Black, 2001).

The mRNA splicing is a multi-step process catalyzed by various small nuclear ribonucleoprotein (snRNP) particles that dynamically assemble, along with other proteins, in a macromolecular machinery called the spliceosome. Splicing starts with the recognition of specific sequences at the exon-intron boundaries by the spliceosome. Alternative splicing additionally involves cis-acting regulatory sequences, that, through their interaction with trans-acting splicing factors, modulate the activity of nearby splice sites. Splice site selection

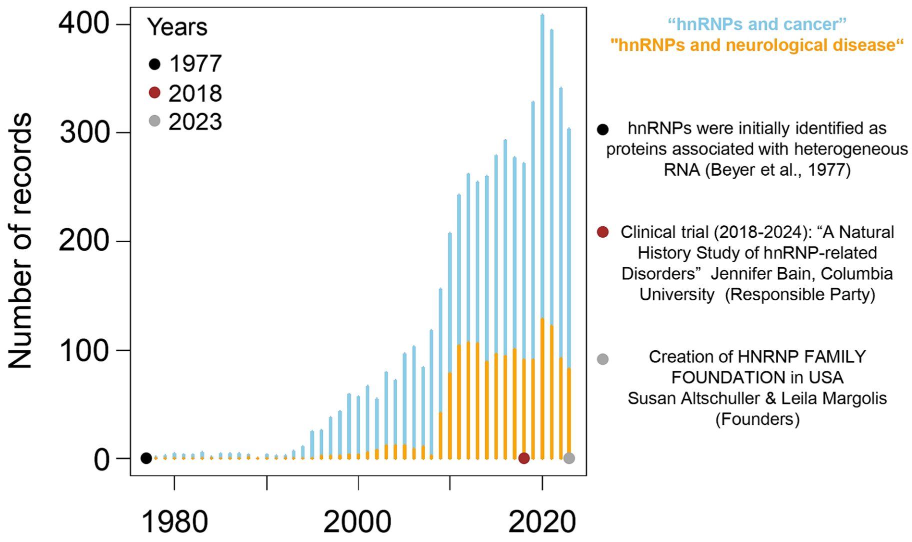


FIGURE 1
Number of records retrieved from PubMed including keywords "hnRNPs and cancer" or "hnRNPs and neurological disease" in the period 1977–2023. Date of search: March 11, 2024.

is followed by two successive transesterification reactions that lead to the removal of the intron and the joining of neighboring exons, ultimately yielding to mature mRNA (reviewed by Wilkinson et al., 2020).

Among the two major classes of splicing factors, one finds the heterogeneous nuclear ribonucleoproteins (hnRNPs). hnRNPs constitute a family of 20 canonical multifunctional RNA-binding proteins (RBPs) in mammals, named from A (HNRNP A1) to U (HNRNP U) (Chaudhury et al., 2010). As components of the spliceosomal assembly, these proteins modulate alternative splicing. Strikingly, although genes encoding those canonical hnRNPs are ubiquitously expressed, genetic variants altering their sequence mainly lead to neurodevelopmental or neurodegenerative disorders, such as intellectual disability, epilepsy, microcephaly, amyotrophic lateral sclerosis, or dementias, pointing out their key role in the central nervous system (Purice and Taylor, 2018; Low et al., 2021). Yet, compared to their dysfunction in cancer, the significance of hnRNPs in neurological disorders remains largely unexplored (Figure 1). Nevertheless, two recent developments mark a growing interest in hnRNPs brain-related disorders (Figure 1): (i) the initiation, in 2018, of a clinical study (Natural History Study of hnRNP-related Disorders; ClinicalTrials.gov ID: NCT03492060), that aims to examine neurological traits in individuals with variants in any hnRNP genes with the ultimate goal to define a hnRNP neurodevelopmental syndrome and propose common therapeutic interventions; and (ii) the creation, in 2023 and 2024, of two foundations, the HNRNP Family Foundation in USA[1] and the HNRNP Japan,[2] dedicated to support patients and families living with hnRNP-related neurodevelopmental disorders.

1 https://www.hnrnp.org
2 https://hnrnpjapan.org

In this review, we provide updated insights into the implications of hnRNPs in neurodevelopmental and neurodegenerative disorders, by exploring the evolution of hnRNPs in mammalian genomes, their differential expression and localization and their physiological roles with a particular focus on the developing and aging brain.

2 Evolution of the hnRNP family

2.1 Identification of the major protein members of the hnRNP family

The hnRNPs, which belong to the RNA-binding protein family, have been named after their initially identified role in packaging heterogeneous nuclear RNA (hnRNA) (Beyer et al., 1977). The classification of hnRNP members started with the recognition of the "core" hnRNP proteins (categorized into the A, B, and C groups) as major components of this family (Beyer et al., 1977). However, the wide range of molecular weights, spanning from 34 to 120 kDa (Choi and Dreyfuss, 1984) as well as similarities in the structure and sequence of hnRNPs with the same molecular weight have severely hampered identification of other members. Thanks to extensive sequence and structural analyses, hnRNP family is now defined as 20 canonical hnRNP sub-families designated from A (hnRNP A1) to U (hnRNP U) (Piñol-Roma et al., 1988), each of them being composed of several paralogues and in some cases, even distantly related proteins (Martinez-Contreras et al., 2007; Figure 2A). Yet, the classification of some hnRNP members is still under debate (Han et al., 2010b; Busch and Hertel, 2012; Geuens et al., 2016).

In the following sections, we will expand the discussion beyond the major hnRNPs initially identified by the Dreyfuss Lab (Dreyfuss et al., 1993). We will emphasize the emergence of hnRNP-like or minor members due to their conserved structure compared to canonical hnRNPs, but also highlight other mechanisms, such as

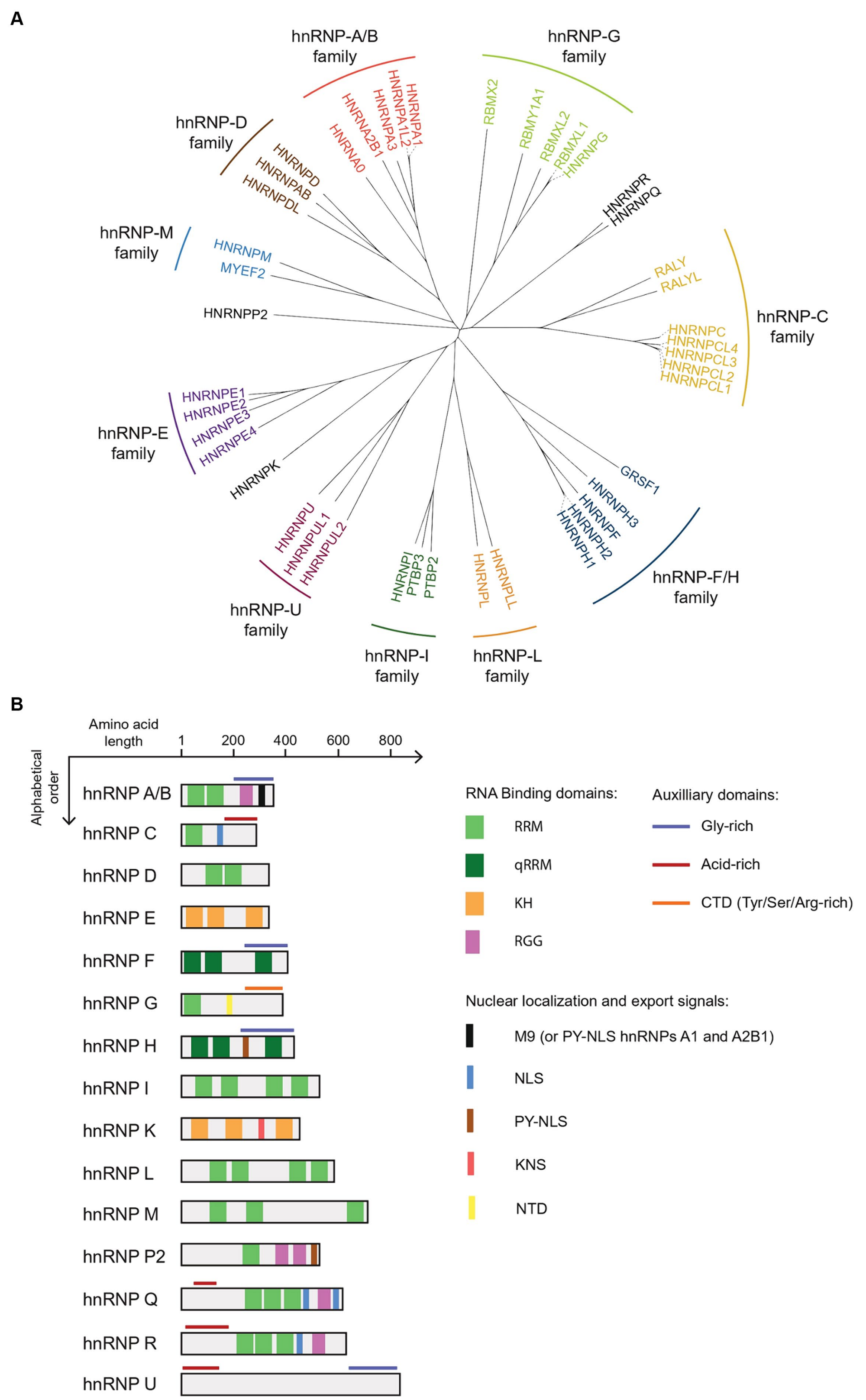


FIGURE 2
Structure and identity of members of hnRNP families. **(A)** Protein sequence comparison of hnRNPs by multiple sequence alignment program, Clustal Omega (https://www.ebi.ac.uk/jdispatcher/msa/clustalo). The protein sequences of the hnRNPs used correspond, for each member, to the highest expressed hnRNP isoform in the human cerebral cortex, identified via a ENST reference number (Ensembl Transcrit number) using the GTEx Transcript Browser program (https://www.gtexportal.org/home/transcriptPage) (Supplementary Table S1). The protein sequences corresponding to these isoforms were identified on the NCBI database from the ENST reference number (Supplementary Table S1). The percentage identity between members

(Continued)

FIGURE 2 (Continued)

of hnRNP families can be found in Supplementary Table S2. **(B)** Schematic representation of the canonical structure for hnRNP sub-families. The schematic illustrates various domains: RRM (RNA recognition motif), qRRM (quasi RNA recognition motif), KH (K-homology domain), RGG (Arg-Gly-Gly repeat domain), NLS (Nuclear localization signal), PY-NLS (Proline/Tyrosine Nuclear localization signal), NTD (Nascent transcripts targeting domain), and KNS (hnRNP K nuclear shuttling).

alternative splicing and gene duplication, that further extend and add complexity to the exhaustive characterization of the hnRNP family.

2.2 Conserved structure across members of the hnRNP family

The analysis of the amino acid (aa) sequence of hnRNP members revealed multiple distinct RNA-binding domains (RBD), including RNA recognition motifs (RRM), quasi-RNA recognition motifs (qRRM), Arg-Gly-Gly repeat domain (RGG), or K homology domains (KH) (Dreyfuss et al., 1993), in all major members of the hnRNP family, except hnRNP U (Figure 2B). Close to the RBDs, hnRNP proteins also typically feature unstructured auxiliary domains with clusters rich in certain aa, such as acidic aa, glycine or proline (Geuens et al., 2016). Those auxiliary domains play dual roles in regulating protein–protein interactions and, in certain cases, subcellular localization. As example, the hnRNP A1 contains a nucleo-cytoplasmic shuttling (NS) domain named M9 within its auxiliary domain, characterized by its glycine-rich composition (Siomi and Dreyfuss, 1995). As such, hnRNPs show a modular composition arising from the combinations and arrangements of various domains, such as RBD and auxiliary domains, that increase their functional diversity (Han et al., 2010a).

The presence of several RBDs confer to hnRNPs the ability to bind multiple RNA sequences simultaneously (Singh and Valcárcel, 2005). Moreover, in addition to their binding to RNA, hnRNPs are concurrently engaged in protein–protein interactions. The interactions of hnRNPs with both proteins and RNA partners/targets are facilitated by their RBDs but also likely by low complexity domains (LCDs) within intrinsically disordered regions (IDRs) (Calabretta and Richard, 2015) or auxiliary domains (Biamonti and Riva, 1994; Cartegni et al., 1996).

As such, the model depicting the assembly of hnRNP G (RBMX) on exon 7 of SMN2 pre-mRNA showed that hnRNP G (RBMX) binds to RNA directly through its N-terminal RRM, and indirectly via the interaction of its C-terminal LCD with the splicing factor Tra2-β1 (Moursy et al., 2014). More recently, Van Lindt and collaborators demonstrated that hnRNP A2 interacts with various RNA molecules through a Try/Gly-rich motif located in the middle of IDR (Van Lindt et al., 2022), as previously suggested for the IDR LCDs of hnRNP A1 that is 72% identical to the IDR of hnRNP A2 (Abdul-Manan et al., 1996). Of note, LCDs present in hnRNPs are thought to participate in liquid–liquid phase separation resulting in the formation of membraneless organelles like nuclear speckles, processing bodies, and stress granules (discussed section 3.1).

Given the conserved RBD structure observed across various hnRNPs, RBPs with such domains have been proposed as members of the hnRNP family. Accordingly, the extensively studied TAR DNA binding protein 43 (TDP-43) is often associated to the hnRNP family and is well documented as a protein partner of many other hnRNPs (D'Ambrogio et al., 2009). On the same line, Raver1, that displays three RRM and that forms complexes with other hnRNP proteins, has also been qualified as a multidomain hnRNP-like protein. Further investigations have revealed, based on sequence similarities within RRM and their general domain organization, that *Raver1* has a related gene called *Raver2*, therefore classified as new member of the hnRNP family (Hüttelmaier et al., 2001; Kleinhenz et al., 2005). Two other RBPs, Msi2 and Msi1, are considered as members of the hnRNP family due to their structurally conserved sequences with hnRNP A/B and hnRNP D (AUF1), which are notably characterized by the presence of two copies of RRMs and one auxiliary domain (Sakakibara et al., 2001). Finally, a RBP known as cold-inducible RNA-binding protein (CIRBP) (Nishiyama et al., 1997), initially recognized for its role in response to cold stress, is also referred to as hnRNP A18 due to high sequence homology with members of the hnRNP family (Sheikh et al., 1997). Indeed, the human hnRNP A18 (CIRBP) comprises a structured N-terminal domain with an RRM, and a C-terminal low-complexity region containing the RGG and RSY regions (Bourgeois et al., 2020).

As RBDs, IDRs, or LCDs can also bind single-stranded DNA (ssDNA) (Dettori et al., 2021), hnRNPs have been thought to interact with DNA. As such hnRNP E1 (PCBP1) and members of the hnRNP A/B family, including hnRNPs A1, A2/B1, and A3, have been documented to associate with single-stranded telomeric DNA and therefore participate in telomere biogenesis (McKay and Cooke, 1992; LaBranche et al., 1998; Ding et al., 1999; Moran-Jones et al., 2005; Tanaka et al., 2007; Mohanty et al., 2021). *In vitro* experiments have demonstrated that hnRNP U can bind ssDNA through its C-terminal glycine-rich region (Kiledjian and Dreyfuss, 1992). A binding affinity test of TDP-43 has also revealed an interaction with single-stranded DNA fragments derived from the HIV-1 TAR sequences (Kuo et al., 2009). Other evidence comes from hnRNP G (RBMX) that is recruited, in response to replication stress, to repetitive DNA sites where it activates the genome surveillance pathway (Zheng et al., 2020). This function is independent of hnRNP G (RBMX) interaction with nascent RNA but involved a poorly characterized RBD, termed RBM1CTR and located within the middle of the hnRNP G (RBMX) protein (Zheng et al., 2020). Of note, like hnRNP G (RBMX) (Adamson et al., 2012), the RRM of many other hnRNPs, including hnRNP R (Ghanawi et al., 2021), hnRNP U (Britton et al., 2014), hnRNP P2 (FUS) (Mamontova et al., 2023) and hnRNP D (Alfano et al., 2019), mediates their recruitment to ssDNA sites upon DNA damage to ultimately facilitate DNA damage response. These roles do not always require a direct binding to DNA but are rather dependent of β-H2AX or PARP1 proteins, that are known to mediate the recruitment of repair proteins to the DNA lesion. Furthermore, the knockdown of hnRNP K leads to DNA repair defects and initiates a DNA damage response (DDR) upon gamma irradiation. This process is facilitated by the upregulation of DDR genes such as *p21* and *p53* (Wiesmann et al., 2017). Although there is increasing evidence for roles of hnRNPs in the regulation of genome stability, as highlighted in recent reviews (Klaric et al., 2021; Provasek et al., 2022), we will focus, in the next section of this review, on their function within the spliceosome.

2.3 Factors contributing to the large membership of the hnRNP family

2.3.1 Alternative splicing of *hnRNP* transcripts

Transcripts encoding major hnRNPs are themselves subjected to alternative splicing (Ezkurdia et al., 2012). It emerges that: (1) nearly all hnRNP members exhibit various isoforms, (2) one isoform frequently appears dominant in expression, and (3) different isoforms are expressed depending on the tissue (see section 3). The *hnRNP I* gene (also known as *PTBP1*) comprises 15 exons. Exon 9 undergoes alternative splicing, leading to the generation of multiple isoforms (Romanelli et al., 2000). The exclusion of exon 9 decreases the inhibitory function of hnRNP I (PTBP1) and enables the initiation of a specialized alternative splicing program specific to the brain (Gueroussov et al., 2015). The *hnRNP R* gene is also subjected to alternative splicing, resulting in the production of two unique protein isoforms, hnRNP R1 and hnRNP R2. hnRNP R1 comprises 633 aa, whereas hnRNP R2 lacks 38 aa distributed across its acidic domain and RRM. The expression patterns of hnRNP R1 and hnRNR R2 vary significantly depending on the tissue. While hnRNP R1 exhibits ubiquitous expression and significantly higher levels compared to hnRNP R2, the latter shows low expression levels specifically in neural tissue (Huang et al., 2005; Cappelli et al., 2018). hnRNP Q exhibits close structural similarities to hnRNP R and undergoes alternative splicing, resulting in three isoforms of hnRNP Q denoted as Q1–Q3 (Mourelatos et al., 2001). Several alternatively spliced hnRNP E2 (PCBP2) mRNAs exist, with the full transcript isoform serving as a model for the retrotransposition event that gave rise to the *hnRNP E1* (*PCBP1*) intronless gene (Makeyev et al., 1999). The principal constituents of the hnRNP A family (hnRNP A1, hnRNP A2/B1, hnRNP A3) are also alternative spliced. hnRNP A1 produces transcripts A1 and A1b, hnRNP A2/B1 is spliced into transcripts B1, A2, A2b, and B1b, and hnRNP A3 generates transcripts A3a and A3b (Han et al., 2010a). Interestingly, a tissue-specific expression patterns of hnRNP A3 isoforms were observed in mice. hnRNP A3b is the predominant isoform in all assessed rodent tissues, except in the brain, where the unspliced A3a isoform exhibited significant overexpression (Han et al., 2010a; Papadopoulou et al., 2012). hnRNP D encompasses four isoforms (p45, p42, p40, and p37) with common structural elements generated through alternative splicing of a shared pre-mRNA. The p42 and p45 isoforms of hnRNP D are predominantly located in the nucleus, while the smaller variants (p40 and p37) are present in both the nuclear and cytoplasmic compartments (White et al., 2013). Strikingly, data of hnRNPs expression that can be found in the Genotype-Tissue Expression (GTEx) portal confirmed that nearly all *hnRNP* genes express multiple isoforms. For those that do not, this phenomenon is attributed to intronless *hnRNP* genes, such as *hnRNP E1* (*PCBP1*) or *RBMXL1*, or to genes that are not expressed in this tissue, such as *hnRNP CL1-4*. Finally, it has been shown that the alternative splicing of the exon 2 of the hnRNP R transcript results in an isoform with a truncated N-terminus, that loses its interaction with Yb1 and the associated function in DNA damage repair (Ghanawi et al., 2021).

Collectively, it appears that alternative splicing largely contributes to the diversity of hnRNPs, by leading to specific expression patterns and/or modifying functions through removal or partial alteration of some functional domains in the spliced isoforms. Thanks to the emergence of the long-read sequencing, we foresee the discovery of many other hnRNP isoforms. As a proof of principle, such technology has revealed a previously uncharacterized isoform of hnRNP A18 (CIRBP) and a shift from the canonical CIRBP-201 isoform to the new CIRBP-210 isoform in infected epithelial cells (Corre et al., 2023).

2.3.2 Evolution: gene duplication and retrotransposition events

The fact that the number of families and number of members within a given family expanded with the emergence of more complex multicellular organism suggests: (i) that hnRNPs have evolved from a common ancestor gene mainly through gene duplication (Busch and Hertel, 2012); and (ii) the presence of strong selective pressures acting on duplicated hnRNP genes (Busch and Hertel, 2012). The increase in the number of major hnRNPs as well as the emergence of additional members that could be designated as minor hnRNP members have been also attributed to retrotransposition events (Chaudhury et al., 2010). Retrocopied genes originate from insertion of retro-transcribed mRNA into the genome. As such, retrocopies lack introns and regulatory sequences found in their parent genes and are often non-functional or qualified as “processed pseudogenes” (Hatfield et al., 2002). However, in some cases, retrocopies may acquire novel functions through the acquisition of mutations or regulatory elements present in their genomic location, thereby contributing to genetic diversity and evolution (Seczynska and Lehner, 2023).

A striking illustration of the intricate evolutionary processes entailing gene duplication and retrotransposition events is exemplified by *RBMX*, encoding hnRNP G on the X chromosome. hnRNP G is part of the hnRNP sub-family with the highest number of paralogs (duplicated genes within the same species). These paralogs comprise duplicated genes with similar intron/exon organizations located on the Y chromosome, originating from an ancestral pair of X/Y chromosomes (Lingenfelter et al., 2001; Elliott et al., 2019). In humans, the long arm of chromosome Y harbors six functional, nearly identical copies (RBMY1A1, RBMY1B, RBMY1D, RBMY1E, RBMY1F, and RBMY1J), along with over 20 pseudogenes (Elliott et al., 2000; Elliott, 2004). Unlike hnRNP G (RBMX), which shows ubiquitous expression, *RBMY* genes display a specific expression pattern, primarily in the testes (Elliott et al., 1998). Yet, they are both involved in Tra2β-dependent pre-mRNA splicing (Venables et al., 2000). In addition to the duplicated genes on chromosome Y, *hnRNP G* (*RBMX*) has undergone multiple retrocopies throughout evolution, resulting in at least nine intronless copies present in the human genome (Lingenfelter et al., 2001; Elliott et al., 2019). Some of the earliest gene duplications and retrocopies, such as the *RBMXP1-5* pseudogenes (Stelzer et al., 2016), are nonfunctional, while others have retained functionality but have adopted new expression patterns, as seen for *RBMXL2* and *RBMXL9* whose expression is restricted to the testes and brain (Lingenfelter et al., 2001; Ehrmann et al., 2008). In humans, the most recent retrocopy located on chromosome 1 (*RBMXL1*) is ubiquitously expressed and encodes a protein that shares 96% identity with hnRNP G (RBMX) (Figure 2A; Lingenfelter et al., 2001).

Various examples of gene duplication and retrotransposition events can be also found in other hnRNP sub-families: (1) Like *hnRNP G* (*RBMX*), *hnRNP E* has 3 paralogues that arose from two duplication events. Interestingly, one of those, *PCBP2* (*hnRNP E2*), has been subjected to two evolutionary independent retrotransposition events, generating 3 retrocopies, *PCBP1* (*hnRNP E1*), *PCBP2P1* and *PCBP2P2* (Makeyev et al., 1999; Makeyev and Liebhaber, 2000). (2) Comparison of the domain architecture of the hnRNP A/B family members revealed that hnRNP A1 and hnRNP A2, that exhibit a 68% aa identity (Biamonti et al., 1994; Mayeda et al., 1994), arose from the duplication of a common ancestral gene, rather than from an independent assembly of domains (Biamonti et al., 1994). Further investigation into this sub-family has revealed in mice that the *hnRNP A2* (that gives rise to four isoforms, A2, B0a, B0b, and B1) and *hnRNP A3* genes have 5 and 7 (14 in humans) processed pseudogenes, respectively, most of them being non-functional (Hatfield et al., 2002; Makeyev et al., 2005), except one *hnRNP A2* pseudogene that contains putative promoter sequences and may potentially produce a functional protein (Hatfield et al., 2002). (3) One isoform of *hnRNP I* (*PTBP1*), known as *PTBP3* has been retrotranscribed and inserted in the genome to give rise to the *ψhnRNP I* pseudogene whose activity remains uncertain (Romanelli et al., 2000). (4) Four processed pseudogenes have been identified in the hnRNP K sub-family, though none of them seem to be functional (Leopoldino et al., 2007).

Although duplication and retrotransposition events clearly participate to the expansion of the number of hnRNPs throughout evolution, it is puzzling that only few paralogues have been shown to be functional. Also, strong sequence homology (Figure 2A) raises the possibility of redundant function among paralogues (discussed in section 5). Interestingly, expression of most duplicated genes and retrocopies is several folds lower than the parent gene (Figure 3), suggesting that processed pseudogenes might become critical in specific context, in particular when the parent gene is not expressed.

Finally, comparison of the sequences of the highest expressed hnRNP isoforms in the cerebral cortex question the need of revising the classification of hnRNPs, notably in sub-families. For instance, hnRNP R and hnRNP Q that share 82% of sequence homology belong to two distinct families, while RALY and RALYL that show up to 47% of similarities with hnRNP C are considered as members of the hnRNP C family (Figure 2A). In addition, the minimum homology within a family ranges from 43% (hnRNP C) to up to 90% [hnRNPs C and G (RBMX)] (Figure 2A), suggesting that the homology and organization of specific domains might preferentially account to define the members of a family.

We anticipate the number of hnRNPs to greatly increase in the future thanks to the advent of next-generation sequencing, advanced bioinformatics analyses and structural methods, which would be determinant to identify genes resulting from duplication or retrotransposition events (Vollger et al., 2019; Feng and Li, 2021).

2.3.3 The relationship between viruses, retroviruses and hnRNPs: causes of retrotransposition events?

The hnRNP families are involved in various steps of the viral life cycle, including biosynthesis (i.e., RNA synthesis, RNA translation) and release stages (Wang et al., 2022). For their replication and propagation, viruses rely on host molecular components such as splicing factors like hnRNP proteins and SR proteins (Bolinger and Boris-Lawrie, 2009; Boudreault et al., 2019). In accordance, it has been shown that the expression of hnRNPs are modified following viral infection. Interestingly, hnRNPs could be either upregulated or downregulated upon a same viral infection. As an example, during HIV-1 infection, hnRNP A1 expression is increased (Monette et al., 2009) while hnRNPs A2/B1 and H are decreased (Dowling et al., 2008). Conversely, expression of a given hnRNP could be either increase or decrease depending on the nature of the virus as shown for hnRNP A1 that is upregulated during influenza A virus (IAV), HIV-1 (Monette et al., 2009), and HPV16 (Cheunim et al., 2008) infections, and downregulated during infections with porcine epidemic diarrhea

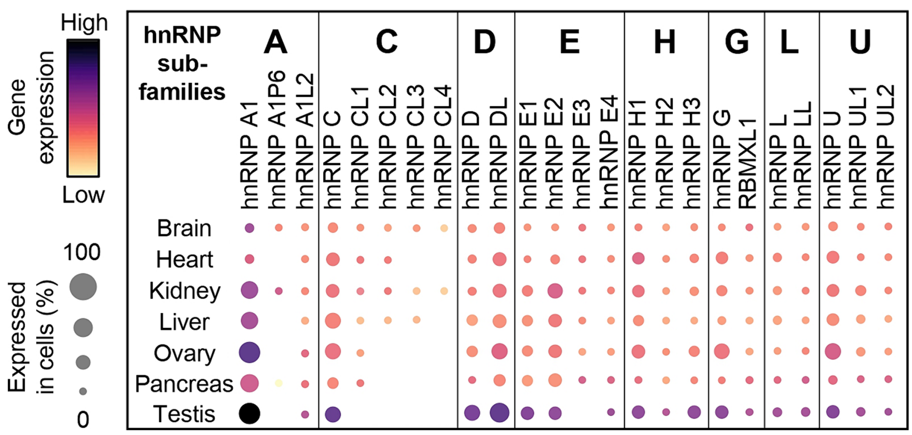


FIGURE 3
Gene expression of the parental hnRNP members, along with their duplicated and retrotransposed genes within the sub-family across multiple organs. Expression profiles of hnRNP sub-family members (A, C, D, E, H, G, L, and U) across various human organs, including the brain, heart, kidney, liver, ovary, pancreas, and testis, obtained from CZ CellxGene Discover platform and showing higher expression of the parental genes. The dot plot was made using the gene expression normalized as described in the CZ CellxGene Discover platform (https://cellxgene.cziscience.com/).

virus or snakehead vesiculovirus (Li et al., 2018; Kaur et al., 2022; Liu et al., 2023). Moreover many hnRNPs [hnRNP D (Lund et al., 2012), hnRNP A1, hnRNP K, hnRNP C1/C2 (Pettit Kneller et al., 2009), hnRNP K (Burnham et al., 2007; Brunetti et al., 2015), hnRNP H (Redondo et al., 2015), and hnRNP M (Jagdeo et al., 2015)] have been shown to relocalize to the cytoplasm following infection with various viruses. In the cytoplasm, those hnRNPs likely interact with viroplasmic proteins NSP2 and NSP5 that serve as the primary site for viral replication and assembly (Dhillon et al., 2018).

Several hnRNPs have also been shown to modulate the viral propagation within infected cells. As such, hnRNP G (RBMX) has been shown to interact with HIV-1 long terminal repeat (LTR) promoter region, where it sustains repressive trimethylation of histone H3 lysine 9 (H3K9me3), prevents the recruitment of the RNA polymerase II and consequently inhibits HIV-1 transcription (Ma et al., 2020). Conversely, hnRNP A2/B1 interacts with LTR G-quadruplexes, functioning as an activator of HIV-1 transcription (Scalabrin et al., 2017). hnRNP H1 is crucial for HIV-1 replication, as it binds to purine-rich sequences on the viral RNA. Depletion or mutation of its binding sites leads to decreased expression of Vif protein, hindering viral replication efficiency (Kutluay et al., 2019). Furthermore, many hnRNPs have been demonstrated to regulate HIV-1 Gag expression: (i) the four isoforms of hnRNP D exert distinct effects on HIV-1 Gag expression, with the longest isoforms, p45 and p42, enhancing viral Gag synthesis, and the shorter isoforms, p40 and p37, inhibiting it (Lund et al., 2012); (ii) hnRNP E1 (PCBP1) reduces cap-dependent translation initiation of HIV-1 viral RNA, resulting in decreased Gag synthesis (Woolaway et al., 2007); (iii) 21 hnRNPs have been identified in at least on affinity purification/mass spectrometry screenings that aimed at discovering potential cellular interaction partners of HIV-1 Gag (Engeland et al., 2014). Interestingly, 6 of them were also shown to bind the HIV-1 5' UTR (Stake et al., 2015).

Altogether, there data indicate that, on one side, hnRNPs are hijacked by viruses for their replication in the host cells, and on the other side, this class of protein is very prone to duplication and retrotransposition events, raising the possibility that those events are correlated.

3 Localization, expression, and regulation

3.1 Intracellular localization

Consistent with their well-described role in splicing, the majority of hnRNP proteins are found in the nucleus under physiological conditions (Piñol-Roma, 1997). For that matter, hnRNPs represent one of the most abundant family of proteins in the nucleus (Dreyfuss et al., 2002). Nuclear localization of hnRNPs is mediated by classical nuclear localization sequence (NLS) as well as non-classical PY-NLS (proline-tyrosine NLS, also known as M9 domain) (Piñol-Roma, 1997; Purice and Taylor, 2018; Khalil et al., 2024). Although, hnRNP proteins have been long thought to be excluded from the nucleolus, proteomic analysis of the human nucleolus revealed that hnRNPs A1, A3, A2/B1, C, G (RBMX), H1, H3, and K are components of the nucleolar proteome (Andersen et al., 2002). Strikingly nucleolar association of some of them [hnRNPs K, G (RBMX), and A2/B1] is enhanced when transcription is inhibited (Andersen et al., 2002). In addition, one recent study used immunofluorescence to show a colocalization of hnRNP UL1 with nucleolin, the major nucleolar protein, in HeLa cells (Cichocka et al., 2022). However, it is worth mentioning that immunogold electron or immunofluorescence microscopy did not show any labeling of hnRNP C and A2/B1 in the nucleolus of human cells (Romero et al., 1998; Friend et al., 2008), the discrepancy with the proteomic data likely coming from the different sensitivity in the methods used. On the same line, tagged version of hnRNP G (RBMX) (Matsunaga et al., 2012) or hnRNP P2 (FUS) (Yang et al., 2014) are not found in the nucleolus after overexpression. Although one can argue that tagging or overexpression of hnRNPs hamper their nucleolar localization, it is puzzling that hnRNP G tagged-proteins that lack its tyrosine-rich region (TRR) but not the ones that lack the RRM, localized to the nucleolus (Matsunaga et al., 2012). Finally, one might anticipate that the advent of highly sensitive proteomic methods will increase, in the future, the number of hnRNPs associated to the nucleolus.

As shown for pre-mRNA splicing factors, several hnRNPs have been found in nuclear speckles. The monoclonal antibody SC35, frequently used to mark nuclear speckles, has been used to perform immunoprecipitation coupled with mass spectrometry on the leukemia human HAP1 cell line. The results revealed numerous hnRNPs as interactants, including hnRNP M, hnRNP C, hnRNP K, hnRNP G (RBMX), and hnRNP U in the top 50 hits (Ilik et al., 2020). A study also reported several hnRNPs [A1, A1L, F, G (RBMX), H1, H3, K, R, and UL1] as key components of paraspeckles (Naganuma et al., 2012), that are typically located in close proximity to nuclear speckles and enriched in specific long non-coding RNAs and RBPs.

It has long been established through pioneering research that certain hnRNP proteins exhibit continuous shuttling between the nucleus and cytoplasm, rather than remaining exclusively within the nucleus (Piñol-Roma and Dreyfuss, 1992). One of the first hnRNP proteins described to undergo nucleocytoplasmic shuttling is hnRNP A1 (Weighardt et al., 1995), through its M9 domain (also known as the PY-NLS) (Izaurralde et al., 1997; Soniat and Chook, 2016). This has been then expanded to other hnRNPs (D, E, I, and K). However, some hnRNPs are exclusively retained in the nucleus such as hnRNPs C and U (Piñol-Roma, 1997) or hnRNP DL (Zhang et al., 2021). In the cytoplasm, hnRNPs can have opposite effects on mRNA stability, promoting either stabilization or degradation. Indeed, many hnRNPs can regulate either positively or negatively rapid mRNA decay. For instance, it has been shown that all the hnRNP D isoforms promote decay by binding to mRNA-destabilization sequence (Loflin et al., 1999; Xu et al., 2001; Fialcowitz et al., 2005). Likewise, hnRNP A2/B1 and hnRNP A1 have been demonstrated to initiate mRNA degradation by facilitating the recruitment of the CCR4-NOT deadenylase complex through their binding to UAASUUAU sequence in the mRNA 3′UTR (Geissler et al., 2016). hnRNPs can also cooperate with RBPs from different families to induce mRNA decay, as demonstrated for hnRNP F, that serves as a co-factor in TTP/BRF1-dependent mRNA degradation (Reznik et al., 2014). Interestingly, this role of hnRNP F is independent of its binding to the mRNA targeted for decay (Reznik et al., 2014). In contrast, hnRNP I (PTBP1) protects mRNAs from degradation by binding to their 3' UTR and preventing the binding of the NMD helicase UPF1 to the 3'UTRs (Ge et al., 2016). Two other hnRNPs, hnRNP L, and hnRNP I (PCBP1), possess the capability to remove the UPF1 NMD factor from the 3' UTR of particular mRNAs, including CFTR mRNA (Siddiqui et al., 2023), safeguarding these transcripts against NMD (Kishor et al., 2019). Interestingly, various hnRNPs can regulate differently the same mRNA. This is exemplified by the regulation of the mouse *Period3* (m*Per3*) mRNA, that is a binding target for several hnRNPs [D, K, I (PTBP1), and Q]: while hnRNP K preserves m*Per3* stability, hnRNPs D and Q promote its degradation and hnRNP I (PTBP1) show no impact on m*Per3* stability (Kim et al., 2011, 2015). Interestingly, hnRNPs Q, D, and I (PTBP1) as well as hnRNP R, also contribute to the oscillation of the circadian mRNAs *Per2*, *Cry1*, and *Nat* (Kim et al., 2005; Woo et al., 2009, 2010). Increase stabilization of mRNA has been also demonstrated for several hnRNPs, although the underlying mechanisms have not been clearly elucidated yet: (i) the stability of *APP* mRNA can be increased by the binding of hnRNPs (F, H1, and C) (Rajagopalan et al., 1998; Khan et al., 2021); (ii) the interaction between hnRNP H/F and the G-quadruplex located at the 3′ end of *p53* mRNA reinforces the binding of hnRNP H/F to *p53* mRNA, increasing its expression in response to DNA damage (Decorsière et al., 2011); (iii) hnRNP L binds and stabilizes the *BCL2* mRNA, which plays a critical role in regulating apoptosis (Lim et al., 2010); (iv) hnRNP U has been demonstrated to modulate the expression of TNFα and several other mRNAs (*GADD45A, HEXIM1, HOXA2, IER3, NHLH2*, and *ZFY*) by promoting mRNA stability (Yugami et al., 2007); (v) hnRNPs E1 (PCBP1) and E2 (PCBP2) regulate the stability of the androgen receptor mRNA (Yeap et al., 2002). Several other cases of hnRNP E1's role in the regulation of mRNA stability have been reviewed by Chaudhury et al. (2010); (vi) hnRNP E1 (PCBP1) controls *p63* mRNA stability by binding to its 3'UTR, particularly the CU-rich element (Cho et al., 2013).

Another important cytoplasmic function resides in the control of translation. First, it has been shown that hnRNP E1 (PCBP1) promotes translation by interaction with the IRES of some mRNAs (Gamarnik and Andino, 1997; Evans et al., 2003; Pickering et al., 2003). This function is shared with many other hnRNP members, as described in this review by Godet et al. which extensively analyzes IRES trans-acting factors (ITAFs) regulating cellular IRESs. Among ITAFs, one can find nuclear proteins capable of shuttling between the nucleus and cytoplasm to govern IRES-dependent translation, including hnRNPs (A1, C, D, E, H2, I, K, L, M, Q, and R) (Godet et al., 2019). Second, several evidence highlight the association of several hnRNPs with ribosomes: (i) hnRNP C, hnRNP G (RBMX), hnRNP H3, and RALY have been found enriched in polysomes fraction during mitosis (Aviner et al., 2017); (ii) other hnRNPs [such as hnRNP E1 (PCBP1), hnRNP E2 (PCBP2), hnRNP A2/B1, and hnRNP I (PTBP1)] have been shown to be associated with polysome under hypoxic conditions, while others showed either no change (hnRNP A3) or reduced translational engagement (hnRNP C) (Ho et al., 2020); and (iii) hnRNP M distribution shifts from monosome fractions under normoxia to polysome fractions under hypoxia, suggesting increased translation activity in response to low oxygen levels (Chen et al., 2019).

Cytoplasmic hnRNPs can also orchestrate the transport of mRNA molecules to precise locations, notably in axons. A recent study has shown that PTBP2 binds and facilitates the trafficking of *hnRNP R* mRNA into axons, consequently enabling the local synthesis of hnRNP R within axons (Salehi et al., 2023). Interestingly, hnRNP R itself may play a role in the axonal translocation of *β-actin* mRNA (Glinka et al., 2010) or of the non-coding RNA 7SK (Glinka et al., 2010; Briese et al., 2018), functions that both sustain axonal growth (Glinka et al., 2010; Briese et al., 2018). RNA co-immunoprecipitation (RIP) with axonal hnRNPs further revealed that various hnRNP proteins [AB, A1, A2/B1, A3, D, DL, E2 (PCBP2), E3, F, H1, H2, I, PTBP2, PTBP3, K, L, R, and U] work together to regulate mRNA transport within axons through their binding to specific mRNA motifs (Lee et al., 2018). Strikingly, axotomy increased the axonal transport of RNA granules containing hnRNPs (H1, F, and K), which exhibit a preference for binding to mRNAs essential for axon regeneration (*nrn1* and *hmgb1*) (Lee et al., 2018). On the same line, hnRNP A/B interacts with mRNAs encoding proteins involved in axon projection and synapse assembly, thereby promoting their local translation and accurate expression of the encoded protein at axon terminals in olfactory sensory neurons (Fukuda et al., 2023). Although it becomes clear that hnRNPs could promote the trafficking of some mRNAs, whether hnRNPs bind fully mature mRNAs or whether those are spliced or processed while transported remain to be determined.

Cytoplasmic hnRNPs are also involved in the formation of membraneless organelles, such as stress granules (SGs). This function is conferred by their LCDs, which facilitate liquid–liquid phase separation mechanisms responsible for the formation of these SGs (Purice and Taylor, 2018). For instance, the LCD of hnRNP A1 induces liquid–liquid phase separation (LLPS) *in vitro* and is sufficient for recruitment into SGs in cells. Notably, elevating the cytoplasmic concentration of hnRNP A1 and closely related RBPs is enough to trigger SG formation, supporting the idea of an LLPS-mediated mechanism (Molliex et al., 2015). Interestingly, some hnRNPs, including hnRNP A1, hnRNP K and hnRNP H relocalize, under cellular stress conditions, to the cytoplasm, where they accumulate in SGs, playing a crucial role in the cellular stress recovery (Guil et al., 2006; Fukuda et al., 2009; Wall et al., 2020). SGs are hallmarks of neurodegenerative disorders, particularly amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) (Dudman and Qi, 2020; Nedelsky and Taylor, 2022). Interestingly, in an effort to characterize the protein composition of SGs, a comprehensive analysis of the available dataset by Asadi et al. revealed that many hnRNPs localized to SGs in various neurodegenerative conditions including the ALS/FTD continuum, Alzheimer's disease (AD), Multiple sclerosis (MS) and Motor neuron disease (MND): TDP-43 and hnRNP P2 (FUS) are found in many if not all pathological conditions, while hnRNP A2/B1 and hnRNP A0 are associated to SG in ALS and AD, respectively (Asadi et al., 2021). Through bioinformatics analyses, hnRNP C, hnRNP DL, hnRNP H1, hnRNP F, hnRNP A2/B1, and hnRNP I (PTBP1) were also predicted to interact with SGs (Asadi et al., 2021).

Altogether, these findings suggest that nucleo-cytoplasmic shuttling of hnRNPs might play critical role in regulating the localization and/or translation of numerous mRNAs in both physiological and pathological contexts.

3.2 hnRNPs expression in the brain

The initial observations came from Dreyfuss and colleagues, who reported a higher expression of several hnRNP proteins (A1, C, D, F/H, K/J, L, and U) in the brain, ovary, and testis compared to other organs (Kamma et al., 1995). More specifically, they also demonstrated, using immunostaining, that neurons exhibit significantly stronger staining intensity than glial cells for all hnRNP proteins. In particular, cerebellar Purkinje cells and large ganglion cells of the basal ganglia expressed more hnRNP proteins than small neuronal cells or glial cells

(Kamma et al., 1995, 1999). The high expression of hnRNP proteins in brain tissues, that correlates with the fact that alternative splicing occurs at the highest frequency in the brain (Mazin et al., 2021), has been confirmed by genome-wide transcriptomic analyses performed in seven different organs (brain, cerebellum, heart, kidney, liver, ovary, and testis) at various developmental stages spanning from early organogenesis to adulthood in humans (Cardoso-Moreira et al., 2019).[3] Interestingly, in all organs including brain, the expression of *hnRNP* transcripts strikingly decreases during the perinatal period (Cardoso-Moreira et al., 2019; Figure 4). These transcriptomics data, along with the GTEX data reanalysis by Gillentine et al. (2021) confirmed that hnRNPs A0, A1, A2/B1, DL, E1 (PCBP1), K, G (RBMX), U, and P2 (FUS) are the most highly expressed hnRNPs in all brain regions analyzed (Figure 4, ≥150RPKM), with a decrease in expression observed during the brain development for almost all of them, except for RALYL, GRSF1, hnRNP H2, and hnRNP UL2 (Cardoso-Moreira et al., 2019; Figure 4). Notably, cerebellum tends to show a higher expression of all hnRNP members (Gillentine et al., 2021). An important point is the different pattern of expression observed between the members of a same hnRNP family, as shown for hnRNP C, hnRNP I (PTBP1), hnRNP F/H, and U (Cardoso-Moreira et al., 2019; Figure 4). Collectively, hnRNP expression in the human brain is subject to spatial and temporal regulation. The spatiotemporal regulation of hnRNPs is illustrated by: (i) PTBP1 (hnRNP I) and PTBP2 (nPTB), whose expressions are almost mutually exclusive. During brain development, cells switch from expressing hnRNP I (PTBP1) to PTBP2, thereby contributing to the neuronal differentiation process (Boutz et al., 2007), and (ii) hnRNP A1 and one of its isoforms, hnRNP A1B show different expression pattern and subcellular localization with hnRNP A1B more restricted to the central nervous system and found in neuronal processes compared to hnRNP A1 (Gagné et al., 2021).

Interestingly, the spatial regulation of hnRNP members differs depending on both the brain structure and the cell type. To illustrate this, we examine the transcriptional landscape of hnRNPs throughout murine corticogenesis (Telley et al., 2019; Figure 5). The findings revealed that the majority of hnRNP members are expressed throughout the cortical development in various cell populations, including neuronal progenitors (VZ), migrating neurons (IZ), and post-migratory neurons (CP). Strikingly, hnRNPs appear to be more expressed in neuronal progenitors, as evidenced by hnRNPs F, G (RBMX), H2, GRSF1, hnRNP I (PTBP1), L, LL, and M, compared to neurons localized in the CP, except for RALYL from the hnRNP C sub-family (Telley et al., 2019). Again, difference of expression throughout the cortical plate could be noted for different members of the same hnRNP family (Figure 5). This raises the possibility of not completely overlapping function of close paralogues. To further investigate the spatial expression of hnRNPs in different cell types within the brain, we took advantage of the single-cell data resource called CZ CellxGene Discover to compare their expression among cortical progenitors (radial glial cells), cortical neurons (excitatory neurons from different cortical layers, inhibitory interneurons) and glia cells (astrocytes, oligodendrocytes, oligodendrocyte precursors) in human[4] (Figure 6). The results confirmed the findings from murine corticogenesis, demonstrating that radial glial cells exhibited a higher expression of hnRNPs compared to cerebral cortex neurons. Among the different subtypes of projection neurons (from layer I to layer VI), hnRNPs seem to be similarly expressed except hnRNP DL that is enriched in deep layer neurons (Figure 6). Interestingly, GABAergic interneurons show slightly higher expression of hnRNPs than pyramidal neurons, potentially corroborating the distinct splicing programs identified in glutamatergic and GABAergic neurons (Feng et al., 2021). Specifically, there is higher expression in interneurons for hnRNPs (A0, A1, DL, K, and R), whereas RALYL is more expressed in pyramidal neurons. Neurons from various layers demonstrate robust expression of RALYL. Given the differential expression pattern of RALYL in the different neuronal subtypes, one might consider this hnRNP as a specific marker to transcriptionally differentiate and classify the layer-specific cortical neurons. No significant difference is observed between oligodendrocytes and oligodendrocyte precursors. However, astrocytes clearly exhibit lower expression of all hnRNPs compared to other cell types, thereby corroborating previous observations by Kamma et al., who noted reduced staining in glial cells compare to Purkinje cells (Kamma et al., 1995; Figure 6).

3.3 Auto- and cross-regulation

The expression of hnRNP proteins is precisely regulated. hnRNPs expression is occasionally regulated by others splicing factors such as SRp30c (also known as SFRS9) that modulates the alternative splicing of *hnRNP A1* by inhibiting the use of a 3′ splice site (Simard and Chabot, 2002). However, in most cases hnRNP proteins can undergo auto- and cross-regulation, notably through AS-NMD, a mechanism that couples alternative splicing to nonsense-mediated decay to force the production of NMD-sensitive isoforms and thereby adjust the level of protein expression (Ni et al., 2007; Müller-McNicoll et al., 2019). It has been shown that hnRNP A2/B1 alters the splicing of the 3'UTR of its own mRNA, leading to the production of NMD-targeted isoforms (McGlincy et al., 2010). hnRNP I (also known as PTBP1) also binds to its own pre-mRNA to suppress the inclusion of exon 11. This induces a frameshift, resulting in the creation of a premature termination codon in the subsequent exon, consequently directing the mRNA for nonsense-mediated decay (Wollerton et al., 2004). Interestingly, hnRNP I can also regulate the level of its PTBP2 (nPTB) paralogue by promoting the skipping of *PTBP2* (*nPTB*) exon 10 and the subsequent production of an NMD substrate, so that only one of the two paralogues are expressed when both genes are transcribed (Spellman et al., 2007). On the same line, the closely related paralogs hnRNP L and LL (Rossbach et al., 2009), along with hnRNP D (also known as AUF1) and hnRNP DL (Kemmerer et al., 2018) have also been reported to control their own expression as well as that of each other hnRNPs through AS-NMD (Müller-McNicoll et al., 2019). In accordance with cross-regulation among the hnRNP families, analysis of binding sites for various hnRNPs within the genes encoding the different hnRNPs revealed a large network of cross-regulatory interactions between hnRNPs (Huelga et al., 2012). Enlarging the

3 https://apps.kaessmannlab.org/evodevoapp/

4 https://cellxgene.cziscience.com/gene-expression

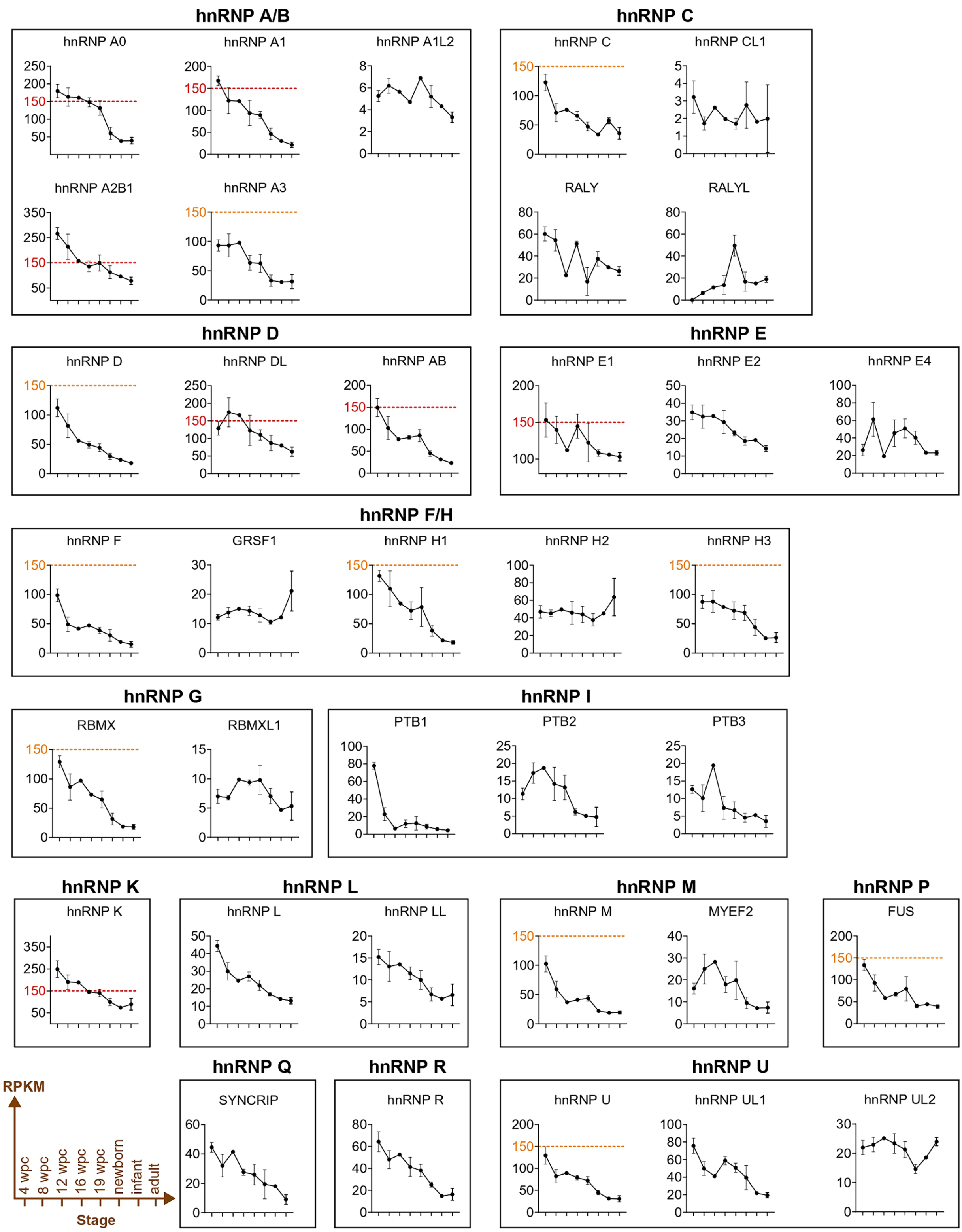


FIGURE 4
The gene expression profiles of hnRNP proteins in the human brain across various developmental stages. Overview of gene expression profiles of the hnRNPs members across human brain from a selection of developmental stages [4-, 8-, 12-, 16-, or 19-wpc (weeks post-conception)], newborn, infant, and young adults (25–32 years) using the resource provided by the Kaessmann Lab (Cardoso-Moreira et al., 2019). Expression levels were calculated in million mapped reads per kilobase of exon (RPKM). The red dashed line corresponds to hnRNPs that reach at least 150 RPKM. The orange dashed line refers to hnRNPs that are close to 150 RPKM. No expression was detected for hnRNP A1L1 (also known as hnRNP A1P6), hnRNP CL2, hnRNP CL3, and hnRNP E3.

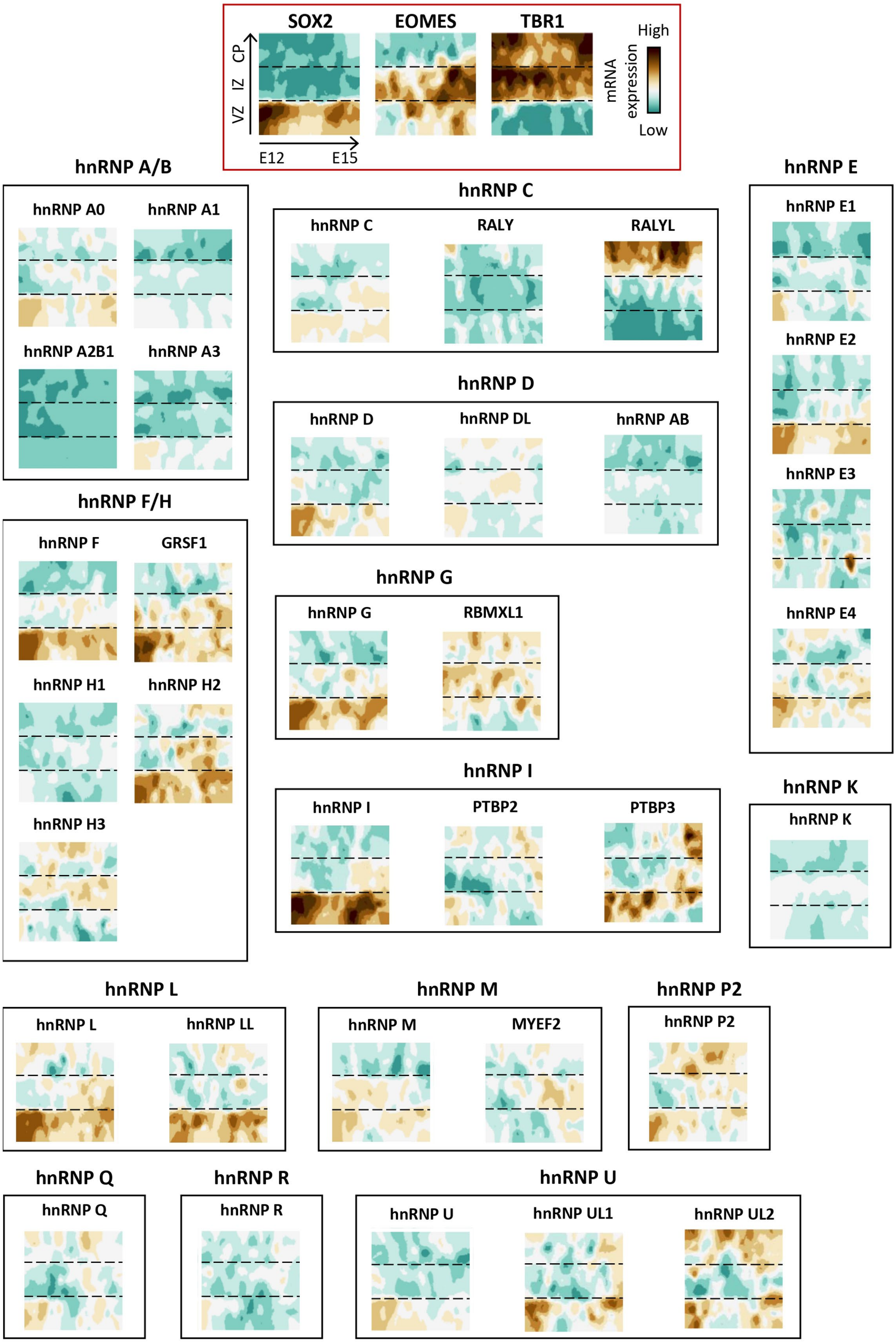


FIGURE 5
Spatio-temporal expression of hnRNP members during corticogenesis in mice. Spatio-temporal expression of hnRNP members from a single-cell RNAseq analysis in mouse developing cortices (Telley et al., 2019). The data were obtained from the open website (http://genebrowser.unige.ch/telagirdon/). X axis is time of apical progenitor birth, Y axis represents time of neuron differentiation. SOX2, EOMES (TBR2), and TBR1 have been utilized as markers to delineate the ventricular zone (VZ) progenitor, newly generated neurons in intermediate zone (IZ), and post-migratory neurons in the cortical plate (CP) respectively.

modes of regulation of hnRNPs, it has been shown that TDP-43 binds to its own mRNA through sequences within the CDS and the 3'UTR to target *TDP-43* transcripts to degradation likely via the exosome system (Ayala et al., 2011).

4 hnRNPs splicing function in the brain

In this section, we will focus on the historically described function of hnRNPs, the regulation of splicing (Dreyfuss et al., 1993), specifically in the developing brain. Extensive discussion of the other functions of hnRNPs in transcriptional regulation, nucleocytoplasmic transport, mRNA biogenesis (stability, metabolism, localization) and decay, translational regulation, chromatin remodeling or telomere maintenance, can be found in recent reviews (Geuens et al., 2016; Purice and Taylor, 2018; Bampton et al., 2020; Low et al., 2021; Brandão-Teles et al., 2023).

Among hnRNPs, hnRNP I (PTBP1) and PTBP2, members of the hnRNP I family, are widely described as key splicing factors during brain development. One of their main roles is to promote the timely expression of synaptic genes during brain maturation. Indeed, both hnRNP I (PTBP1) and PTBP2 are involved in the regulation of the expression of PSD95, that plays a key role in synapse maturation. In progenitors, they both promote PSD95 mRNA decay by suppressing the splicing of its exon 18. As progenitors differentiate into neurons, both genes are progressively silenced, resulting in exon 18 splicing and subsequent PSD95 expression. In accordance, reintroduction of hnRNP I (PTBP1) or PTBP2 in differentiated neurons inhibits PSD95 expression, impairing glutamatergic synapse development (Zheng et al., 2012). Recent PTBP2 CLIP-seq analysis in both human cortical tissue and neurons derived from induced pluripotent stem cells revealed other synaptic genes as novel PTBP2 targets. This includes *SYNGAP1*, a synaptic gene implicated in a neurodevelopmental disorder (Dawicki-McKenna et al., 2023). It was shown that PTBP2 promotes the inclusion of an alternative 3′ splice site in exon 11 of *SYNGAP1* resulting in the introduction of a premature stop codon and degradation of the *SYNGAP1* mRNA. Of note, hnRNP I (PTBP1) also regulates this splicing event. As for PSD95, progressive downregulation of PTBP proteins as neurons mature drives the increased expression of SYNGAP1 (Dawicki-McKenna et al., 2023). Among the other synapse-associated targets, Dawicki-McKenna et al. also found the glutamate receptor gene *GRIN1* to be spliced by PTBP2. This event involves the inclusion of a previously unannotated alternative exon, resulting in a frameshift in the canonical transcript and reduced expression. Yet, the role of hnRNP I (PTBP1) in the regulation of *GRIN1* has not been addressed (Dawicki-McKenna et al., 2023). Notably, hnRNP I (PTBP1) and PTBP2 could also regulate synapse formation through the regulation of expression of the different neurexins isoforms, the adhesion molecules that shape neuronal synapses (Resnick et al., 2008). Aside from its roles in synapse formation and maturation, PTBP2 plays a role in regulating the timing of axonogenesis, notably by regulating the switch from the long to the short isoforms of Shootin1, that sequentially regulate axon formation and elongation through distinct function on actin cytoskeleton (Zhang et al., 2019). Strikingly, this role is unique to PTBP2. Interestingly, the differences in the splicing regulation patterns of hnRNP I (PTBP1) and PTBP2 have been shown to arise from shift in the expression levels of hnRNP I (PTBP1) and PTBP2 proteins during neuronal differentiation. Indeed, hnRNP I (PTBP1) suppresses the inclusion of alternative exon 10 in the *PTBP2* pre-mRNA, leading to the generation of a premature termination codon and its degradation through NMD (Boutz et al., 2007). Upon differentiation of progenitors to neurons, the repression of hnRNP I (PTBP1) expression is facilitated by the miRNA miR124 (Makeyev et al., 2007) releasing the negative regulation of hnRNP I (PTBP1) on PTBP2 (Boutz et al.,

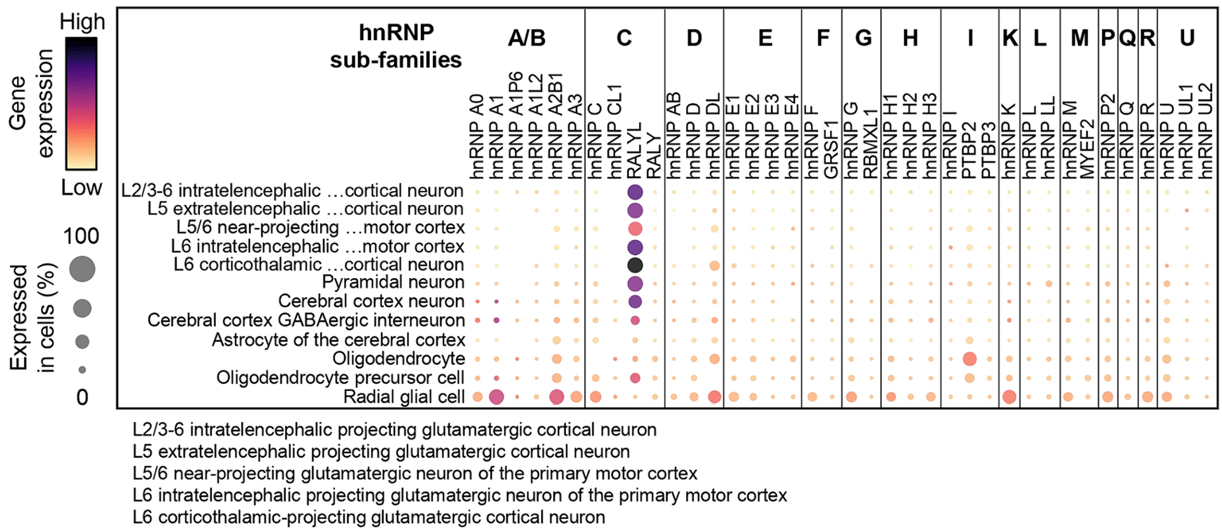


FIGURE 6
hnRNP expression patterns across various neuronal cell types in the human brain. Expression profiles of hnRNP sub-families in cortical progenitors (radial glial cells), cortical neurons (excitatory neurons from different cortical layers, inhibitory interneurons) and glia cells (astrocytes, oligodendrocytes, oligodendrocyte precursors) in the human brain. The dot plot was made using the gene expression normalized as described in the CZ CellxGene Discover platform (https://cellxgene.cziscience.com/).

2007). Also, hnRNP I (PTBP1) is critical to maintain the pool of neural progenitor cells (NPCs) by repressing a poison exon in filamin A specifically in NPCs. Not least, a human intronic mutation within a hnRNP I (PTBP1) binding site in the *FLNA* gene that prevents the usual exclusion of the *FLNA* poison exon in NPCs, results in a brain-specific malformation (Zhang et al., 2016). Like hnRNP I (PTBP1), hnRNPs H1/H2 have been shown to regulate the ability of the progenitors to generate neurons. Indeed, hnRNP H1/H2 proteins bind to *TRF2* (telomere repeat-binding factor 2) exon 7, inhibiting its splicing and thereby inhibiting the production of the exon 7 truncated TRF2-S short isoform, that is essential to promote neurogenesis (Grammatikakis et al., 2016). Interestingly, as neurogenesis progresses, there is a gradual decline in the levels of hnRNP H1/H2 proteins, that coincides with an increase in the abundance of TRF2-S. Notably, experimental silencing of hnRNPs H1/H2 leads to elevated levels of TRF2-S, thereby promoting neurogenesis (Grammatikakis et al., 2016).

Other hnRNPs have been identified as important for splicing in a physiological context: (i) *hnRNP U* knockout in mouse dorsal telencephalon leads to numerous alternative splicing events, notably in Doublecortin that controls axon growth and guidance, Siva1 that regulates neural apoptosis and synaptic function, and MDM2, a p53 negative regulator, which is targeted in brain tumor therapy (Sapir et al., 2022); (ii) hnRNP K competes with the constitutive splicing factor U2AF65 to control the splicing of several neuronal genes including *Snap25* (synaptosomal-associated protein 25) during neuronal differentiation (Cao et al., 2012); (iii) whole-genome investigation of alternative splicing has revealed the significant involvement of hnRNP F/H sub-family in the proliferation and differentiation processes of oligodendrocytes (Wang et al., 2012). In addition, hnRNPs F/H play a crucial role in regulating the major proteolipid protein in oligodendrocytes, underscoring its importance in the development and functioning of myelinating cells (Wang et al., 2007).

Splicing function of hnRNPs is also clearly associated to neurodegeneration condition. First, Spinal Muscular Atrophy (SMA), that primarily affects the motor neurons in the spinal cord, involves the splicing function of hnRNPs as key factors. The *SMN2* gene, which encodes the survival motor neuron 2 protein undergoes complex splicing regulation, notably splicing of exon 7. This process involves the intricate interplay of several hnRNP proteins. Among these, hnRNP G (RBMX), hnRNP M, and hnRNP Q, facilitate the inclusion of exon 7 in *SMN2*. Conversely, the depletion of hnRNPs A1/A2 promotes exon 7 inclusion in *SMN2*. Remarkably, *SMN2* is almost identical to *SMN1* gene, which is mutated in SMA. Interestingly, promoting expression of the full SMN2 isoform containing exon 7 in a SMN1 mutated context reduces the severity of SMA (Singh and Singh, 2018; Wirth et al., 2020; Wirth, 2021). Second, as extensively reviewed by Corsi et al. (2022), there are many hnRNP proteins [D, A3, H1, C, R, A2/B1, A1, G (RBMX), E2 (PCBP2), I (PTBP1), and PTBP2] that intricately regulate *MAPT* splicing, impacting the balance between various tau isoforms crucial for normal neuronal function and implicated in neurodegenerative diseases like Alzheimer's disease. Third, TDP-43 is a central player in the pathogenesis of the neurodegenerative disorder Frontotemporal Dementia-Amyotrophic Lateral Sclerosis (FTD-ALS). Specifically, the mislocalization of TDP-43 in the cytoplasm induces aberrant splicing of several genes: (i) activation of a cryptic splice site in the first intron of *STMN2* gene (encoding Stathmin-2) that compromises axon repair following motor neuron injury in ALS (Klim et al., 2019; Melamed et al., 2019; Baughn et al., 2023), (ii) insertion of a cryptic exon between exon 20 and 21 within the *UNC13A* transcript, a gene that plays important roles in neurotransmitter release at synapses. Consequently, this alternative splicing event generates a premature stop codon and triggers the NMD mechanism to degrade *UNC13A* pre-mRNA (Brown et al., 2022; Ma et al., 2022). Of note, TDP-43 interacts with some hnRNP members (A1, A2/B1, and L) that have been recently shown to also bind *UNC13A* RNA and repress cryptic exon inclusion, independently of TDP-43 (Koike et al., 2023). Accordingly, this has recently been corroborated using a genetically modified neuronal cell line that overexpresses either hnRNP L or a GFP control. They demonstrated that overexpression of hnRNP L decreases the abnormal inclusion of the *UNC13A* cryptic exon in a siRNA *TDP-43* condition and elevates the levels of full-length *UNC13A* in a siRNA scramble condition (Agra Almeida Quadros et al., 2024). They also demonstrated that overexpression of hnRNP L does not correct the splicing defect of the *STMN2* transcripts in a siRNA *TDP-43* condition (Agra Almeida Quadros et al., 2024).

5 Functional compensation between hnRNP members

As seen in the previous sections, members of hnRNP sub-families share various structural and functional properties raising the possibility that hnRNPs might have redundant functions. Recent evidence supports a functional compensation between close paralogues. Whether compensatory mechanisms exist across hnRNPs from different sub-families need to be demonstrated.

Mouse genetics have suggested some compensatory mechanisms among hnRNP proteins. First knockin mouse models carrying *HnRnp H2* variants found in patients presenting with neurodevelopmental disorder have been generated, along with *HnRnp H2*-KO mice (Korff et al., 2023). While the knockin mice recapitulated key clinical features observed in human patients, including reduced survival, impaired motor and cognitive functions, the *HnRnp H2*-KO mice displayed no discernible phenotypes. Intriguingly, the KO mice exhibited upregulated expression of hnRNP H1 while knockin mice failed to upregulate hnRNP H1. These findings suggest a compensatory mechanism by hnRNP H1 to counteract the loss of hnRNP H2, implying that the hnRNP H2-related disorder may result from a toxic gain of function or a complex loss of hnRNP H2 function with impaired compensation by hnRNP H1 (Korff et al., 2023). Second, Vuong et al. demonstrated that overexpression of PTBP1 (hnRNP I) rescues the lethality and brain degenerative phenotypes induced by the inactivation of *PTBP2* (*nPTB*) in mice. They further showed that hnRNP I (PTBP1) partly compensates for splicing defect occurring upon *Ptbp2* depletion. More importantly, this compensation occurs when *Ptbp2* is inactivated in dorsal progenitors (Emx1 + cells) but not when *Ptbp2* is inactivated in the whole brain (Nestin + cells), suggesting that the redundancy of the two proteins could be restricted to specific cell types during brain development (Vuong et al., 2016).

Additional work in cellular model confirm a potential redundancy of hnRNPs: (i) in the context of hnRNP G (RBMX) sub-family, the work of David Elliott's Lab has shown that the defects in splicing induced by the loss of *hnRNP G* (*RBMX*) in HEK293 cells is compensated by the exogenous expression of its 73% identical RBMXL2 retrocopy and even by the more divergent RBMY1A1 protein (Ehrmann et al., 2019; Siachisumo et al., 2023). The fact that two testis-specific proteins can rescue hnRNP G (RBMX) function in a different cellular context, strongly argue for common and conserved function through evolution. Interestingly, several patients carrying mutations in the *hnRNP G* (*RBMX*) gene have been reported to manifest various syndromes, such as Shashi syndrome and Gustavson syndrome (Shashi et al., 2015; Johansson et al., 2023). Yet, there is little phenotypic overlap between the two syndromes, suggesting a distinct disease-causing mechanism. It has been proposed without being demonstrated that phenotypic variations could be linked to hnRNP G (RBMX) retrocopies, particularly RBMXL1 and RBMXL9, which are known to be expressed in the brain (Johansson et al., 2023). As demonstrated for RBMXL2 (Siachisumo et al., 2023), one can hypothesize that those two retrocopies could also compensate for some splicing defects in those two hnRNP G (RBMX) related brain disorders; (ii) in a search for regulator of cell growth, He et al. showed that while knockdown of hnRNP A2 leads to growth defects, hnRNP A1 and hnRNP A3 depletion did not alter growth unless they are simultaneously depleted (He et al., 2005). This result suggests that these two hnRNP proteins that show the highest sequence identity (Ma et al., 2002) may functionally compensate for each other (He et al., 2005); and (iii) hnRNP L (Yu et al., 2009) and its paralog hnRNP LL (Liu et al., 2012) redundantly modulates the splicing of the calcium/calmodulin-dependent protein kinase IV (CaMKIV), a crucial enzyme involved in signal transduction and gene expression regulation. However, it is important to keep in mind that the redundancy could be specific to some mRNAs. Indeed, although the domain architecture between both proteins is highly conserved, with each containing four very similar RRMs, hnRNP L and hnRNP LL are very different in respect of their binding preferences: while hnRNP LL prefers binding to the CANRCA sequence, hnRNP L shows a broader range of preferred target sequences (CANRCA, CAN_2RCA, and CACA). The biological consequence of the differential sequences preferences of hnRNP L and LL can be evidenced by their distinct binding to the CD45 regulatory element ESS1, that present seven "CA repeat" known to differentially regulate CD45 splicing repression: while both hnRNP L and LL can bind CA6-7 repeat, only hnRNP L binds to CA2-4 repeats (Smith et al., 2013). Another example illustrating the opposite effects of hnRNP L and hnRNP LL is the splicing of the *CHRNA1* gene. While hnRNP L promotes the exclusion of exon P3A in the *CHRNA1* pre-mRNA, hnRNP LL tends to favor its inclusion (Rahman et al., 2013).

In sum, the compensatory mechanisms among hnRNPs seem very complex ranging from broad overlap in their function to compensation of specific splicing event or in specific cellular context. As such, the full characterization of functional compensation between hnRNPs represent a challenge that can be only met by extensive bench work.

6 hnRNPs and neurodevelopmental/neurodegenerative disorders

6.1 Neurodevelopmental disorders

Growing evidence link variants in multiple *hnRNP* genes to neurodevelopmental disorders (NDD). These disorders encompass a wide spectrum of neurodevelopmental symptoms, including developmental delay, microcephaly, brain anomalies, intellectual disability, and epilepsy (Gillentine et al., 2021), and have been referred as HNRNP-Related Rare Neurodevelopmental Disorders (HNRNP-RNDDs) by the hnRNP family foundation (see text footnote 1) (Gillentine et al., 2021). Though the association with HNRNP-RNDDs have been clearly shown for 8 hnRNPs (detailed below), several other hnRNPs (AB, D, F, H3, UL1, and UL2) are relevant candidate for NDDs (Gillentine et al., 2021), but this needs to be formally demonstrated. Notably, these candidates do not show a similar expression pattern neither in time or space (Figures 4, 5), that would explain their association to disease. Interestingly, although the molecular mechanism underlying HNRNP-RNDDs have not been fully investigated, to date, most of the studies converge toward loss of function effect of the identified variants in hnRNPs. Whether all these disorders are solely caused by the alteration of the canonical splicing function of hnRNPs and how the variants lead to brain phenotype at the cellular level is not known.

6.1.1 hnRNP G

hnRNP G is a X-linked gene located at the genetic locus Xq26.3. In line with a key function of hnRNP G (RBMX) in brain development, a hemizygous 23-base pair deletion, resulting in a frameshift mutation and premature termination in the last exon of *hnRNP G* (*RBMX*), has been identified in males from a large family in North Carolina. All affected males present with intellectual disability, craniofacial dysmorphism, and other neurological features. This syndrome was characterized as the Intellectual developmental disorder, X-linked syndromic, Shashi type (Phenotype MIM number: 300986) or HNRNPG-RNDD (Shashi et al., 2000, 2015). At the molecular level the mode of action of these variants has not been identified yet.

Recently, three affected males from a large Swedish family carrying a hemizygous 3-base pair in-frame deletion within exon 5 of the *hnRNP G* gene were diagnosed with Gustavson-type X-linked syndromic intellectual developmental disorder (Gustavson et al., 1993; Johansson et al., 2023). Gustavson syndrome is characterized by microcephaly, severe intellectual disabilities, optic atrophy with visual impairment, hearing loss, spasticity, seizures, and restricted joint mobility and therefore differ from the Shashi syndrome (Shashi et al., 2000, 2015; Johansson et al., 2023). HNRNPG-RNDD spectrum has thus been expanded to Intellectual Developmental Disorder, X-linked syndromic, Gustavson type (Phenotype MIM number: 309555). The 3 bp deletion leads to the removal of the proline at position 162. This in frame deletion of a single aa could impair protein–protein interaction as Pro162 is part of a tri-proline stretch that has been shown to facilitate interaction with SH3 domain-containing proteins. RNA sequencing of SH-SY5Y overexpressing GFP-tagged WT or DelPro162 hnRNP G (RBMX) proteins revealed an enrichment of genes involved in RNA polymerase II transcription among the differentially expressed genes (Gillentine, 2023; Johansson et al., 2023). As mentioned earlier, the most recent retrocopy of hnRNP G, RBMXL1, found on chromosome 1, encodes a protein (Q96E39) with similar expression pattern and high homology with hnRNP G (RBMX) (P38159) (Lingenfelter et al., 2001). Interestingly, although the proline 162 is conserved in RBMXL1, the proline 161 that forms the tri-proline motif in hnRNP G (RBMX) (PPP–160–162) is mutated into a serine (PSP–160–162). It has been suggested that the disruption of the tri-proline motif in the retrocopy excludes the possibility that

RBMXL1 compensates for the effects of the hnRNP G (RBMX) variant observed in the patients (Johansson et al., 2023).

6.1.2 hnRNP H2

As *hnRNP G* (*RBMX*), *hnRNP H2* map to the chromosome X (Xq22.1 locus). Unrelated females carrying distinct variants in the *hnRNP H2* gene were identified with developmental delay, intellectual disability, and autism. They were classified under the Intellectual Developmental Disorder, X-linked syndromic, Bain type (Phenotype MIM number: 300986) or HNRNPH2-RNDD (Bain et al., 2016, 2021; Peron et al., 2020). Interestingly, variants affecting conserved residues within the NLS are associated with more severe phenotype compare to variants located outside the NLS (Bain et al., 2016), suggesting that gain of cytoplasmic localization of the protein severely hampers brain development (Bain et al., 2021). Although, no toxic nuclear or cytoplasmic accumulation was observed in muscle tissue biopsies (Bain et al., 2021), investigations of mouse models carrying hnRNP H2 variants, revealed an accumulation of mutant proteins within cytoplasmic RNA granules, confirming a possible gain of function mechanism due to the mislocalization of mutant hnRNP H2 protein (Korff et al., 2023).

While initial studies only reported females patients (Bain et al., 2016, 2021; Peron et al., 2020), some recent studies have documented males carrying pathogenic variants in hnRNP H2 (Harmsen et al., 2019; Jepsen et al., 2019; Somashekar et al., 2020; Kreienkamp et al., 2022). For instance, Harmsen et al. identified a hemizygous *de novo* missense mutation in a young male diagnosed with the Bain type of X-linked syndromic intellectual developmental disorder and presenting with developmental delay, intellectual disability, and progressive microcephaly. This contradicts the initial hypothesis according to which variants in hnRNP H2 are lethal in males during embryonic development (Bain et al., 2016, 2021; Peron et al., 2020).

6.1.3 hnRNP H1

hnRNP H1 that encodes the close paralogue of hnRNP H2, is found on chromosome 5. A *de novo* heterozygous variant in the PY-NLS (p.R206W) of hnRNP H1 protein has been identified in a young boy diagnosed with a neurodevelopmental disorder characterized by craniofacial dysmorphism and skeletal and ophthalmological defects (Pilch et al., 2018). These latter clinical features being unique to individuals with hnRNP H1 variant, this initial patient (Pilch et al., 2018) as well as 7 other patients carrying the same R206W variant, frameshift variant, in frame deletion or gene duplication (Reichert et al., 2020) were categorized under a related but distinct condition: neurodevelopmental disorder with craniofacial dysmorphism and skeletal defects (Phenotype MIM number: 620083) or HNRNPH1-RNDD. Strikingly, a variant at the corresponding arginine position in hnRNP H2 has also been found to be mutated in individuals with Bain Syndrome (Phenotype MIM number 300986) (Bain et al., 2016), confirming that variants in those two close paralogues lead to distinct NDDs. Of note, the severity of the phenotypes due to pathogenic variants in hnRNP H1 is variable, with variants in the NLS associated with the more severe phenotypes. Mode of action of those variants has not been addressed.

6.1.4 hnRNP C

Recently, hnRNP C has been added to the list of HNRNP-RNDDs and classified under Intellectual developmental disorder, autosomal dominant 74 (Phenotype MIM number: 620688). Two independent studies have identified 13 young individuals, ranging from 17 months to 15 years old (7 males and 6 females), carrying deletions in the C-terminal (5 patients) or N-terminal region (1 patient), frameshift mutations (4 patients), and missense mutations (3 patients) in the *hnRNP C* gene, all variants being found at the heterozygous levels (Kaplanis et al., 2020; Niggl et al., 2023). All patients present with motor and speech delay, intellectual disability, and facial dysmorphisms (Kaplanis et al., 2020; Niggl et al., 2023). Analysis of hnRNP C protein level in iPSCs derived from PBMCs obtained from a patient carrying a C-terminal deletion revealed haploinsufficiency. At the molecular level, *hnRNP C* knockdown in human cell lines or haploinsufficiency in fibroblast cells obtained from a patient with a frameshift mutation lead to defects in the alternative splicing of 60 genes associated with intellectual disability (Niggl et al., 2023). Moreover, *in utero* electroporation (IUE) experiments in mice of two distinct siRNAs against *hnRNP C* gene to deplete *hnRNP C* in cells destined to form the somatosensory cortex at embryonic day E14.5, demonstrated that *hnRNP C*-deficient neurons failed to properly reach the cortical plate compared to the control condition. Further *in vitro* and *in vivo* experiments showed that overexpression of WT hnRNP C phenocopies the loss of hnRNP C function, suggesting that the dose of hnRNP C is critical for proper cortical development (Niggl et al., 2023).

6.1.5 hnRNP U

HNRNPU-related neurodevelopmental disorder (HNRNPU-RNDD) has been extensively studied and documented in numerous publications. Patients were associated to Developmental and epileptic encephalopathy 54 (Phenotype MIM number: 617391) and develop a range of symptoms, typically including moderate to severe intellectual disability, seizures, behavioral abnormalities, speech and language delay as well as craniofacial dysmorphism and agenesis of the corpus callosum (Caliebe et al., 2010; Ballif et al., 2012; Bramswig et al., 2017; Depienne et al., 2017; Leduc et al., 2017; Yates et al., 2017; Durkin et al., 2020; Taylor et al., 2022). A wide range of *de novo* variants have been identified in NDD patients. This includes splice site variants (9), nonsense (14), missense (5), in frame deletion (2), frameshift duplications (3), Frameshift deletion (26) and larger deletion (1), for a total of 57 variants identified to date (Taylor et al., 2022). Those genetics studies strongly suggest that haplinsufficiency is the main mechansism of pathogenecity in HNRNPU variants.

6.1.6 hnRNP R

One study reported four unrelated patients who present with developmental delay, microcephaly, facial dysmorphism and skeletal and brain abnormalities (Phenotype MIM number: 620073) (Duijkers et al., 2019). Authors have identified one missense variant and 2 frameshift variants in the last exon, shown to lead to the production of truncated proteins lacking most of the hnRNP R RGG domain. RNAseq analysis preformed in cells from patients carrying the frameshift variants revealed a strong enrichment of homeobox genes, known for their role in development, among the most deregulated genes. Further candidates-based analysis attributed this HOX deregulation to impaired splicing (Duijkers et al., 2019). To note, a nonsense variant in the last exon has also been identified in a patient presenting with epileptic encephalopathy but also some clinical features overlapping with the 4 other variants (Helbig et al., 2016; Duijkers et al., 2019). The fact that truncated variants leading to very similar proteins lead to different syndrome is puzzling and hamper a clear classification as HNRNPR-related neurodevelopmental disorder (HNRNPR-RNDD).

6.1.7 hnRNP Q

SYNCRIP, also known as hnRNP Q, is also associated with a neurodevelopmental disorder (HNRNPQ-RNDD), characterized by developmental delay, intellectual disability, and autism spectrum disorder accompanied in some cases by malformations of cortical development and myoclonic-atonic epilepsy (Phenotype MIM number: 616686). Eight patients have been identified so far. They all carry *de novo* variants, including frameshift variant (5 patients), missense variant (2), in frame deletion (1) and whole gene deletion (1), suggesting loss of function mechanism (Firth et al., 2009; Rauch et al., 2012; Lelieveld et al., 2016; Guo et al., 2019; Semino et al., 2021). Yet, this has not been tested. To note, SYNCRIP (HNRNP Q) is also part of the proximal 6q loci that have been shown to be deleted in 20 individuals with moderate to severe NDDs (Engwerda et al., 2018).

6.1.8 hnRNP K

Au-Kline syndrome (AKS) or also known as Okamoto syndrome (Phenotype MIM number: 616580), named after the clinicians who first described the pathology (Au et al., 2018; Okamoto, 2019) and characterized by intellectual disability, facial dysmorphisms, and skeletal malformations is caused by mutation in *hnRNP K* and has therefore been added to the HNRNP-RNDD list (Gillentine et al., 2021). The identified *de novo* variants include deletion of a region encompassing hnRNP K (3 individuals), 3 frameshift variants from which two have been experimentally proven to lead to mRNA degradation of the mutant mRNA by NMD and 1 missense mutation, indicating that *hnRNP K* haploinsufficiency is driving neurodevelopmental phenotypes (Lange et al., 2016; Miyake et al., 2017; Au et al., 2018; Okamoto, 2019; Maystadt et al., 2020).

6.2 Neurodegenerative disorders

6.2.1 FTLD-ALS spectrum

To date, genetic studies have linked 4 hnRNPs [A1, A2/B1, FUS (hnRNP P2) and TDP-43] to neurodegenerative diseases, including Amyotrophic lateral sclerosis (ALS), Frontotemporal dementia (FTD) and Frontotemporal lobar degeneration (FTLD) that form a clinical disease continuum from motor neuron degenerative disease to dementia (Van Langenhove et al., 2012; Purice and Taylor, 2018). Given that TDP-43 and FUS (hnRNP P2) are key pathological proteins in FTLD-ALS spectrum, they represent the most extensively studied hnRNP proteins associated with neurodegenerative disorders (Bampton et al., 2020).

Although TDP-43 cytoplasmic and nuclear inclusions have been recognized as hallmarks of both FTD and ALS for a long time, it is now shown that genetic variants in TDP-43 account for 1% of all ALS cases and a small number of FTD cases (Sreedharan et al., 2008; Keating et al., 2022). Most of the identified ALS-FTD mutations are missense variants in the TDP-43 C-terminal low complexity domain (LCD) that is involved in protein–protein interaction and phase separation (Keating et al., 2022). The pathogenic effect of those TDP-43 variants has been associated to loss of the nuclear function of TDP-43 as well as gain-of-function in the cytoplasm where it sequesters mRNAs in inclusions (Halliday et al., 2012; Wood et al., 2021). Notably, TDP-43 does not operate independently to facilitate neurodegeneration. Indeed, multiple mass spectrometry analyses have uncovered a close interaction between TDP-43 and numerous hnRNP members [A0, A1, A2/B1, A3, DL, C, E1 (PCBP1), E2 (PCBP2), G (RBMX), H1, I (PTBP1), K, M, P2 (FUS), Q, R, U, UL1, and UL2] (Freibaum et al., 2010; Romano and Buratti, 2013; García Morato et al., 2023). Among these 19 hnRNPs, 11 have been identified as TDP-43 interactors in at least two independent studies. As this intricate interplay between TDP-43 and other hnRNPs plays a critical role in co-regulating RNA splicing targets (see section 4), and as expression levels of certain hnRNPs vary significantly among individuals with FTLD-TDP and control patients (Mohagheghi et al., 2016), TDP-43-hnRNPs cooperation could be central in ALS-FTD disorder. To note, although not associated to any TDP-43 variants, FTLD-TDP, or frontotemporal lobar degeneration with TDP-43 pathology, that is a subtype of FTD, is characterized by the presence of abnormal accumulations of aggregated cytoplasmic TDP-43 in neurons and glia (Chen-Plotkin et al., 2010).

Mutations in hnRNP P2 (FUS) have been identified in approximately 1% of all ALS cases. In the case of FTD, the genetic and pathological involvement of hnRNP P2 (FUS) is still debated (Josephs et al., 2011; Gami-Patel et al., 2016; Nolan et al., 2016; Ishigaki and Sobue, 2018; Kwok et al., 2020). Mutations associated with ALS are distributed all along the *hnRNP P2* (*FUS*) gene. However, there is a cluster of variants in the C-terminal region encompassing the PY-NLS (495–526 aa), whose pathogenicity has been linked to an abnormal accumulation of hnRNP P2 (FUS) in the cytosol (Khalil et al., 2024). As such, FUS-mediated toxicity and associated neurodegeneration is predominantly associated with gain-of-function mechanisms (Sun et al., 2015; Suzuki and Matsuoka, 2015; Scekic-Zahirovic et al., 2016; Sharma et al., 2016; Devoy et al., 2017; Sama et al., 2017; López-Erauskin et al., 2018; An et al., 2019; Tsai et al., 2020). Like TDP-43, hnRNP P2 (FUS) interacts with many hnRNPs [A1, A2/B1, A3, C, D, G (RBMX), H1, H2, K, M, R, U, UL1, hnRNP P2 (FUS) itself] (Kamelgarn et al., 2016; Reber et al., 2016). hnRNPs represent a quarter of the high-confidence hnRNP P2 (FUS) interactors, suggesting a potential collaboration between hnRNPs and hnRNP P2 (FUS) to bind mRNA (Reber et al., 2016). Accordingly, several hnRNPs, like hnRNP A1, C, D, and G (RBMX) were identified in some but not all hnRNP P2 (FUS) pathological deposits in specific brain regions like entorhinal cortex region or hippocampus in postmortem FTD brain (Gami-Patel et al., 2016).

Mutations occurring within the LCD of hnRNP A1 and hnRNP A2/B1 have been linked to both familial and sporadic cases of ALS. However, they represent a very small subset (less than 1%) of both familial and sporadic ALS cases (Bampton et al., 2020; Khalil et al., 2024). Wild-type hnRNP A2/B1 and hnRNP A1 proteins tend to form self-seeding fibrils, a tendency worsened by disease mutations. The identified missense mutations speed up fibril formation and leading to excessive incorporation of hnRNP A2 and hnRNP A1 into stress granules. They also induce the formation of cytoplasmic inclusions in animal models, mimicking human pathology (Kim et al., 2013). However, it is worth noting that mutations in this region are more frequently associated with the pleiotropic degenerative disorder known as multisystem proteinopathy (Kim et al., 2013; Le Ber et al., 2014; Suzuki et al., 2023). Patients with multiple sclerosis (MS) commonly exhibit genomic single nucleotide variants (SNVs) within the nucleocytoplasmic transport M9 domain of the *hnRNP A1* gene, indicating that disrupted hnRNP A1-mediated nucleocytoplasmic transport may contribute to MS pathology (Lee and Levin, 2014). In samples from MS patients, immunofluorescence analysis demonstrates a significant colocalization of hnRNP A1 and TDP-43 within the cytoplasm of neurons in the brain, contrasting with controls (Salapa et al., 2020). Moreover, RNA sequencing in MS brains (Salapa et al., 2024) revealed differential expression of around 550 genes between control and MS samples. 80% of these differentially expressed transcripts had previously shown binding to hnRNP A1. Overall, the findings endorse the notion that issues with RNA regulation stemming from dysfunctional hnRNP A1 play a pivotal role in driving neurodegeneration in MS (Salapa et al., 2024). In addition, hnRNP A1 and hnRNP B1 levels have been shown to be increased in the cerebrospinal fluid of MS patients compared to patients with other neurological disorders (Sueoka et al., 2004). Although hnRNP A3 belongs to the same sub-family as hnRNP A1 and hnRNP A2/B1, it has not yet been linked to multiple sclerosis (Low et al., 2021).

6.2.2 hnRNPs and Alzheimer's disease

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive cognitive decline, memory loss, and neuropathological features including the accumulation of amyloid-beta plaques and tangled proteins called Tau fibrils. Multiple lines of evidence have linked hnRNPs to AD: (1) Although there are no reported cases of hnRNP A1-related mutations that lead to AD (Clarke et al., 2021), expression of hnRNP A1 is markedly diminished in the brains of individuals with Alzheimer's disease (Berson et al., 2012). This could lead to direct impairment of APP and Tau proteins as hnRNP A1 binding sites have been found in introns 6 and 8 of the *APP* pre-mRNA (Donev et al., 2007) and that hnRNP A1 regulates the splicing of Tau (Liu et al., 2020). (2) A proteomic study of 16 human brain tissues from AD patients and age-matched controls revealed a significantly increased expression of hnRNPs C, K, L, M, R, U and UL2, in AD, while the expression level of TDP-43, and hnRNPs AB, A3, DL, and E1 (PCBP1) were decreased (Zhang et al., 2018). (3) Cytoplasmic mis-localization of hnRNP K in neurons of the dentate nucleus was shown in AD postmortem brain samples (Sidhu et al., 2022). Of note, similar hnRNP K mislocalization has been observed in FTLD brain tissue (Sidhu et al., 2022). (4) hnRNP A/B loss in AD is not due to Aβ or tau but rather to deficits in cholinergic signaling and likely triggers the large changes in alternative splicing observed in AD (Berson et al., 2012). (5) hnRNP C competes with FMRP for mRNA binding sites, leading to the upregulation of APP synthesis (Lee et al., 2010). (6) Computational analysis shows hnRNP Q lncRNAs crucial in protein folding and AD association (Ashraf et al., 2019).

7 Discussion

Despite significant progress in identifying and classifying hnRNP members, defining their functions in the context of the brain remains challenging due to their multifunctional nature. While splicing regulation is the most well-described function, others remain poorly understood, particularly their roles in the cytoplasm under physiological or pathological conditions such as in ALS and FTD. Recent evidence has highlighted their significance in neurodevelopmental disorders, although they have been less extensively investigated compared to their role in cancer (Figure 1), where hnRNPs serve as promising biomarkers (Zhou et al., 2021; Li et al., 2022; Lu et al., 2022; Mo et al., 2022; Tuersun et al., 2023). Indeed, there is a 41-year gap between the initial discovery of hnRNP proteins (Beyer et al., 1977) and the initiation of the first clinical trial in 2018 (Natural History Study of hnRNP-related Disorders; ClinicalTrials.gov ID: NCT03492060), involving individuals with hnRNP genetic variants and associated neurological comorbidities (Figure 1). Concomitantly to those first clinical trials, the establishment of two HNRNP family foundations, one in the USA (see text footnote 1) and one in Japan (see text footnote 2), gave a significant boost to the hnRNPs research and promises major breakthroughs, as it is in the field of cancer.

hnRNP members not only regulate their own expression but also that of other hnRNP proteins, whether closely related or not, revealing the complexity of interactions within this RNA-binding protein family. Increasing evidence suggests that hnRNPs can compensate for certain functions of closely related members. This observation extends to another organ with high cellular and molecular similarities to the brain: the testis (Matos et al., 2021). For instance, RBMXL2 compensates for the absence of hnRNP G (RBMX) in somatic cells (Siachisumo et al., 2023). This finding aligns with a recent model proposing that RBMXL2 takes over hnRNP G (RBMX) function during meiosis due to the transcriptional inactivation of the X chromosome (Ehrmann et al., 2019). The intricate interplay network among hnRNP proteins not only complicates our understanding of the mechanisms underlying neurological disorders, but also meets a challenge for the development of targeted therapy. On the other hand, the functional redundancy among hnRNP proteins also instills hope for potential treatments using ASO therapeutic strategy, as recently commented by Kelvington and Abel (2023). Finally, it is noteworthy mentioning that the use of hnRNP as new tool for therapeutic strategies is starting to emerge. Indeed, novel CRISPR-Cas9 applications aim to induce specific RNA splicing by fusing a RNA-targeted CAS9 (dCasRx) to hnRNPs, such as hnRNP A1 (Konermann et al., 2018). This was used in patient-derived iPSCs to modify alternative splicing in the *MAPT* gene, aiming to counteract pathogenic mutations associated with Frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17). First results in human cortical neurons show that this strategy successfully restored the balance between the two major Tau isoforms, Tau-4R and Tau-3R (Konermann et al., 2018). This highlights the need for a sound understanding of the physiological function of hnRNPs and the mechanisms related to the alteration of their normal function in pathological conditions.

Author contributions

PT: Writing – original draft. SF: Writing – review & editing. JG: Writing – review & editing, Supervision.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This work was supported by grants from the French state funds through the Agence Nationale de la Recherche [ANR-10-IDEX-0002-02 and ANR-10-LABX-0030-INRT, SFRI-STRAT'US project (ANR 20-SFRI-0012)], INSERM/CNRS and University of Strasbourg. PT is supported by the "*Ministère de l'Enseignement supérieur et de la Recherche et de l'innovation*" (MESRI), the EUR IMCBio (ANR-17-EURE-0023), and the University of Strasbourg. SF is supported by the French National Research Agency (ANR-22-CE92-0072, RETRO-RBMX). JG is an INSERM investigator.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2024.1411639/full#supplementary-material

References

Abdul-Manan, N., O'Malley, S. M., and Williams, K. R. (1996). Origins of binding specificity of the A1 heterogeneous nuclear ribonucleoprotein. *Biochemistry* 35, 3545–3554. doi: 10.1021/bi952298p

Adamson, B., Smogorzewska, A., Sigoillot, F. D., King, R. W., and Elledge, S. J. (2012). A genome-wide homologous recombination screen identifies the RNA-binding protein RBMX as a component of the DNA-damage response. *Nat. Cell Biol.* 14, 318–328. doi: 10.1038/ncb2426

Aebersold, R., Agar, J. N., Amster, I. J., Baker, M. S., Bertozzi, C. R., Boja, E. S., et al. (2018). How many human proteoforms are there? *Nat. Chem. Biol.* 14, 206–214. doi: 10.1038/nchembio.2576

Agra Almeida Quadros, A. R., Li, Z., Wang, X., Ndayambaje, I. S., Aryal, S., Ramesh, N., et al. (2024). Cryptic splicing of stathmin-2 and UNC13A mRNAs is a pathological hallmark of TDP-43-associated Alzheimer's disease. *Acta Neuropathol.* 147:9. doi: 10.1007/s00401-023-02655-0

Alfano, L., Caporaso, A., Altieri, A., Dell'aquila, M., Landi, C., Bini, L., et al. (2019). Depletion of the RNA binding protein HNRNPD impairs homologous recombination by inhibiting DNA-end resection and inducing R-loop accumulation. *Nucleic Acids Res.* 47, 4068–4085. doi: 10.1093/nar/gkz076

An, H., Skelt, L., Notaro, A., Highley, J. R., Fox, A. H., La Bella, V., et al. (2019). ALS-linked FUS mutations confer loss and gain of function in the nucleus by promoting excessive formation of dysfunctional paraspeckles. *Acta Neuropathol. Commun.* 7:7. doi: 10.1186/s40478-019-0658-x

Andersen, J. S., Lyon, C. E., Fox, A. H., Leung, A. K., Lam, Y. W., Steen, H., et al. (2002). Directed proteomic analysis of the human nucleolus. *Curr. Biol.* 12, 1–11. doi: 10.1016/S0960-9822(01)00650-9

Asadi, M. R., Sadat Moslehian, M., Sabaie, H., Jalaiei, A., Ghafouri-Fard, S., Taheri, M., et al. (2021). Stress granules and neurodegenerative disorders: a scoping review. *Front. Aging Neurosci.* 13:650740. doi: 10.3389/fnagi.2021.650740

Ashraf, G. M., Ganash, M., and Athanasios, A. (2019). Computational analysis of non-coding RNAs in Alzheimer's disease. *Bioinformation* 15, 351–357. doi: 10.6026/97320630015351

Au, P. Y. B., Goedhart, C., Ferguson, M., Breckpot, J., Devriendt, K., Wierenga, K., et al. (2018). Phenotypic spectrum of Au-Kline syndrome: a report of six new cases and review of the literature. *Eur. J. Hum. Genet.* 26, 1272–1281. doi: 10.1038/s41431-018-0187-2

Aviner, R., Hofmann, S., Elman, T., Shenoy, A., Geiger, T., Elkon, R., et al. (2017). Proteomic analysis of polyribosomes identifies splicing factors as potential regulators of translation during mitosis. *Nucleic Acids Res.* 45, 5945–5957. doi: 10.1093/nar/gkx326

Ayala, Y. M., De Conti, L., Avendaño-Vázquez, S. E., Dhir, A., Romano, M., D'ambrogio, A., et al. (2011). TDP-43 regulates its mRNA levels through a negative feedback loop. *EMBO J.* 30, 277–288. doi: 10.1038/emboj.2010.310

Bain, J. M., Cho, M. T., Telegrafi, A., Wilson, A., Brooks, S., Botti, C., et al. (2016). Variants in HNRNPH2 on the X chromosome are associated with a neurodevelopmental disorder in females. *Am. J. Hum. Genet.* 99, 728–734. doi: 10.1016/j.ajhg.2016.06.028

Bain, J. M., Thornburg, O., Pan, C., Rome-Martin, D., Boyle, L., Fan, X., et al. (2021). Detailed clinical and psychological phenotype of the X-linked HNRNPH2-related neurodevelopmental disorder. *Neurol Genet* 7:e551. doi: 10.1212/NXG.0000000000000551

Ballif, B. C., Rosenfeld, J. A., Traylor, R., Theisen, A., Bader, P. I., Ladda, R. L., et al. (2012). High-resolution array CGH defines critical regions and candidate genes for microcephaly, abnormalities of the corpus callosum, and seizure phenotypes in patients with microdeletions of 1q43q44. *Hum. Genet.* 131, 145–156. doi: 10.1007/s00439-011-1073-y

Bampton, A., Gittings, L. M., Fratta, P., Lashley, T., and Gatt, A. (2020). The role of hnRNPs in frontotemporal dementia and amyotrophic lateral sclerosis. *Acta Neuropathol.* 140, 599–623. doi: 10.1007/s00401-020-02203-0

Baughn, M. W., Melamed, Z., López-Erauskin, J., Beccari, M. S., Ling, K., Zuberi, A., et al. (2023). Mechanism of STMN2 cryptic splice-polyadenylation and its correction for TDP-43 proteinopathies. *Science* 379, 1140–1149. doi: 10.1126/science.abq5622

Berson, A., Barbash, S., Shaltiel, G., Goll, Y., Hanin, G., Greenberg, D. S., et al. (2012). Cholinergic-associated loss of hnRNP-A/B in Alzheimer's disease impairs cortical splicing and cognitive function in mice. *EMBO Mol. Med.* 4, 730–742. doi: 10.1002/emmm.201100995

Beyer, A. L., Christensen, M. E., Walker, B. W., and Lestourgeon, W. M. (1977). Identification and characterization of the packaging proteins of core 40S hnRNP particles. *Cell* 11, 127–138. doi: 10.1016/0092-8674(77)90323-3

Biamonti, G., and Riva, S. (1994). New insights into the auxiliary domains of eukaryotic RNA binding proteins. *FEBS Lett.* 340, 1–8. doi: 10.1016/0014-5793(94)80162-2

Biamonti, G., Ruggiu, M., Saccone, S., Della Valle, G., and Riva, S. (1994). Two homologous genes, originated by duplication, encode the human hnRNP proteins A2 and A1. *Nucleic Acids Res.* 22, 1996–2002. doi: 10.1093/nar/22.11.1996

Bolinger, C., and Boris-Lawrie, K. (2009). Mechanisms employed by retroviruses to exploit host factors for translational control of a complicated proteome. *Retrovirology* 6:8. doi: 10.1186/1742-4690-6-8

Boudreault, S., Roy, P., Lemay, G., and Bisaillon, M. (2019). Viral modulation of cellular RNA alternative splicing: A new key player in virus-host interactions? *Wiley Interdiscip Rev RNA* 10:e1543. doi: 10.1002/wrna.1543

Bourgeois, B., Hutten, S., Gottschalk, B., Hofweber, M., Richter, G., Sternat, J., et al. (2020). Nonclassical nuclear localization signals mediate nuclear import of CIRBP. *Proc. Natl. Acad. Sci. USA* 117, 8503–8514. doi: 10.1073/pnas.1918944117

Boutz, P. L., Stoilov, P., Li, Q., Lin, C. H., Chawla, G., Ostrow, K., et al. (2007). A post-transcriptional regulatory switch in polypyrimidine tract-binding proteins reprograms alternative splicing in developing neurons. *Genes Dev.* 21, 1636–1652. doi: 10.1101/gad.1558107

Bramswig, N. C., Lüdecke, H. J., Hamdan, F. F., Altmüller, J., Beleggia, F., Elcioglu, N. H., et al. (2017). Heterozygous HNRNPU variants cause early onset epilepsy and severe intellectual disability. *Hum. Genet.* 136, 821–834. doi: 10.1007/s00439-017-1795-6

Brandão-Teles, C., Antunes, A., De Moraes Vrechi, T. A., and Martins-De-Souza, D. (2023). The roles of hnRNP family in the brain and brain-related disorders. *Mol. Neurobiol.* 61, 3578–3595. doi: 10.1007/s12035-023-03747-4

Briese, M., Saal-Bauernschubert, L., Ji, C., Moradi, M., Ghanawi, H., Uhl, M., et al. (2018). hnRNP R and its main interactor, the noncoding RNA 7SK, coregulate the axonal transcriptome of motoneurons. *Proc. Natl. Acad. Sci. USA* 115, E2859–e2868. doi: 10.1073/pnas.1721670115

Britton, S., Dernoncourt, E., Delteil, C., Froment, C., Schiltz, O., Salles, B., et al. (2014). DNA damage triggers SAF-A and RNA biogenesis factors exclusion from chromatin coupled to R-loops removal. *Nucleic Acids Res.* 42, 9047–9062. doi: 10.1093/nar/gku601

Brown, A. L., Wilkins, O. G., Keuss, M. J., Hill, S. E., Zanovello, M., Lee, W. C., et al. (2022). TDP-43 loss and ALS-risk SNPs drive mis-splicing and depletion of UNC13A. *Nature* 603, 131–137. doi: 10.1038/s41586-022-04436-3

Brunetti, J. E., Scolaro, L. A., and Castilla, V. (2015). The heterogeneous nuclear ribonucleoprotein K (hnRNP K) is a host factor required for dengue virus and Junín virus multiplication. *Virus Res.* 203, 84–91. doi: 10.1016/j.virusres.2015.04.001

Burnham, A. J., Gong, L., and Hardy, R. W. (2007). Heterogeneous nuclear ribonuclear protein K interacts with Sindbis virus nonstructural proteins and viral subgenomic mRNA. *Virology* 367, 212–221. doi: 10.1016/j.virol.2007.05.008

Busch, A., and Hertel, K. J. (2012). Evolution of SR protein and hnRNP splicing regulatory factors. *Wiley Interdiscip Rev RNA* 3, 1–12. doi: 10.1002/wrna.100

Calabretta, S., and Richard, S. (2015). Emerging roles of disordered sequences in RNA-binding proteins. *Trends Biochem. Sci.* 40, 662–672. doi: 10.1016/j.tibs.2015.08.012

Caliebe, A., Kroes, H. Y., van der Smagt, J. J., Martin-Subero, J. I., Tönnies, H., van 't Slot, R., et al. (2010). Four patients with speech delay, seizures and variable corpus callosum thickness sharing a 0.440 Mb deletion in region 1q44 containing the HNRPU gene. *Eur. J. Med. Genet.* 53, 179–185. doi: 10.1016/j.ejmg.2010.04.001

Cao, W., Razanau, A., Feng, D., Lobo, V. G., and Xie, J. (2012). Control of alternative splicing by forskolin through hnRNP K during neuronal differentiation. *Nucleic Acids Res.* 40, 8059–8071. doi: 10.1093/nar/gks504

Cappelli, S., Romano, M., and Buratti, E. (2018). Systematic analysis of gene expression profiles controlled by hnRNP Q and hnRNP R, two closely related human RNA binding proteins implicated in mRNA processing mechanisms. *Front. Mol. Biosci.* 5:79. doi: 10.3389/fmolb.2018.00079

Cardoso-Moreira, M., Halbert, J., Valloton, D., Velten, B., Chen, C., Shao, Y., et al. (2019). Gene expression across mammalian organ development. *Nature* 571, 505–509. doi: 10.1038/s41586-019-1338-5

Cartegni, L., Maconi, M., Morandi, E., Cobianchi, F., Riva, S., and Biamonti, G. (1996). hnRNP A1 selectively interacts through its Gly-rich domain with different RNA-binding proteins. *J. Mol. Biol.* 259, 337–348. doi: 10.1006/jmbi.1996.0324

Chaudhury, A., Chander, P., and Howe, P. H. (2010). Heterogeneous nuclear ribonucleoproteins (hnRNPs) in cellular processes: focus on hnRNP E1's multifunctional regulatory roles. *RNA* 16, 1449–1462. doi: 10.1261/rna.2254110

Chen, T. M., Lai, M. C., Li, Y. H., Chan, Y. L., Wu, C. H., Wang, Y. M., et al. (2019). hnRNPM induces translation switch under hypoxia to promote colon cancer development. *EBioMedicine* 41, 299–309. doi: 10.1016/j.ebiom.2019.02.059

Chen-Plotkin, A. S., Lee, V. M., and Trojanowski, J. Q. (2010). TAR DNA-binding protein 43 in neurodegenerative disease. *Nat. Rev. Neurol.* 6, 211–220. doi: 10.1038/nrneurol.2010.18

Cheunim, T., Zhang, J., Milligan, S. G., Mcphillips, M. G., and Graham, S. V. (2008). The alternative splicing factor hnRNP A1 is up-regulated during virus-infected epithelial cell differentiation and binds the human papillomavirus type 16 late regulatory element. *Virus Res.* 131, 189–198. doi: 10.1016/j.virusres.2007.09.006

Cho, S. J., Jung, Y. S., and Chen, X. (2013). Poly (C)-binding protein 1 regulates p63 expression through mRNA stability. *PLoS One* 8:e71724. doi: 10.1371/journal.pone.0071724

Choi, Y. D., and Dreyfuss, G. (1984). Isolation of the heterogeneous nuclear RNA-ribonucleoprotein complex (hnRNP): a unique supramolecular assembly. *Proc. Natl. Acad. Sci. USA* 81, 7471–7475. doi: 10.1073/pnas.81.23.7471

Cichocka, M., Karlik, A., Plewka, P., Gawade, K., Stępień, A., Świergiel, P., et al. (2022). The novel role of hnRNP UL1 in human cell nucleoli. *Int. J. Biol. Sci.* 18, 4809–4823. doi: 10.7150/ijbs.75084

Clarke, J. P., Thibault, P. A., Salapa, H. E., and Levin, M. C. (2021). A comprehensive analysis of the role of hnRNP A1 function and dysfunction in the pathogenesis of neurodegenerative disease. *Front. Mol. Biosci.* 8:659610. doi: 10.3389/fmolb.2021.659610

Corre, M., Boehm, V., Besic, V., Kurowska, A., Viry, A., Mohammad, A., et al. (2023). Alternative splicing induced by bacterial pore-forming toxins sharpens CIRBP-mediated cell response to Listeria infection. *Nucleic Acids Res.* 51, 12459–12475. doi: 10.1093/nar/gkad1033

Corsi, A., Bombieri, C., Valenti, M. T., and Romanelli, M. G. (2022). Tau Isoforms: Gaining Insight into MAPT Alternative Splicing. *International Journal Of Molecular Sciences* 23, 15383. doi: 10.3390/ijms232315383

D'ambrogio, A., Buratti, E., Stuani, C., Guarnaccia, C., Romano, M., Ayala, Y. M., et al. (2009). Functional mapping of the interaction between TDP-43 and hnRNP A2 *in vivo*. *Nucleic Acids Res.* 37, 4116–4126. doi: 10.1093/nar/gkp342

Dawicki-Mckenna, J. M., Felix, A. J., Waxman, E. A., Cheng, C., Amado, D. A., Ranum, P. T., et al. (2023). Mapping PTBP2 binding in human brain identifies SYNGAP1 as a target for therapeutic splice switching. *Nat. Commun.* 14:2628. doi: 10.1038/s41467-023-38273-3

Decorsière, A., Cayrel, A., Vagner, S., and Millevoi, S. (2011). Essential role for the interaction between hnRNP H/F and a G quadruplex in maintaining p53 pre-mRNA 3′-end processing and function during DNA damage. *Genes Dev.* 25, 220–225. doi: 10.1101/gad.607011

Depienne, C., Nava, C., Keren, B., Heide, S., Rastetter, A., Passemard, S., et al. (2017). Genetic and phenotypic dissection of 1q43q44 microdeletion syndrome and neurodevelopmental phenotypes associated with mutations in ZBTB18 and HNRNPU. *Hum. Genet.* 136, 463–479. doi: 10.1007/s00439-017-1772-0

Dettori, L. G., Torrejon, D., Chakraborty, A., Dutta, A., Mohamed, M., Papp, C., et al. (2021). A tale of loops and tails: the role of intrinsically disordered protein regions in R-loop recognition and phase separation. *Front. Mol. Biosci.* 8:691694. doi: 10.3389/fmolb.2021.691694

Devoy, A., Kalmar, B., Stewart, M., Park, H., Burke, B., Noy, S. J., et al. (2017). Humanized mutant FUS drives progressive motor neuron degeneration without aggregation in 'FUSDelta14' knockin mice. *Brain* 140, 2797–2805. doi: 10.1093/brain/awx248

Dhillon, P., Tandra, V. N., Chorghade, S. G., Namsa, N. D., Sahoo, L., and Rao, C. D. (2018). Cytoplasmic relocalization and colocalization with viroplasms of host cell proteins, and their role in rotavirus infection. *J. Virol.* 92:e00612-18. doi: 10.1128/JVI.00612-18

Ding, J., Hayashi, M. K., Zhang, Y., Manche, L., Krainer, A. R., and Xu, R. M. (1999). Crystal structure of the two-RRM domain of hnRNP A1 (UP1) complexed with single-stranded telomeric DNA. *Genes Dev.* 13, 1102–1115. doi: 10.1101/gad.13.9.1102

Donev, R., Newall, A., Thome, J., and Sheer, D. (2007). A role for SC35 and hnRNPA1 in the determination of amyloid precursor protein isoforms. *Mol. Psychiatry* 12, 681–690. doi: 10.1038/sj.mp.4001971

Dowling, D., Nasr-Esfahani, S., Tan, C. H., O'Brien, K., Howard, J. L., Jans, D. A., et al. (2008). HIV-1 infection induces changes in expression of cellular splicing factors that regulate alternative viral splicing and virus production in macrophages. *Retrovirology* 5:18. doi: 10.1186/1742-4690-5-18

Dreyfuss, G., Kim, V. N., and Kataoka, N. (2002). Messenger-RNA-binding proteins and the messages they carry. *Nat. Rev. Mol. Cell Biol.* 3, 195–205. doi: 10.1038/nrm760

Dreyfuss, G., Matunis, M. J., Piñol-Roma, S., and Burd, C. G. (1993). hnRNP proteins and the biogenesis of mRNA. *Annu. Rev. Biochem.* 62, 289–321. doi: 10.1146/annurev.bi.62.070193.001445

Dudman, J., and Qi, X. (2020). Stress granule dysregulation in amyotrophic lateral sclerosis. *Front. Cell. Neurosci.* 14:598517. doi: 10.3389/fncel.2020.598517

Duijkers, F. A., Mcdonald, A., Janssens, G. E., Lezzerini, M., Jongejan, A., Van Koningsbruggen, S., et al. (2019). HNRNPR variants that impair Homeobox gene expression drive developmental disorders in humans. *Am. J. Hum. Genet.* 104, 1040–1059. doi: 10.1016/j.ajhg.2019.03.024

Durkin, A., Albaba, S., Fry, A. E., Morton, J. E., Douglas, A., Beleza, A., et al. (2020). Clinical findings of 21 previously unreported probands with HNRNPU-related syndrome and comprehensive literature review. *Am. J. Med. Genet. A* 182, 1637–1654. doi: 10.1002/ajmg.a.61599

Ehrmann, I., Crichton, J. H., Gazzara, M. R., James, K., Liu, Y., Grellscheid, S. N., et al. (2019). An ancient germ cell-specific RNA-binding protein protects the germline from cryptic splice site poisoning. *eLife* 8:e39304. doi: 10.7554/eLife.39304

Ehrmann, I., Dalgliesh, C., Tsaousi, A., Paronetto, M. P., Heinrich, B., Kist, R., et al. (2008). Haploinsufficiency of the germ cell-specific nuclear RNA binding protein hnRNP G-T prevents functional spermatogenesis in the mouse. *Hum. Mol. Genet.* 17, 2803–2818. doi: 10.1093/hmg/ddn179

Elliott, D. J. (2004). The role of potential splicing factors including RBMY, RBMX, hnRNPG-T and STAR proteins in spermatogenesis. *Int. J. Androl.* 27, 328–334. doi: 10.1111/j.1365-2605.2004.00496.x

Elliott, D. J., Dalgliesh, C., Hysenaj, G., and Ehrmann, I. (2019). RBMX family proteins connect the fields of nuclear RNA processing, disease and sex chromosome biology. *Int. J. Biochem. Cell Biol.* 108, 1–6. doi: 10.1016/j.biocel.2018.12.014

Elliott, D. J., Oghene, K., Makarov, G., Makarova, O., Hargreave, T. B., Chandley, A. C., et al. (1998). Dynamic changes in the subnuclear organisation of pre-mRNA splicing proteins and RBM during human germ cell development. *J. Cell Sci.* 111, 1255–1265. doi: 10.1242/jcs.111.9.1255

Elliott, D. J., Venables, J. P., Newton, C. S., Lawson, D., Boyle, S., Eperon, I. C., et al. (2000). An evolutionarily conserved germ cell-specific hnRNP is encoded by a retrotransposed gene. *Hum. Mol. Genet.* 9, 2117–2124. doi: 10.1093/hmg/9.14.2117

Engeland, C. E., Brown, N. P., Börner, K., Schümann, M., Krause, E., Kaderali, L., et al. (2014). Proteome analysis of the HIV-1 gag interactome. *Virology* 460-461, 194–206. doi: 10.1016/j.virol.2014.04.038

Engwerda, A., Frentz, B., Den Ouden, A. L., Flapper, B. C. T., Swertz, M. A., Gerkes, E. H., et al. (2018). The phenotypic spectrum of proximal 6q deletions based on a large cohort derived from social media and literature reports. *Eur. J. Hum. Genet.* 26, 1478–1489. doi: 10.1038/s41431-018-0172-9

Evans, J. R., Mitchell, S. A., Spriggs, K. A., Ostrowski, J., Bomsztyk, K., Ostarek, D., et al. (2003). Members of the poly (rC) binding protein family stimulate the activity of the c-myc internal ribosome entry segment in vitro and in vivo. *Oncogene* 22, 8012–8020. doi: 10.1038/sj.onc.1206645

Ezkurdia, I., Del Pozo, A., Frankish, A., Rodriguez, J. M., Harrow, J., Ashman, K., et al. (2012). Comparative proteomics reveals a significant bias toward alternative protein isoforms with conserved structure and function. *Mol. Biol. Evol.* 29, 2265–2283. doi: 10.1093/molbev/mss100

Feng, X., and Li, H. (2021). Higher rates of processed pseudogene Acquisition in Humans and Three Great Apes Revealed by Long-read assemblies. *Mol. Biol. Evol.* 38, 2958–2966. doi: 10.1093/molbev/msab062

Feng, H., Moakley, D. F., Chen, S., Mckenzie, M. G., Menon, V., and Zhang, C. (2021). Complexity and graded regulation of neuronal cell-type-specific alternative splicing revealed by single-cell RNA sequencing. *Proc. Natl. Acad. Sci. USA* 118:e2013056118. doi: 10.1073/pnas.2013056118

Fialcowitz, E. J., Brewer, B. Y., Keenan, B. P., and Wilson, G. M. (2005). A hairpin-like structure within an AU-rich mRNA-destabilizing element regulates trans-factor binding selectivity and mRNA decay kinetics. *J. Biol. Chem.* 280, 22406–22417. doi: 10.1074/jbc.M500618200

Firth, H. V., Richards, S. M., Bevan, A. P., Clayton, S., Corpas, M., Rajan, D., et al. (2009). DECIPHER: database of chromosomal imbalance and phenotype in humans using Ensembl resources. *Am. J. Hum. Genet.* 84, 524–533. doi: 10.1016/j.ajhg.2009.03.010

Freibaum, B. D., Chitta, R. K., High, A. A., and Taylor, J. P. (2010). Global analysis of TDP-43 interacting proteins reveals strong association with RNA splicing and translation machinery. *J. Proteome Res.* 9, 1104–1120. doi: 10.1021/pr901076y

Friend, L. R., Han, S. P., Rothnagel, J. A., and Smith, R. (2008). Differential subnuclear localisation of hnRNPs A/B is dependent on transcription and cell cycle stage. *Biochim. Biophys. Acta* 1783, 1972–1980. doi: 10.1016/j.bbamcr.2008.05.021

Fukuda, N., Fukuda, T., Percipalle, P., Oda, K., Takei, N., Czaplinski, K., et al. (2023). Axonal mRNA binding of hnRNP A/B is crucial for axon targeting and maturation of olfactory sensory neurons. *Cell Rep.* 42:112398. doi: 10.1016/j.celrep.2023.112398

Fukuda, T., Naiki, T., Saito, M., and Irie, K. (2009). hnRNP K interacts with RNA binding motif protein 42 and functions in the maintenance of cellular ATP level during stress conditions. *Genes Cells* 14, 113–128. doi: 10.1111/j.1365-2443.2008.01256.x

Gagné, M., Deshaies, J. E., Sidibé, H., Benchaar, Y., Arbour, D., Dubinski, A., et al. (2021). hnRNP A1B, a splice variant of HNRNPA1, is spatially and temporally regulated. *Front. Neurosci.* 15:724307. doi: 10.3389/fnins.2021.724307

Gamarnik, A. V., and Andino, R. (1997). Two functional complexes formed by KH domain containing proteins with the 5′ noncoding region of poliovirus RNA. *RNA* 3, 882–892

Gami-Patel, P., Bandopadhyay, R., Brelstaff, J., Revesz, T., and Lashley, T. (2016). The presence of heterogeneous nuclear ribonucleoproteins in frontotemporal lobar degeneration with FUS-positive inclusions. *Neurobiol. Aging* 46, 192–203. doi: 10.1016/j.neurobiolaging.2016.07.004

García Morato, J., Gloeckner, C. J., and Kahle, P. J. (2023). Proteomics elucidating physiological and pathological functions of TDP-43. *Proteomics* 23:e2200410. doi: 10.1002/pmic.202200410

Ge, Z., Quek, B. L., Beemon, K. L., and Hogg, J. R. (2016). Polypyrimidine tract binding protein 1 protects mRNAs from recognition by the nonsense-mediated mRNA decay pathway. *eLife* 5:e11155. doi: 10.7554/eLife.11155

Geissler, R., Simkin, A., Floss, D., Patel, R., Fogarty, E. A., Scheller, J., et al. (2016). A widespread sequence-specific mRNA decay pathway mediated by hnRNPs A1 and A2/B1. *Genes Dev.* 30, 1070–1085. doi: 10.1101/gad.277392.116

Geuens, T., Bouhy, D., and Timmerman, V. (2016). The hnRNP family: insights into their role in health and disease. *Hum. Genet.* 135, 851–867. doi: 10.1007/s00439-016-1683-5

Ghanawi, H., Hennlein, L., Zare, A., Bader, J., Salehi, S., Hornburg, D., et al. (2021). Loss of full-length hnRNP R isoform impairs DNA damage response in motoneurons by inhibiting Yb1 recruitment to chromatin. *Nucleic Acids Res.* 49, 12284–12305. doi: 10.1093/nar/gkab1120

Gillentine, M. A. (2023). Comment on Gustavson syndrome is caused by an in-frame deletion in RBMX associated with potentially disturbed SH3 domain interactions. *Eur. J. Hum. Genet.* 32, 253–256.

Gillentine, M. A., Wang, T., Hoekzema, K., Rosenfeld, J., Liu, P., Guo, H., et al. (2021). Rare deleterious mutations of HNRNP genes result in shared neurodevelopmental disorders. *Genome Med.* 13:63. doi: 10.1186/s13073-021-00870-6

Glinka, M., Herrmann, T., Funk, N., Havlicek, S., Rossoll, W., Winkler, C., et al. (2010). The heterogeneous nuclear ribonucleoprotein-R is necessary for axonal beta-actin mRNA translocation in spinal motor neurons. *Hum. Mol. Genet.* 19, 1951–1966. doi: 10.1093/hmg/ddq073

Godet, A. C., David, F., Hantelys, F., Tatin, F., Lacazette, E., Garmy-Susini, B., et al. (2019). IRES trans-acting factors, key actors of the stress response. *Int. J. Mol. Sci.* 20:924. doi: 10.3390/ijms20040924

Grabowski, P. J., and Black, D. L. (2001). Alternative RNA splicing in the nervous system. *Prog. Neurobiol.* 65, 289–308. doi: 10.1016/S0301-0082(01)00007-7

Grammatikakis, I., Zhang, P., Panda, A. C., Kim, J., Maudsley, S., Abdelmohsen, K., et al. (2016). Alternative splicing of neuronal differentiation factor TRF2 regulated by HNRNPH1/H2. *Cell Rep.* 15, 926–934. doi: 10.1016/j.celrep.2016.03.080

Gueroussov, S., Gonatopoulos-Pournatzis, T., Irimia, M., Raj, B., Lin, Z. Y., Gingras, A. C., et al. (2015). An alternative splicing event amplifies evolutionary differences between vertebrates. *Science* 349, 868–873. doi: 10.1126/science.aaa8381

Guil, S., Long, J. C., and Cáceres, J. F. (2006). hnRNP A1 relocalization to the stress granules reflects a role in the stress response. *Mol. Cell. Biol.* 26, 5744–5758. doi: 10.1128/MCB.00224-06

Guo, H., Duyzend, M. H., Coe, B. P., Baker, C., Hoekzema, K., Gerdts, J., et al. (2019). Genome sequencing identifies multiple deleterious variants in autism patients with more severe phenotypes. *Genet. Med.* 21, 1611–1620. doi: 10.1038/s41436-018-0380-2

Gustavson, K. H., Annerén, G., Malmgren, H., Dahl, N., Ljunggren, C. G., and Bäckman, H. (1993). New X-linked syndrome with severe mental retardation, severely impaired vision, severe hearing defect, epileptic seizures, spasticity, restricted joint mobility, and early death. *Am. J. Med. Genet.* 45, 654–658. doi: 10.1002/ajmg.1320450527

Halliday, G., Bigio, E. H., Cairns, N. J., Neumann, M., Mackenzie, I. R., and Mann, D. M. (2012). Mechanisms of disease in frontotemporal lobar degeneration: gain of function versus loss of function effects. *Acta Neuropathol.* 124, 373–382. doi: 10.1007/s00401-012-1030-4

Han, S. P., Kassahn, K. S., Skarshewski, A., Ragan, M. A., Rothnagel, J. A., and Smith, R. (2010a). Functional implications of the emergence of alternative splicing in hnRNP A/B transcripts. *RNA* 16, 1760–1768. doi: 10.1261/rna.2142810

Han, S. P., Tang, Y. H., and Smith, R. (2010b). Functional diversity of the hnRNPs: past, present and perspectives. *Biochem. J.* 430, 379–392. doi: 10.1042/BJ20100396

Harmsen, S., Buchert, R., Mayatepek, E., Haack, T. B., and Distelmaier, F. (2019). Bain type of X-linked syndromic mental retardation in boys. *Clin. Genet.* 95, 734–735. doi: 10.1111/cge.13524

Hatfield, J. T., Rothnagel, J. A., and Smith, R. (2002). Characterization of the mouse hnRNP A2/B1/B0 gene and identification of processed pseudogenes. *Gene* 295, 33–42. doi: 10.1016/S0378-1119(02)00800-4

He, Y., Brown, M. A., Rothnagel, J. A., Saunders, N. A., and Smith, R. (2005). Roles of heterogeneous nuclear ribonucleoproteins A and B in cell proliferation. *J. Cell Sci.* 118, 3173–3183. doi: 10.1242/jcs.02448

Helbig, K. L., Farwell Hagman, K. D., Shinde, D. N., Mroske, C., Powis, Z., Li, S., et al. (2016). Diagnostic exome sequencing provides a molecular diagnosis for a significant proportion of patients with epilepsy. *Genet. Med.* 18, 898–905. doi: 10.1038/gim.2015.186

Ho, J. J. D., Balukoff, N. C., Theodoridis, P. R., Wang, M., Krieger, J. R., Schatz, J. H., et al. (2020). A network of RNA-binding proteins controls translation efficiency to activate anaerobic metabolism. *Nat. Commun.* 11:2677. doi: 10.1038/s41467-020-16504-1

Huang, J., Chen, X. H., Wu, K., and Xu, P. (2005). Cloning and expression of a novel isoform of heterogeneous nuclear ribonucleoprotein-R. *Neuroreport* 16, 727–730. doi: 10.1097/00001756-200505120-00014

Huelga, S. C., Vu, A. Q., Arnold, J. D., Liang, T. Y., Liu, P. P., Yan, B. Y., et al. (2012). Integrative genome-wide analysis reveals cooperative regulation of alternative splicing by hnRNP proteins. *Cell Rep.* 1, 167–178. doi: 10.1016/j.celrep.2012.02.001

Hüttelmaier, S., Illenberger, S., Grosheva, I., Rüdiger, M., Singer, R. H., and Jockusch, B. M. (2001). Raver1, a dual compartment protein, is a ligand for PTB/hnRNPI and microfilament attachment proteins. *J. Cell Biol.* 155, 775–786. doi: 10.1083/jcb.200105044

Ilik, İ., Malszycki, M., Lübke, A. K., Schade, C., Meierhofer, D., and Aktaş, T. (2020). SON and SRRM2 are essential for nuclear speckle formation. *eLife* 9:e60579. doi: 10.7554/eLife.60579

Ishigaki, S., and Sobue, G. (2018). Importance of functional loss of FUS in FTLD/ALS. *Front. Mol. Biosci.* 5:44. doi: 10.3389/fmolb.2018.00044

Izaurralde, E., Jarmolowski, A., Beisel, C., Mattaj, I. W., Dreyfuss, G., and Fischer, U. (1997). A role for the M9 transport signal of hnRNP A1 in mRNA nuclear export. *J. Cell Biol.* 137, 27–35. doi: 10.1083/jcb.137.1.27

Jagdeo, J. M., Dufour, A., Fung, G., Luo, H., Kleifeld, O., Overall, C. M., et al. (2015). Heterogeneous nuclear ribonucleoprotein M facilitates enterovirus infection. *J. Virol.* 89, 7064–7078. doi: 10.1128/JVI.02977-14

Jepsen, W. M., Ramsey, K., Szelinger, S., Llaci, L., Balak, C., Belnap, N., et al. (2019). Two additional males with X-linked, syndromic mental retardation carry de novo mutations in HNRNPH2. *Clin. Genet.* 96, 183–185. doi: 10.1111/cge.13580

Johansson, J., Lidéus, S., Frykholm, C., Gunnarsson, C., Mihalic, F., Gudmundsson, S., et al. (2023). Gustavson syndrome is caused by an in-frame deletion in RBMX associated with potentially disturbed SH3 domain interactions. *Eur. J. Hum. Genet.* 32, 253–256. doi: 10.1038/s41431-023-01498-3

Josephs, K. A., Hodges, J. R., Snowden, J. S., Mackenzie, I. R., Neumann, M., Mann, D. M., et al. (2011). Neuropathological background of phenotypical variability in frontotemporal dementia. *Acta Neuropathol.* 122, 137–153. doi: 10.1007/s00401-011-0839-6

Kamelgarn, M., Chen, J., Kuang, L., Arenas, A., Zhai, J., Zhu, H., et al. (2016). Proteomic analysis of FUS interacting proteins provides insights into FUS function and its role in ALS. *Biochim. Biophys. Acta* 1862, 2004–2014. doi: 10.1016/j.bbadis.2016.07.015

Kamma, H., Horiguchi, H., Wan, L., Matsui, M., Fujiwara, M., Fujimoto, M., et al. (1999). Molecular characterization of the hnRNP A2/B1 proteins: tissue-specific expression and novel isoforms. *Exp. Cell Res.* 246, 399–411. doi: 10.1006/excr.1998.4323

Kamma, H., Portman, D. S., and Dreyfuss, G. (1995). Cell type-specific expression of hnRNP proteins. *Exp. Cell Res.* 221, 187–196. doi: 10.1006/excr.1995.1366

Kaplanis, J., Samocha, K. E., Wiel, L., Zhang, Z., Arvai, K. J., Eberhardt, R. Y., et al. (2020). Evidence for 28 genetic disorders discovered by combining healthcare and research data. *Nature* 586, 757–762. doi: 10.1038/s41586-020-2832-5

Kaur, R., Batra, J., Stuchlik, O., Reed, M. S., Pohl, J., Sambhara, S., et al. (2022). Heterogeneous ribonucleoprotein A1 (hnRNPA1) interacts with the nucleoprotein of the influenza a virus and impedes virus replication. *Viruses* 14:199. doi: 10.3390/v14020199

Keating, S. S., San Gil, R., Swanson, M. E. V., Scotter, E. L., and Walker, A. K. (2022). TDP-43 pathology: from noxious assembly to therapeutic removal. *Prog. Neurobiol.* 211:102229. doi: 10.1016/j.pneurobio.2022.102229

Kelvington, B. A., and Abel, T. (2023). hnRNPH2 gain-of-function mutations reveal therapeutic strategies and a role for RNA granules in neurodevelopmental disorders. *J. Clin. Invest.* 133:e171499. doi: 10.1172/JCI171499

Kemmerer, K., Fischer, S., and Weigand, J. E. (2018). Auto- and cross-regulation of the hnRNPs D and DL. *RNA* 24, 324–331. doi: 10.1261/rna.063420.117

Khalil, B., Linsenmeier, M., Smith, C. L., Shorter, J., and Rossoll, W. (2024). Nuclear-import receptors as gatekeepers of pathological phase transitions in ALS/FTD. *Mol. Neurodegener.* 19:8. doi: 10.1186/s13024-023-00698-1

Khan, M. I., Zhang, J., and Liu, Q. (2021). HnRNP F and hnRNP H1 regulate mRNA stability of amyloid precursor protein. *Neuroreport* 32, 824–832. doi: 10.1097/WNR.0000000000001662

Kiledjian, M., and Dreyfuss, G. (1992). Primary structure and binding activity of the hnRNP U protein: binding RNA through RGG box. *EMBO J.* 11, 2655–2664. doi: 10.1002/j.1460-2075.1992.tb05331.x

Kim, T. D., Kim, J. S., Kim, J. H., Myung, J., Chae, H. D., Woo, K. C., et al. (2005). Rhythmic serotonin N-acetyltransferase mRNA degradation is essential for the maintenance of its circadian oscillation. *Mol. Cell. Biol.* 25, 3232–3246. doi: 10.1128/MCB.25.8.3232-3246.2005

Kim, H. J., Kim, N. C., Wang, Y. D., Scarborough, E. A., Moore, J., Diaz, Z., et al. (2013). Mutations in prion-like domains in hnRNPA2B1 and hnRNPA1 cause multisystem proteinopathy and ALS. *Nature* 495, 467–473. doi: 10.1038/nature11922

Kim, D. Y., Kwak, E., Kim, S. H., Lee, K. H., Woo, K. C., and Kim, K. T. (2011). hnRNP Q mediates a phase-dependent translation-coupled mRNA decay of mouse Period3. *Nucleic Acids Res.* 39, 8901–8914. doi: 10.1093/nar/gkr605

Kim, S. H., Lee, K. H., Kim, D. Y., Kwak, E., Kim, S., and Kim, K. T. (2015). Rhythmic control of mRNA stability modulates circadian amplitude of mouse Period3 mRNA. *J. Neurochem.* 132, 642–656. doi: 10.1111/jnc.13027

Kishor, A., Ge, Z., and Hogg, J. R. (2019). hnRNP L-dependent protection of normal mRNAs from NMD subverts quality control in B cell lymphoma. *EMBO J.* 38:e99128. doi: 10.15252/embj.201899128

Klaric, J. A., Wüst, S., and Panier, S. (2021). New faces of old friends: emerging new roles of RNA-binding proteins in the DNA double-Strand break response. *Front. Mol. Biosci.* 8:668821. doi: 10.3389/fmolb.2021.668821

Kleinhenz, B., Fabienke, M., Swiniarski, S., Wittenmayer, N., Kirsch, J., Jockusch, B. M., et al. (2005). Raver2, a new member of the hnRNP family. *FEBS Lett.* 579, 4254–4258. doi: 10.1016/j.febslet.2005.07.001

Klim, J. R., Williams, L. A., Limone, F., Guerra San Juan, I., Davis-Dusenbery, B. N., Mordes, D. A., et al. (2019). ALS-implicated protein TDP-43 sustains levels of STMN2, a mediator of motor neuron growth and repair. *Nat. Neurosci.* 22, 167–179. doi: 10.1038/s41593-018-0300-4

Koike, Y., Pickles, S., Ayuso, V. E., Jansen-West, K., Qi, Y. A., Li, Z., et al. (2023). Correction: TDP-43 and other hnRNPs regulate cryptic exon inclusion of a key ALS/FTD risk gene, UNC13A. *PLoS Biol.* 21:e3002228. doi: 10.1371/journal.pbio.3002228

Konermann, S., Lotfy, P., Brideau, N. J., Oki, J., Shokhirev, M. N., and Hsu, P. D. (2018). Transcriptome engineering with RNA-targeting type VI-D CRISPR effectors. *Cell* 173, 665–676.e14. doi: 10.1016/j.cell.2018.02.033

Korff, A., Yang, X., O'donovan, K., Gonzalez, A., Teubner, B. J., Nakamura, H., et al. (2023). A murine model of hnRNPH2-related neurodevelopmental disorder reveals a mechanism for genetic compensation by HNRNPH1. *J. Clin. Invest.* 133:e160309. doi: 10.1172/JCI160309

Kreienkamp, H. J., Wagner, M., Weigand, H., Mcconkie-Rossell, A., Mcdonald, M., Keren, B., et al. (2022). Variant-specific effects define the phenotypic spectrum of HNRNPH2-associated neurodevelopmental disorders in males. *Hum. Genet.* 141, 257–272. doi: 10.1007/s00439-021-02412-x

Kuo, P. H., Doudeva, L. G., Wang, Y. T., Shen, C. K., and Yuan, H. S. (2009). Structural insights into TDP-43 in nucleic-acid binding and domain interactions. *Nucleic Acids Res.* 37, 1799–1808. doi: 10.1093/nar/gkp013

Kutluay, S. B., Emery, A., Penumutchu, S. R., Townsend, D., Tenneti, K., Madison, M. K., et al. (2019). Genome-wide analysis of heterogeneous nuclear ribonucleoprotein (hnRNP) binding to HIV-1 RNA reveals a key role for hnRNP H1 in alternative viral mRNA splicing. *J. Virol.* 93:e01048-19. doi: 10.1128/JVI.01048-19

Kwok, J. B., Loy, C. T., Dobson-Stone, C., and Halliday, G. M. (2020). The complex relationship between genotype, pathology and phenotype in familial dementia. *Neurobiol. Dis.* 145:105082. doi: 10.1016/j.nbd.2020.105082

Labranche, H., Dupuis, S., Ben-David, Y., Bani, M. R., Wellinger, R. J., and Chabot, B. (1998). Telomere elongation by hnRNP A1 and a derivative that interacts with telomeric repeats and telomerase. *Nat. Genet.* 19, 199–202. doi: 10.1038/575

Lander, E. S., Linton, L. M., Birren, B., Nusbaum, C., Zody, M. C., Baldwin, J., et al. (2001). Initial sequencing and analysis of the human genome. *Nature* 409, 860–921. doi: 10.1038/35057062

Lange, L., Pagnamenta, A. T., Lise, S., Clasper, S., Stewart, H., Akha, E. S., et al. (2016). A de novo frameshift in HNRNPK causing a kabuki-like syndrome with nodular heterotopia. *Clin. Genet.* 90, 258–262. doi: 10.1111/cge.12773

Le Ber, I., Van Bortel, I., Nicolas, G., Bouya-Ahmed, K., Camuzat, A., Wallon, D., et al. (2014). hnRNPA2B1 and hnRNPA1 mutations are rare in patients with "multisystem proteinopathy" and frontotemporal lobar degeneration phenotypes. *Neurobiol. Aging* 35:934.e935-6. doi: 10.1016/j.neurobiolaging.2013.09.016

Leduc, M. S., Chao, H. T., Qu, C., Walkiewicz, M., Xiao, R., Magoulas, P., et al. (2017). Clinical and molecular characterization of de novo loss of function variants in HNRNPU. *Am. J. Med. Genet. A* 173, 2680–2689. doi: 10.1002/ajmg.a.38388

Lee, E. K., Kim, H. H., Kuwano, Y., Abdelmohsen, K., Srikantan, S., Subaran, S. S., et al. (2010). hnRNP C promotes APP translation by competing with FMRP for APP mRNA recruitment to P bodies. *Nat. Struct. Mol. Biol.* 17, 732–739. doi: 10.1038/nsmb.1815

Lee, S., and Levin, M. (2014). Novel somatic single nucleotide variants within the RNA binding protein hnRNP A1 in multiple sclerosis patients. *F1000Res* 3:132. doi: 10.12688/f1000research.4436.2

Lee, S. J., Oses-Prieto, J. A., Kawaguchi, R., Sahoo, P. K., Kar, A. N., Rozenbaum, M., et al. (2018). hnRNPs interacting with mRNA localization motifs define axonal RNA regulons. *Mol. Cell. Proteomics* 17, 2091–2106. doi: 10.1074/mcp.RA118.000603

Lelieveld, S. H., Reijnders, M. R., Pfundt, R., Yntema, H. G., Kamsteeg, E. J., De Vries, P., et al. (2016). Meta-analysis of 2,104 trios provides support for 10 new genes for intellectual disability. *Nat. Neurosci.* 19, 1194–1196. doi: 10.1038/nn.4352

Leopoldino, A. M., Carregaro, F., Silva, C. H., Feitosa, O., Mancini, U. M., Freitas, J. M., et al. (2007). Sequence and transcriptional study of HNRPK pseudogenes, and expression and molecular modeling analysis of hnRNP K isoforms. *Genome* 50, 451–462. doi: 10.1139/G07-016

Li, Y., Wang, H., Wan, J., Ma, Q., Qi, Y., and Gu, Z. (2022). The hnRNPK/A1/R/U complex regulates gene transcription and translation and is a favorable prognostic biomarker for human colorectal adenocarcinoma. *Front. Oncol.* 12:845931. doi: 10.3389/fonc.2022.845931

Li, Z., Zeng, W., Ye, S., Lv, J., Nie, A., Zhang, B., et al. (2018). Cellular hnRNP A1 interacts with nucleocapsid protein of porcine epidemic diarrhea virus and impairs viral replication. *Viruses* 10:127. doi: 10.3390/v10030127

Lim, M. H., Lee, D. H., Jung, S. E., Youn, D. Y., Park, C. S., and Lee, J. H. (2010). Effect of modulation of hnRNP L levels on the decay of bcl-2 mRNA in MCF-7 cells. *Korean J. Physiol. Pharmacol.* 14, 15–20. doi: 10.4196/kjpp.2010.14.1.15

Lingenfelter, P. A., Delbridge, M. L., Thomas, S., Hoekstra, H. E., Mitchell, M. J., Graves, J. A., et al. (2001). Expression and conservation of processed copies of the RBMX gene. *Mamm. Genome* 12, 538–545. doi: 10.1007/s00335001-0003-z

Liu, Y., Kim, D., Choi, N., Oh, J., Ha, J., Zhou, J., et al. (2020). hnRNP A1 regulates alternative splicing of tau exon 10 by targeting 3' splice sites. *Cells* 9:936. doi: 10.3390/cells9040936

Liu, A. Q., Qin, X., Wu, H., Feng, H., Zhang, Y. A., and Tu, J. (2023). hnRNPA1 impedes snakehead vesiculovirus replication via competitively disrupting viral phosphoprotein-nucleoprotein interaction and degrading viral phosphoprotein. *Virulence* 14:2196847. doi: 10.1080/21505594.2023.2196847

Liu, G., Razanau, A., Hai, Y., Yu, J., Sohail, M., Lobo, V. G., et al. (2012). A conserved serine of heterogeneous nuclear ribonucleoprotein L (hnRNP L) mediates depolarization-regulated alternative splicing of potassium channels. *J. Biol. Chem.* 287, 22709–22716. doi: 10.1074/jbc.M112.357343

Loflin, P., Chen, C. Y., and Shyu, A. B. (1999). Unraveling a cytoplasmic role for hnRNP D in the in vivo mRNA destabilization directed by the AU-rich element. *Genes Dev.* 13, 1884–1897. doi: 10.1101/gad.13.14.1884

López-Erauskin, J., Tadokoro, T., Baughn, M. W., Myers, B., Mcalonis-Downes, M., Chillon-Marinas, C., et al. (2018). ALS/FTD-linked mutation in FUS suppresses intra-axonal protein synthesis and drives disease without nuclear loss-of-function of FUS. *Neuron* 100, 816–830.e7. doi: 10.1016/j.neuron.2018.09.044

Low, Y. H., Asi, Y., Foti, S. C., and Lashley, T. (2021). Heterogeneous nuclear ribonucleoproteins: implications in neurological diseases. *Mol. Neurobiol.* 58, 631–646. doi: 10.1007/s12035-020-02137-4

Lu, Y., Wang, X., Gu, Q., Wang, J., Sui, Y., Wu, J., et al. (2022). Heterogeneous nuclear ribonucleoprotein A/B: an emerging group of cancer biomarkers and therapeutic targets. *Cell Death Discov.* 8:337. doi: 10.1038/s41420-022-01129-8

Lund, N., Milev, M. P., Wong, R., Sanmuganantham, T., Woolaway, K., Chabot, B., et al. (2012). Differential effects of hnRNP D/AUF1 isoforms on HIV-1 gene expression. *Nucleic Acids Res.* 40, 3663–3675. doi: 10.1093/nar/gkr1238

Ma, L., Jiang, Q. A., Sun, L., Yang, X., Huang, H., Jin, X., et al. (2020). X-linked RNA-binding motif protein modulates HIV-1 infection of CD4(+) T cells by maintaining the Trimethylation of histone H3 lysine 9 at the downstream region of the 5′ Long terminal repeat of HIV Proviral DNA. *MBio* 11:e03424-19. doi: 10.1128/mBio.03424-19

Ma, A. S., Moran-Jones, K., Shan, J., Munro, T. P., Snee, M. J., Hoek, K. S., et al. (2002). Heterogeneous nuclear ribonucleoprotein A3, a novel RNA trafficking response element-binding protein. *J. Biol. Chem.* 277, 18010–18020. doi: 10.1074/jbc.M200050200

Ma, X. R., Prudencio, M., Koike, Y., Vatsavayai, S. C., Kim, G., Harbinski, F., et al. (2022). TDP-43 represses cryptic exon inclusion in the FTD-ALS gene UNC13A. *Nature* 603, 124–130. doi: 10.1038/s41586-022-04424-7

Makeyev, A. V., Chkheidze, A. N., and Liebhaber, S. A. (1999). A set of highly conserved RNA-binding proteins, alphaCP-1 and alphaCP-2, implicated in mRNA stabilization, are coexpressed from an intronless gene and its intron-containing paralog. *J. Biol. Chem.* 274, 24849–24857. doi: 10.1074/jbc.274.35.24849

Makeyev, A. V., Kim, C. B., Ruddle, F. H., Enkhmandakh, B., Erdenechimeg, L., and Bayarsaihan, D. (2005). HnRNP A3 genes and pseudogenes in the vertebrate genomes. *J. Exp. Zool. A Comp. Exp. Biol.* 303A, 259–271. doi: 10.1002/jez.a.164

Makeyev, A. V., and Liebhaber, S. A. (2000). Identification of two novel mammalian genes establishes a subfamily of KH-domain RNA-binding proteins. *Genomics* 67, 301–316. doi: 10.1006/geno.2000.6244

Makeyev, E. V., Zhang, J., Carrasco, M. A., and Maniatis, T. (2007). The MicroRNA miR-124 promotes neuronal differentiation by triggering brain-specific alternative pre-mRNA splicing. *Mol. Cell* 27, 435–448. doi: 10.1016/j.molcel.2007.07.015

Mamontova, E. M., Clément, M. J., Sukhanova, M. V., Joshi, V., Bouhss, A., Rengifo-Gonzalez, J. C., et al. (2023). FUS RRM regulates poly(ADP-ribose) levels after transcriptional arrest and PARP-1 activation on DNA damage. *Cell Rep.* 42:113199. doi: 10.1016/j.celrep.2023.113199

Martinez-Contreras, R., Cloutier, P., Shkreta, L., Fisette, J. F., Revil, T., and Chabot, B. (2007). hnRNP proteins and splicing control. *Adv. Exp. Med. Biol.* 623, 123–147. doi: 10.1007/978-0-387-77374-2_8

Matos, B., Publicover, S. J., Castro, L. F. C., Esteves, P. J., and Fardilha, M. (2021). Brain and testis: more alike than previously thought? *Open Biol.* 11:200322. doi: 10.1098/rsob.200322

Matsunaga, S., Takata, H., Morimoto, A., Hayashihara, K., Higashi, T., Akatsuchi, K., et al. (2012). RBMX: a regulator for maintenance and centromeric protection of sister chromatid cohesion. *Cell Rep.* 1, 299–308. doi: 10.1016/j.celrep.2012.02.005

Mayeda, A., Munroe, S. H., Cáceres, J. F., and Krainer, A. R. (1994). Function of conserved domains of hnRNP A1 and other hnRNP A/B proteins. *EMBO J.* 13, 5483–5495. doi: 10.1002/j.1460-2075.1994.tb06883.x

Maystadt, I., Deprez, M., Moortgat, S., Benoît, V., and Karadurmus, D. (2020). A second case of Okamoto syndrome caused by HNRNPK mutation. *Am. J. Med. Genet. A* 182, 1537–1539. doi: 10.1002/ajmg.a.61568

Mazin, P. V., Khaitovich, P., Cardoso-Moreira, M., and Kaessmann, H. (2021). Alternative splicing during mammalian organ development. *Nat. Genet.* 53, 925–934. doi: 10.1038/s41588-021-00851-w

Mcglincy, N. J., Tan, L. Y., Paul, N., Zavolan, M., Lilley, K. S., and Smith, C. W. (2010). Expression proteomics of UPF1 knockdown in HeLa cells reveals autoregulation of hnRNP A2/B1 mediated by alternative splicing resulting in nonsense-mediated mRNA decay. *BMC Genomics* 11:565. doi: 10.1186/1471-2164-11-565

Mckay, S. J., and Cooke, H. (1992). hnRNP A2/B1 binds specifically to single stranded vertebrate telomeric repeat TTAGGGn. *Nucleic Acids Res.* 20, 6461–6464. doi: 10.1093/nar/20.24.6461

Melamed, Z., López-Erauskin, J., Baughn, M. W., Zhang, O., Drenner, K., Sun, Y., et al. (2019). Premature polyadenylation-mediated loss of stathmin-2 is a hallmark of TDP-43-dependent neurodegeneration. *Nat. Neurosci.* 22, 180–190. doi: 10.1038/s41593-018-0293-z

Miyake, N., Inaba, M., Mizuno, S., Shiina, M., Imagawa, E., Miyatake, S., et al. (2017). A case of atypical kabuki syndrome arising from a novel missense variant in HNRNPK. *Clin. Genet.* 92, 554–555. doi: 10.1111/cge.13023

Mo, L., Meng, L., Huang, Z., Yi, L., Yang, N., and Li, G. (2022). An analysis of the role of HnRNP C dysregulation in cancers. *Biomark. Res.* 10:19. doi: 10.1186/s40364-022-00366-4

Mohagheghi, F., Prudencio, M., Stuani, C., Cook, C., Jansen-West, K., Dickson, D. W., et al. (2016). TDP-43 functions within a network of hnRNP proteins to inhibit the production of a truncated human SORT1 receptor. *Hum. Mol. Genet.* 25, 534–545. doi: 10.1093/hmg/ddv491

Mohanty, B. K., Karam, J. A., Howley, B. V., Dalton, A. C., Grelet, S., Dincman, T., et al. (2021). Heterogeneous nuclear ribonucleoprotein E1 binds polycytosine DNA and monitors genome integrity. *Life Sci. Alliance* 4:e202000995. doi: 10.26508/lsa.202000995

Molliex, A., Temirov, J., Lee, J., Coughlin, M., Kanagaraj, A. P., Kim, H. J., et al. (2015). Phase separation by low complexity domains promotes stress granule assembly and drives pathological fibrillization. *Cell* 163, 123–133. doi: 10.1016/j.cell.2015.09.015

Monette, A., Ajamian, L., López-Lastra, M., and Mouland, A. J. (2009). Human immunodeficiency virus type 1 (HIV-1) induces the cytoplasmic retention of heterogeneous nuclear ribonucleoprotein A1 by disrupting nuclear import: implications for HIV-1 gene expression. *J. Biol. Chem.* 284, 31350–31362. doi: 10.1074/jbc.M109.048736

Morales, J., Pujar, S., Loveland, J. E., Astashyn, A., Bennett, R., Berry, A., et al. (2022). A joint NCBI and EMBL-EBI transcript set for clinical genomics and research. *Nature* 604, 310–315. doi: 10.1038/s41586-022-04558-8

Moran-Jones, K., Wayman, L., Kennedy, D. D., Reddel, R. R., Sara, S., Snee, M. J., et al. (2005). hnRNP A2, a potential ssDNA/RNA molecular adapter at the telomere. *Nucleic Acids Res.* 33, 486–496. doi: 10.1093/nar/gki203

Mourelatos, Z., Abel, L., Yong, J., Kataoka, N., and Dreyfuss, G. (2001). SMN interacts with a novel family of hnRNP and spliceosomal proteins. *EMBO J.* 20, 5443–5452. doi: 10.1093/emboj/20.19.5443

Moursy, A., Allain, F. H., and Cléry, A. (2014). Characterization of the RNA recognition mode of hnRNP G extends its role in SMN2 splicing regulation. *Nucleic Acids Res.* 42, 6659–6672. doi: 10.1093/nar/gku244

Müller-Mcnicoll, M., Rossbach, O., Hui, J., and Medenbach, J. (2019). Auto-regulatory feedback by RNA-binding proteins. *J. Mol. Cell Biol.* 11, 930–939. doi: 10.1093/jmcb/mjz043

Naganuma, T., Nakagawa, S., Tanigawa, A., Sasaki, Y. F., Goshima, N., and Hirose, T. (2012). Alternative 3′-end processing of long noncoding RNA initiates construction of nuclear paraspeckles. *EMBO J.* 31, 4020–4034. doi: 10.1038/emboj.2012.251

Nedelsky, N. B., and Taylor, J. P. (2022). Pathological phase transitions in ALS-FTD impair dynamic RNA-protein granules. *RNA* 28, 97–113. doi: 10.1261/rna.079001.121

Ni, J. Z., Grate, L., Donohue, J. P., Preston, C., Nobida, N., O'Brien, G., et al. (2007). Ultraconserved elements are associated with homeostatic control of splicing regulators by alternative splicing and nonsense-mediated decay. *Genes Dev.* 21, 708–718. doi: 10.1101/gad.1525507

Niggl, E., Bouman, A., Briere, L. C., Hoogenboezem, R. M., Wallaard, I., Park, J., et al. (2023). HNRNPC haploinsufficiency affects alternative splicing of intellectual disability-associated genes and causes a neurodevelopmental disorder. *Am. J. Hum. Genet.* 110, 1414–1435. doi: 10.1016/j.ajhg.2023.07.005

Nishiyama, H., Itoh, K., Kaneko, Y., Kishishita, M., Yoshida, O., and Fujita, J. (1997). A glycine-rich RNA-binding protein mediating cold-inducible suppression of mammalian cell growth. *J. Cell Biol.* 137, 899–908. doi: 10.1083/jcb.137.4.899

Nolan, M., Talbot, K., and Ansorge, O. (2016). Pathogenesis of FUS-associated ALS and FTD: insights from rodent models. *Acta Neuropathol. Commun.* 4:99. doi: 10.1186/s40478-016-0358-8

Nurk, S., Koren, S., Rhie, A., Rautiainen, M., Bzikadze, A. V., Mikheenko, A., et al. (2022). The complete sequence of a human genome. *Science* 376, 44–53. doi: 10.1126/science.abj6987

Okamoto, N. (2019). Okamoto syndrome has features overlapping with Au-Kline syndrome and is caused by HNRNPK mutation. *Am. J. Med. Genet. A* 179, 822–826. doi: 10.1002/ajmg.a.61079

Pan, Q., Shai, O., Lee, L. J., Frey, B. J., and Blencowe, B. J. (2008). Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. *Nat. Genet.* 40, 1413–1415. doi: 10.1038/ng.259

Papadopoulou, C., Boukakis, G., Ganou, V., Patrinou-Georgoula, M., and Guialis, A. (2012). Expression profile and interactions of hnRNP A3 within hnRNP/mRNP complexes in mammals. *Arch. Biochem. Biophys.* 523, 151–160. doi: 10.1016/j.abb.2012.04.012

Peron, A., Novara, F., La Briola, F., Merati, E., Giannusa, E., Segalini, E., et al. (2020). Missense variants in the Arg206 residue of HNRNPH2: further evidence of causality and expansion of the phenotype. *Am. J. Med. Genet. A* 182, 823–828. doi: 10.1002/ajmg.a.61486

Pettit Kneller, E. L., Connor, J. H., and Lyles, D. S. (2009). hnRNPs Relocalize to the cytoplasm following infection with vesicular stomatitis virus. *J. Virol.* 83, 770–780. doi: 10.1128/JVI.01279-08

Pickering, B. M., Mitchell, S. A., Evans, J. R., and Willis, A. E. (2003). Polypyrimidine tract binding protein and poly r(C) binding protein 1 interact with the BAG-1 IRES and stimulate its activity in vitro and in vivo. *Nucleic Acids Res.* 31, 639–646. doi: 10.1093/nar/gkg146

Pilch, J., Koppolu, A. A., Walczak, A., Murcia Pienkowski, V. A., Biernacka, A., Skiba, P., et al. (2018). Evidence for HNRNPH1 being another gene for Bain type syndromic mental retardation. *Clin. Genet.* 94, 381–385. doi: 10.1111/cge.13410

Piñol-Roma, S. (1997). HnRNP proteins and the nuclear export of mRNA. *Semin. Cell Dev. Biol.* 8, 57–63. doi: 10.1006/scdb.1996.0122

Piñol-Roma, S., Choi, Y. D., Matunis, M. J., and Dreyfuss, G. (1988). Immunopurification of heterogeneous nuclear ribonucleoprotein particles reveals an assortment of RNA-binding proteins. *Genes Dev.* 2, 215–227. doi: 10.1101/gad.2.2.215

Piñol-Roma, S., and Dreyfuss, G. (1992). Shuttling of pre-mRNA binding proteins between nucleus and cytoplasm. *Nature* 355, 730–732. doi: 10.1038/355730a0

Provasek, V. E., Mitra, J., Malojirao, V. H., and Hegde, M. L. (2022). DNA double-Strand breaks as pathogenic lesions in neurological disorders. *Int. J. Mol. Sci.* 23:4653. doi: 10.3390/ijms23094653

Purice, M. D., and Taylor, J. P. (2018). Linking hnRNP function to ALS and FTD pathology. *Front. Neurosci.* 12:326. doi: 10.3389/fnins.2018.00326

Rahman, M. A., Masuda, A., Ohe, K., Ito, M., Hutchinson, D. O., Mayeda, A., et al. (2013). HnRNP L and hnRNP LL antagonistically modulate PTB-mediated splicing suppression of CHRNA1 pre-mRNA. *Sci. Rep.* 3:2931. doi: 10.1038/srep02931

Rajagopalan, L. E., Westmark, C. J., Jarzembowski, J. A., and Malter, J. S. (1998). hnRNP C increases amyloid precursor protein (APP) production by stabilizing APP mRNA. *Nucleic Acids Res.* 26, 3418–3423. doi: 10.1093/nar/26.14.3418

Rauch, A., Wieczorek, D., Graf, E., Wieland, T., Endele, S., Schwarzmayr, T., et al. (2012). Range of genetic mutations associated with severe non-syndromic sporadic intellectual disability: an exome sequencing study. *Lancet* 380, 1674–1682. doi: 10.1016/S0140-6736(12)61480-9

Reber, S., Stettler, J., Filosa, G., Colombo, M., Jutzi, D., Lenzken, S. C., et al. (2016). Minor intron splicing is regulated by FUS and affected by ALS-associated FUS mutants. *EMBO J.* 35, 1504–1521. doi: 10.15252/embj.201593791

Redondo, N., Madan, V., Alvarez, E., and Carrasco, L. (2015). Impact of vesicular stomatitis virus M proteins on different cellular functions. *PLoS One* 10:e0131137. doi: 10.1371/journal.pone.0131137

Reichert, S. C., Li, R., Turner, S. A., van Jaarsveld, R. H., Massink, M. P. G., van den Boogaard, M. J. H., et al. (2020). HNRNPH1-related syndromic intellectual disability: seven additional cases suggestive of a distinct syndromic neurodevelopmental syndrome. *Clin. Genet.* 98, 91–98. doi: 10.1111/cge.13765

Resnick, M., Segall, A., G, G. R. K., Lupowitz, Z., and Zisapel, N. (2008). Alternative splicing of neurexins: a role for neuronal polypyrimidine tract binding protein. *Neurosci. Lett.* 439, 235–240. doi: 10.1016/j.neulet.2008.05.034

Reznik, B., Clement, S. L., and Lykke-Andersen, J. (2014). hnRNP F complexes with tristetraprolin and stimulates ARE-mRNA decay. *PLoS One* 9:e100992. doi: 10.1371/journal.pone.0100992

Romanelli, M. G., Lorenzi, P., and Morandi, C. (2000). Organization of the human gene encoding heterogeneous nuclear ribonucleoprotein type I (hnRNP I) and characterization of hnRNP I related pseudogene. *Gene* 255, 267–272. doi: 10.1016/S0378-1119(00)00331-0

Romano, M., and Buratti, E. (2013). Targeting RNA binding proteins involved in neurodegeneration. *J. Biomol. Screen.* 18, 967–983. doi: 10.1177/1087057113497256

Romero, F., Germani, A., Puvion, E., Camonis, J., Varin-Blank, N., Gisselbrecht, S., et al. (1998). Vav binding to heterogeneous nuclear ribonucleoprotein (hnRNP) C. Evidence for Vav-hnRNP interactions in an RNA-dependent manner. *J. Biol. Chem.* 273, 5923–5931. doi: 10.1074/jbc.273.10.5923

Rossbach, O., Hung, L. H., Schreiner, S., Grishina, I., Heiner, M., Hui, J., et al. (2009). Auto- and cross-regulation of the hnRNP L proteins by alternative splicing. *Mol. Cell. Biol.* 29, 1442–1451. doi: 10.1128/MCB.01689-08

Sakakibara, S., Nakamura, Y., Satoh, H., and Okano, H. (2001). RNA-binding protein Musashi2: developmentally regulated expression in neural precursor cells and subpopulations of neurons in mammalian CNS. *J. Neurosci.* 21, 8091–8107. doi: 10.1523/JNEUROSCI.21-20-08091.2001

Salapa, H. E., Hutchinson, C., Popescu, B. F., and Levin, M. C. (2020). Neuronal RNA-binding protein dysfunction in multiple sclerosis cortex. *Ann. Clin. Transl. Neurol.* 7, 1214–1224. doi: 10.1002/acn3.51103

Salapa, H. E., Thibault, P. A., Libner, C. D., Ding, Y., Clarke, J. W. E., Denomy, C., et al. (2024). hnRNP A1 dysfunction alters RNA splicing and drives neurodegeneration in multiple sclerosis (MS). *Nat. Commun.* 15:356. doi: 10.1038/s41467-023-44658-1

Salehi, S., Zare, A., Prezza, G., Bader, J., Schneider, C., Fischer, U., et al. (2023). Cytosolic Ptbp2 modulates axon growth in motoneurons through axonal localization and translation of *Hnrnpr*. *Nat. Commun.* 14:4158. doi: 10.1038/s41467-023-39787-6

Sama, R. R., Fallini, C., Gatto, R., Mckeon, J. E., Song, Y., Rotunno, M. S., et al. (2017). ALS-linked FUS exerts a gain of toxic function involving aberrant p38 MAPK activation. *Sci. Rep.* 7:115. doi: 10.1038/s41598-017-00091-1

Sapir, T., Kshirsagar, A., Gorelik, A., Olender, T., Porat, Z., Scheffer, I. E., et al. (2022). Heterogeneous nuclear ribonucleoprotein U (HNRNPU) safeguards the developing mouse cortex. *Nat. Commun.* 13:4209. doi: 10.1038/s41467-022-31752-z

Scalabrin, M., Frasson, I., Ruggiero, E., Perrone, R., Tosoni, E., Lago, S., et al. (2017). The cellular protein hnRNP A2/B1 enhances HIV-1 transcription by unfolding LTR promoter G-quadruplexes. *Sci. Rep.* 7:45244. doi: 10.1038/srep45244

Scekic-Zahirovic, J., Sendscheid, O., El Oussini, H., Jambeau, M., Sun, Y., Mersmann, S., et al. (2016). Toxic gain of function from mutant FUS protein is crucial to trigger cell autonomous motor neuron loss. *EMBO J.* 35, 1077–1097. doi: 10.15252/embj.201592559

Seczynska, M., and Lehner, P. J. (2023). The sound of silence: mechanisms and implications of HUSH complex function. *Trends Genet.* 39, 251–267. doi: 10.1016/j.tig.2022.12.005

Semino, F., Schröter, J., Willemsen, M. H., Bast, T., Biskup, S., Beck-Woedl, S., et al. (2021). Further evidence for de novo variants in SYNCRIP as the cause of a neurodevelopmental disorder. *Hum. Mutat.* 42, 1094–1100. doi: 10.1002/humu.24245

Sharma, A., Lyashchenko, A. K., Lu, L., Nasrabady, S. E., Elmaleh, M., Mendelsohn, M., et al. (2016). ALS-associated mutant FUS induces selective motor neuron degeneration through toxic gain of function. *Nat. Commun.* 7:10465. doi: 10.1038/ncomms10465

Shashi, V., Berry, M. N., Shoaf, S., Sciote, J. J., Goldstein, D., and Hart, T. C. (2000). A unique form of mental retardation with a distinctive phenotype maps to Xq26-q27. *Am. J. Hum. Genet.* 66, 469–479. doi: 10.1086/302772

Shashi, V., Xie, P., Schoch, K., Goldstein, D. B., Howard, T. D., Berry, M. N., et al. (2015). The RBMX gene as a candidate for the Shashi X-linked intellectual disability syndrome. *Clin. Genet.* 88, 386–390. doi: 10.1111/cge.12511

Sheikh, M. S., Carrier, F., Papathanasiou, M. A., Hollander, M. C., Zhan, Q., Yu, K., et al. (1997). Identification of several human homologs of hamster DNA damage-inducible transcripts. Cloning and characterization of a novel UV-inducible cDNA that codes for a putative RNA-binding protein. *J. Biol. Chem.* 272, 26720–26726. doi: 10.1074/jbc.272.42.26720

Siachisumo, C., Luzzi, S., Aldalaqan, S., Hysenaj, G., Dalgliesh, C., Cheung, K., et al. (2023). An anciently diverged family of RNA binding proteins maintain correct splicing of a class of ultra-long exons through cryptic splice site repression: Cold Spring Harbor Laboratory.

Siddiqui, A., Saxena, A., Echols, J., Havasi, V., Fu, L., and Keeling, K. M. (2023). RNA binding proteins PTBP1 and HNRNPL regulate CFTR mRNA decay. *Heliyon* 9:e22281. doi: 10.1016/j.heliyon.2023.e22281

Sidhu, R., Gatt, A., Fratta, P., Lashley, T., and Bampton, A. (2022). HnRNP K mislocalisation in neurons of the dentate nucleus is a novel neuropathological feature of neurodegenerative disease and ageing. *Neuropathol. Appl. Neurobiol.* 48:e12793. doi: 10.1111/nan.12793

Simard, M. J., and Chabot, B. (2002). SRp30c is a repressor of 3′ splice site utilization. *Mol. Cell. Biol.* 22, 4001–4010. doi: 10.1128/MCB.22.12.4001-4010.2002

Singh, R. N., and Singh, N. N. (2018). Mechanism of splicing regulation of spinal muscular atrophy genes. *Adv. Neurobiol.* 20, 31–61. doi: 10.1007/978-3-319-89689-2_2

Singh, R., and Valcárcel, J. (2005). Building specificity with nonspecific RNA-binding proteins. *Nat. Struct. Mol. Biol.* 12, 645–653. doi: 10.1038/nsmb961

Siomi, H., and Dreyfuss, G. (1995). A nuclear localization domain in the hnRNP A1 protein. *J. Cell Biol.* 129, 551–560. doi: 10.1083/jcb.129.3.551

Smith, S. A., Ray, D., Cook, K. B., Mallory, M. J., Hughes, T. R., and Lynch, K. W. (2013). Paralogs hnRNP L and hnRNP LL exhibit overlapping but distinct RNA binding constraints. *PLoS One* 8:e80701. doi: 10.1371/journal.pone.0080701

Somashekar, P. H., Narayanan, D. L., Jagadeesh, S., Suresh, B., Vaishnavi, R. D., Bielas, S., et al. (2020). Bain type of X-linked syndromic mental retardation in a male with a pathogenic variant in HNRNPH2. *Am. J. Med. Genet. A* 182, 183–188. doi: 10.1002/ajmg.a.61388

Soniat, M., and Chook, Y. M. (2016). Karyopherin-β2 recognition of a PY-NLS variant that lacks the proline-tyrosine motif. *Structure* 24, 1802–1809. doi: 10.1016/j.str.2016.07.018

Spellman, R., Llorian, M., and Smith, C. W. (2007). Crossregulation and functional redundancy between the splicing regulator PTB and its paralogs nPTB and ROD1. *Mol. Cell* 27, 420–434. doi: 10.1016/j.molcel.2007.06.016

Sreedharan, J., Blair, I. P., Tripathi, V. B., Hu, X., Vance, C., Rogelj, B., et al. (2008). TDP-43 mutations in familial and sporadic amyotrophic lateral sclerosis. *Science* 319, 1668–1672. doi: 10.1126/science.1154584

Stake, M., Singh, D., Singh, G., Marcela Hernandez, J., Kaddis Maldonado, R., Parent, L. J., et al. (2015). HIV-1 and two avian retroviral 5′ untranslated regions bind orthologous human and chicken RNA binding proteins. *Virology* 486, 307–320. doi: 10.1016/j.virol.2015.06.001

Stelzer, G., Rosen, N., Plaschkes, I., Zimmerman, S., Twik, M., Fishilevich, S., et al. (2016). The GeneCards suite: from gene data mining to disease genome sequence analyses. *Curr. Protoc. Bioinformatics* 54, 1.30.31–31.30.33. doi: 10.1002/cpbi.5

Sueoka, E., Yukitake, M., Iwanaga, K., Sueoka, N., Aihara, T., and Kuroda, Y. (2004). Autoantibodies against heterogeneous nuclear ribonucleoprotein B1 in CSF of MS patients. *Ann. Neurol.* 56, 778–786. doi: 10.1002/ana.20276

Sun, S., Ling, S. C., Qiu, J., Albuquerque, C. P., Zhou, Y., Tokunaga, S., et al. (2015). ALS-causative mutations in FUS/TLS confer gain and loss of function by altered association with SMN and U1-snRNP. *Nat. Commun.* 6:6171. doi: 10.1038/ncomms7171

Suzuki, H., and Matsuoka, M. (2015). Overexpression of nuclear FUS induces neuronal cell death. *Neuroscience* 287, 113–124. doi: 10.1016/j.neuroscience.2014.12.007

Suzuki, N., Nishiyama, A., Warita, H., and Aoki, M. (2023). Genetics of amyotrophic lateral sclerosis: seeking therapeutic targets in the era of gene therapy. *J. Hum. Genet.* 68, 131–152. doi: 10.1038/s10038-022-01055-8

Tanaka, E., Fukuda, H., Nakashima, K., Tsuchiya, N., Seimiya, H., and Nakagama, H. (2007). HnRNP A3 binds to and protects mammalian telomeric repeats in vitro. *Biochem. Biophys. Res. Commun.* 358, 608–614. doi: 10.1016/j.bbrc.2007.04.177

Taylor, J., Spiller, M., Ranguin, K., Vitobello, A., Philippe, C., Bruel, A. L., et al. (2022). Expanding the phenotype of HNRNPU-related neurodevelopmental disorder with emphasis on seizure phenotype and review of literature. *Am. J. Med. Genet. A* 188, 1497–1514. doi: 10.1002/ajmg.a.62677

Telley, L., Agirman, G., Prados, J., Amberg, N., Fièvre, S., Oberst, P., et al. (2019). Temporal patterning of apical progenitors and their daughter neurons in the developing neocortex. *Science* 364:eaav2522. doi: 10.1126/science.aav2522

Tsai, Y. L., Coady, T. H., Lu, L., Zheng, D., Alland, I., Tian, B., et al. (2020). ALS/FTD-associated protein FUS induces mitochondrial dysfunction by preferentially sequestering respiratory chain complex mRNAs. *Genes Dev.* 34, 785–805. doi: 10.1101/gad.335836.119

Tuersun, H., Liu, L., Zhang, J., Maimaitizunong, R., Tang, X., and Li, H. (2023). m6A reading protein RBMX as a biomarker for prognosis and tumor progression in esophageal cancer. *Transl. Cancer Res.* 12, 2319–2335. doi: 10.21037/tcr-23-84

Van Langenhove, T., Van Der Zee, J., and Van Broeckhoven, C. (2012). The molecular basis of the frontotemporal lobar degeneration-amyotrophic lateral sclerosis spectrum. *Ann. Med.* 44, 817–828. doi: 10.3109/07853890.2012.665471

Van Lindt, J., Lazar, T., Pakravan, D., Demulder, M., Meszaros, A., Van Den Bosch, L., et al. (2022). F/YGG-motif is an intrinsically disordered nucleic-acid binding motif. *RNA Biol.* 19, 622–635. doi: 10.1080/15476286.2022.2066336

Venables, J. P., Elliott, D. J., Makarova, O. V., Makarov, E. M., Cooke, H. J., and Eperon, I. C. (2000). RBMY, a probable human spermatogenesis factor, and other hnRNP G proteins interact with Tra2beta and affect splicing. *Hum. Mol. Genet.* 9, 685–694. doi: 10.1093/hmg/9.5.685

Venter, J. C., Adams, M. D., Myers, E. W., Li, P. W., Mural, R. J., Sutton, G. G., et al. (2001). The sequence of the human genome. *Science* 291, 1304–1351. doi: 10.1126/science.1058040

Vollger, M. R., Dishuck, P. C., Sorensen, M., Welch, A. E., Dang, V., Dougherty, M. L., et al. (2019). Long-read sequence and assembly of segmental duplications. *Nat. Methods* 16, 88–94. doi: 10.1038/s41592-018-0236-3

Vuong, J. K., Lin, C. H., Zhang, M., Chen, L., Black, D. L., and Zheng, S. (2016). PTBP1 and PTBP2 serve Both specific and redundant functions in neuronal pre-mRNA splicing. *Cell Rep.* 17, 2766–2775. doi: 10.1016/j.celrep.2016.11.034

Wall, M. L., Bera, A., Wong, F. K., and Lewis, S. M. (2020). Cellular stress orchestrates the localization of hnRNP H to stress granules. *Exp. Cell Res.* 394:112111. doi: 10.1016/j.yexcr.2020.112111

Wang, E., Aslanzadeh, V., Papa, F., Zhu, H., De La Grange, P., and Cambi, F. (2012). Global profiling of alternative splicing events and gene expression regulated by hnRNPH/F. *PLoS One* 7:e51266. doi: 10.1371/journal.pone.0051266

Wang, E., Dimova, N., and Cambi, F. (2007). PLP/DM20 ratio is regulated by hnRNPH and F and a novel G-rich enhancer in oligodendrocytes. *Nucleic Acids Res.* 35, 4164–4178. doi: 10.1093/nar/gkm387

Wang, E. T., Sandberg, R., Luo, S., Khrebtukova, I., Zhang, L., Mayr, C., et al. (2008). Alternative isoform regulation in human tissue transcriptomes. *Nature* 456, 470–476. doi: 10.1038/nature07509

Wang, J., Sun, D., Wang, M., Cheng, A., Zhu, Y., Mao, S., et al. (2022). Multiple functions of heterogeneous nuclear ribonucleoproteins in the positive single-stranded RNA virus life cycle. *Front. Immunol.* 13:989298. doi: 10.3389/fimmu.2022.989298

Weighardt, F., Biamonti, G., and Riva, S. (1995). Nucleo-cytoplasmic distribution of human hnRNP proteins: a search for the targeting domains in hnRNP A1. *J. Cell Sci.* 108, 545–555. doi: 10.1242/jcs.108.2.545

White, E. J., Brewer, G., and Wilson, G. M. (2013). Post-transcriptional control of gene expression by AUF1: mechanisms, physiological targets, and regulation. *Biochim. Biophys. Acta* 1829, 680–688. doi: 10.1016/j.bbagrm.2012.12.002

Wiesmann, N., Strozynski, J., Beck, C., Zimmermann, N., Mendler, S., Gieringer, R., et al. (2017). Knockdown of hnRNPK leads to increased DNA damage after irradiation and reduces survival of tumor cells. *Carcinogenesis* 38, 321–328. doi: 10.1093/carcin/bgx006

Wilkinson, M. E., Charenton, C., and Nagai, K. (2020). RNA splicing by the spliceosome. *Annu. Rev. Biochem.* 89, 359–388. doi: 10.1146/annurev-biochem-091719-064225

Wirth, B. (2021). Spinal muscular atrophy: in the challenge lies a solution. *Trends Neurosci.* 44, 306–322. doi: 10.1016/j.tins.2020.11.009

Wirth, B., Karakaya, M., Kye, M. J., and Mendoza-Ferreira, N. (2020). Twenty-five years of spinal muscular atrophy research: from phenotype to genotype to therapy, and what comes next. *Annu. Rev. Genomics Hum. Genet.* 21, 231–261. doi: 10.1146/annurev-genom-102319-103602

Wollerton, M. C., Gooding, C., Wagner, E. J., Garcia-Blanco, M. A., and Smith, C. W. (2004). Autoregulation of polypyrimidine tract binding protein by alternative splicing leading to nonsense-mediated decay. *Mol. Cell* 13, 91–100. doi: 10.1016/S1097-2765(03)00502-1

Woo, K. C., Ha, D. C., Lee, K. H., Kim, D. Y., Kim, T. D., and Kim, K. T. (2010). Circadian amplitude of cryptochrome 1 is modulated by mRNA stability regulation via cytoplasmic hnRNP D oscillation. *Mol. Cell. Biol.* 30, 197–205. doi: 10.1128/MCB.01154-09

Woo, K. C., Kim, T. D., Lee, K. H., Kim, D. Y., Kim, W., Lee, K. Y., et al. (2009). Mouse period 2 mRNA circadian oscillation is modulated by PTB-mediated rhythmic mRNA degradation. *Nucleic Acids Res.* 37, 26–37. doi: 10.1093/nar/gkn893

Wood, A., Gurfinkel, Y., Polain, N., Lamont, W., and Lyn Rea, S. (2021). Molecular mechanisms underlying TDP-43 pathology in cellular and animal models of ALS and FTLD. *Int. J. Mol. Sci.* 22:4705. doi: 10.3390/ijms22094705

Woolaway, K., Asai, K., Emili, A., and Cochrane, A. (2007). hnRNP E1 and E2 have distinct roles in modulating HIV-1 gene expression. *Retrovirology* 4:28. doi: 10.1186/1742-4690-4-28

Xu, N., Chen, C. Y., and Shyu, A. B. (2001). Versatile role for hnRNP D isoforms in the differential regulation of cytoplasmic mRNA turnover. *Mol. Cell. Biol.* 21, 6960–6971. doi: 10.1128/MCB.21.20.6960-6971.2001

Yang, L., Gal, J., Chen, J., and Zhu, H. (2014). Self-assembled FUS binds active chromatin and regulates gene transcription. *Proc. Natl. Acad. Sci. USA* 111, 17809–17814. doi: 10.1073/pnas.1414004111

Yates, T. M., Vasudevan, P. C., Chandler, K. E., Donnelly, D. E., Stark, Z., Sadedin, S., et al. (2017). De novo mutations in HNRNPU result in a neurodevelopmental syndrome. *Am. J. Med. Genet. A* 173, 3003–3012. doi: 10.1002/ajmg.a.38492

Yeap, B. B., Voon, D. C., Vivian, J. P., Mcculloch, R. K., Thomson, A. M., Giles, K. M., et al. (2002). Novel binding of HuR and poly(C)-binding protein to a conserved UC-rich motif within the 3′-untranslated region of the androgen receptor messenger RNA. *J. Biol. Chem.* 277, 27183–27192. doi: 10.1074/jbc.M202883200

Yu, J., Hai, Y., Liu, G., Fang, T., Kung, S. K., and Xie, J. (2009). The heterogeneous nuclear ribonucleoprotein L is an essential component in the Ca2+/calmodulin-dependent protein kinase IV-regulated alternative splicing through cytidine-adenosine repeats. *J. Biol. Chem.* 284, 1505–1513. doi: 10.1074/jbc.M805113200

Yugami, M., Kabe, Y., Yamaguchi, Y., Wada, T., and Handa, H. (2007). hnRNP-U enhances the expression of specific genes by stabilizing mRNA. *FEBS Lett.* 581, 1–7. doi: 10.1016/j.febslet.2006.11.062

Zhang, X., Chen, M. H., Wu, X., Kodani, A., Fan, J., Doan, R., et al. (2016). Cell-type-specific alternative splicing governs cell fate in the developing cerebral cortex. *Cell* 166, 1147–1162.e15. doi: 10.1016/j.cell.2016.07.025

Zhang, M., Ergin, V., Lin, L., Stork, C., Chen, L., and Zheng, S. (2019). Axonogenesis is coordinated by neuron-specific alternative splicing programming and splicing regulator PTBP2. *Neuron* 101, 690–706.e10. doi: 10.1016/j.neuron.2019.01.022

Zhang, Q., Ma, C., Gearing, M., Wang, P. G., Chin, L. S., and Li, L. (2018). Integrated proteomics and network analysis identifies protein hubs and network alterations in Alzheimer's disease. *Acta Neuropathol. Commun.* 6:19. doi: 10.1186/s40478-018-0524-2

Zhang, Q., Zhang, J., Ye, J., Li, X., Liu, H., Ma, X., et al. (2021). Nuclear speckle specific hnRNP D-like prevents age- and AD-related cognitive decline by modulating RNA splicing. *Mol. Neurodegener.* 16:66. doi: 10.1186/s13024-021-00485-w

Zheng, S., Gray, E. E., Chawla, G., Porse, B. T., O'Dell, T. J., and Black, D. L. (2012). PSD-95 is post-transcriptionally repressed during early neural development by PTBP1 and PTBP2. *Nat. Neurosci.* 15:s381, 381–388. doi: 10.1038/nn.3026

Zheng, T., Zhou, H., Li, X., Peng, D., Yang, Y., Zeng, Y., et al. (2020). RBMX is required for activation of ATR on repetitive DNAs to maintain genome stability. *Cell Death Differ.* 27, 3162–3176. doi: 10.1038/s41418-020-0570-8

Zhou, J., Guo, Y., Huo, Z., Xing, Y., Fang, J., Ma, G., et al. (2021). Identification of therapeutic targets and prognostic biomarkers from the hnRNP family in invasive breast carcinoma. *Aging (Albany NY)* 13, 4503–4521. doi: 10.18632/aging.202411

frontiers | Frontiers in Molecular Neuroscience

TYPE Mini Review
PUBLISHED 25 July 2024
DOI 10.3389/fnmol.2024.1412964

Check for updates

OPEN ACCESS

EDITED BY
Oriane Mauger,
Max Planck Institute of Psychiatry, Germany

REVIEWED BY
Florian Heyd,
Free University of Berlin, Germany
Elisabetta Furlanis,
Harvard Medical School, United States

*CORRESPONDENCE
Xiaochang Zhang
✉ xczhang@uchicago.edu

RECEIVED 06 April 2024
ACCEPTED 12 July 2024
PUBLISHED 25 July 2024

CITATION
Zhang X (2024) Splice-switching antisense oligonucleotides for pediatric neurological disorders.
Front. Mol. Neurosci. 17:1412964.
doi: 10.3389/fnmol.2024.1412964

Splice-switching antisense oligonucleotides for pediatric neurological disorders

Xiaochang Zhang*

Department of Human Genetics, The Neuroscience Institute, University of Chicago, Chicago, IL, United States

Pediatric neurological disorders are frequently devastating and present unmet needs for effective medicine. The successful treatment of spinal muscular atrophy with splice-switching antisense oligonucleotides (SSO) indicates a feasible path to targeting neurological disorders by redirecting pre-mRNA splicing. One direct outcome is the development of SSOs to treat haploinsufficient disorders by targeting naturally occurring non-productive splice isoforms. The development of personalized SSO treatment further inspired the therapeutic exploration of rare diseases. This review will discuss the recent advances that utilize SSOs to treat pediatric neurological disorders.

KEYWORDS
ASO, SSO, neurodevelopmental disorder, epilepsy, autism, alternative splicing, nonsense-mediated mRNA decay, Syngap1

Introduction

Over the last two decades, causal variants for pediatric neurological disorders have been increasingly uncovered by high-throughput DNA sequencing. Many clinically comparable disease symptoms, such as developmental and epileptic encephalopathy (DEE), turn out to be caused by mutations in dozens of genes that have different biological functions and pathophysiology. Consequently, human diseases are increasingly classified based on their molecular causes and clinical presentations. Such accumulating genetic evidence offers unique opportunities to develop gene- or variant-specific treatments in addition to generic symptom-oriented drugs. Precision medicine strategies for neurological disorders, such as gene replacement therapy, genome editing, and splicing modulation, have been actively explored (Deverman et al., 2018; Nussbacher et al., 2019; Raguram et al., 2022). Antisense oligonucleotides (ASO) represent one type of such therapeutic means and have shown promising clinical outcomes for spinal muscular atrophy (SMA), Duchenne muscular dystrophy (DMD), and Amyotrophic Lateral Sclerosis (ALS), among other ongoing clinical and preclinical studies (Rinaldi and Wood, 2018).

ASOs are modified short nucleotides that bind to pre-mRNA through Watson-Crick base pairing (Kole et al., 2012). ASOs can be used as steric blockers to intervene in processes such as splicing and protein translation, or as gapmers to promote RNase H1-mediated target mRNA degradation. The nucleobases and the backbone are modified to resist nuclease degradation, enhance the target binding, and boost cellular intake. ASO modifications, such as 2'-O-methoxyethyl-modified (MOE) nucleotides with phosphorothioate (PS) backbone, have been clinically tested and proven to be generally tolerated (Egli and Manoharan, 2023). Various modifications have been developed to enhance efficacy and decrease toxicity. Splice-switching oligonucleotides (SSO) are a specific category of ASOs that bind to pre-mRNA as steric blockers and redirect splicing. SSOs have been successfully developed to treat SMA and DMD (Voit et al., 2014; Finkel et al., 2017). ASO gapmers have been recently approved by the FDA to treat SOD1 ALS. This review focuses on the progress of SSOs in targeting pediatric neurological conditions.

Most human protein-coding genes are split by introns, which are spliced out by the spliceosome (Berget et al., 1977; Chow et al., 1977). Introns are collectively defined by their 5′ splice donor site (5'SS), 3′ acceptor site (3'SS), the branchpoint adenosine, the poly-pyrimidine tract upstream of the 3'SS, and other regulatory sequences. Pre-mRNA splicing allows the reshuffle of different exons (Gilbert, 1978), and RNA-seq analyses showed that over 95% of intron-containing human genes undergo alternative splicing (AS) to generate multiple mRNA isoforms (Pan et al., 2008; Wang et al., 2008). Alternative splicing can lead to skipped exons (SE), alternative 5′ splice site (A5SS), alternative 3′ splice site (A3SS), mutually exclusive exons (MXE), and retained introns (RI) (Graveley, 2001). Alternative splicing can happen in species-, tissue- and cell-type-specific manners (Barbosa-Morais et al., 2012; Merkin et al., 2012; Feng et al., 2021). Alternative splicing is prevalent in the brain, and recent long-read sequencing analyses have uncovered coordinated splicing of distant exons (Gupta et al., 2018; Yang et al., 2023; Zhang et al., 2023). Alternative splicing is modulated by intronic and exonic cis-regulatory sequences and their associated RNA-binding proteins (Black, 2003; Wang and Burge, 2008; Barash et al., 2010; Xiong et al., 2015; Bao et al., 2019; Van Nostrand et al., 2020). The natural occurrence of alternative splicing and the identification of splicing enhancers/suppressors indicate that re-directing splicing holds its own dimension for gene regulation and therapeutic intervention.

About 10% of exonic human mutations are estimated to cause diseases by disrupting pre-mRNA splicing (Soemedi et al., 2017). While whole-exome sequencing detects exonic and splice site mutations for genetically defined disorders, integrating transcriptome and whole-genome analysis uncovers more causal intronic splicing mutations (Cummings et al., 2017; Kim et al., 2023). These splicing mutations frequently introduce aberrant splice sites that lead to loss-of-function or hypomorphic alleles. Disease-causing splicing variants can be suppressed to treat human diseases. Redirecting splicing can also lead to beneficial effects by (1) bypassing nonessential inframe exons that carry pathogenic mutations, (2) bypassing an additional exon to correct the reading frame, and (3) redirecting alternative splicing to promote functional isoform production.

SSOs bind to pre-mRNA through Watson-Crick base pairing and redirect pre-mRNA splicing (Kole et al., 2012; Centa and Hastings, 2022). The SSO binding sites are frequently splicing enhancers or suppressors, and the double-stranded SSO-pre-mRNA can block RNA–RNA or RNA-protein interactions that modulate splice site usage. Since the success of SSOs in treating DMD and SMA (Hua et al., 2011; Finkel et al., 2017), redirecting pre-mRNA splicing has been increasingly recognized as a powerful therapeutic strategy to treat neurological disorders (Hill and Meisler, 2021; Nikom and Zheng, 2023). The sequence flexibility and the clinically proven chemistry have made SSO a fast-growing platform for personalized medicine. The development of the SSO drug Milasen for a girl named Mila is inspirational, and the approach displayed promising progress toward previously undruggable targets and rare mutations (Kim et al., 2019, 2023). This review focuses on recently reported SSO strategies targeting pediatric neurological conditions and the value of genetic tools.

SSOs can promote either exon skipping or inclusion. With a focus on pediatric neurological conditions, recently reported SSO strategies generally fall into three main categories (Figure 1). The most straightforward application of SSOs would be suppressing an abnormal splice site introduced by a specific mutation – a variant-specific SSO (Figure 1A). SSOs have also been developed to skip a nonessential exon that is either inframe and carries deleterious mutations or correct the reading frame caused by frameshift mutations – an exon-specific SSO (Figure 1B). Lastly, SSOs can increase protein expression through a paralog and rescue recessive diseases or boost protein expression from the wild-type allele and rescue haploinsufficiency – such SSOs are independent of mutations and can be considered gene-specific SSOs (Figure 1C). While variant- and exon-specific SSOs play prominent roles in personalized medicine, a gene-specific SSO can be used to treat patients carrying mutations across the same gene. SSO-mediated therapy, or treatments for genetic disorders in general, would be more effective when used for sooner intervention in disease progression than later. Thus, early genetic diagnosis-aided treatment before the existence of irreversible disease presentations, as shown by a recent study (Kim et al., 2023), appears to be a promising practice.

SSOs for recessive diseases

Recessive diseases frequently involve loss-of-function alleles, and several SSO-based therapeutic strategies have been reported (Figure 1). SSO can promote the inclusion or exclusion of specific exons. Thus, it is straightforward to use SSOs to suppress undesired exons, such as abnormal/cryptic splice sites. SSOs can also block splicing silencers and promote exon inclusion to make functional proteins, such as the *SMN2* case below.

Spinal muscular atrophy (SMA) is a motor neuron disorder caused by recessive loss-of-function mutations in *SMN1* (Lefebvre et al., 1995). The loss of spinal cord motor neurons in SMA patients leads to muscle weakness and atrophy, and the disease presentations fall into different clinical categories based on the age of onset and the severity of symptoms. Type 1 SMA, with the disease onset by 6 months of age and an expected life shorter than 2 years, is the most severe form and affects about 50% of all cases. *SMN2* is a hominid-specific duplication of *SMN1*, and increased *SMN2* copy numbers are inversely correlated with SMA severity (Rochette et al., 2001; Calucho et al., 2018). Compared to *SMN1*, *SMN2* carries a single synonymous C-to-T change in exon 7 that causes 90% of *SMN2* mRNA to skip exon 7 and encode an unstable protein (Monani et al., 1999). The exon7-included *SMN2* mRNA encodes an identical protein to SMN1. Multiple strategies, including SSOs and splicing modulatory small molecules, have been developed to promote *SMN2* exon 7 inclusion and treat SMA (Hua et al., 2010). The FDA-approved SSO Spinraza/nusinersen consists of 18 2'-MOE nucleotides with a PS backbone, binds to *SMN2* intron7, and promotes the inclusion of *SMN2* exon7 (Hua et al., 2011). Nusinersen has been shown to significantly improve the motor conditions and life expectancy of SMA patients (Finkel et al., 2017; Mercuri et al., 2018).

A straightforward application of SSOs would be suppressing abnormal/cryptic splice sites introduced by pathogenic mutations (Figure 1A). This strategy has been explored to treat multiple diseases, such as the *USH1C* Usher syndrome (Lentz et al., 2013). Autosomal recessive *USH1C* mutations cause type 1 Usher syndrome

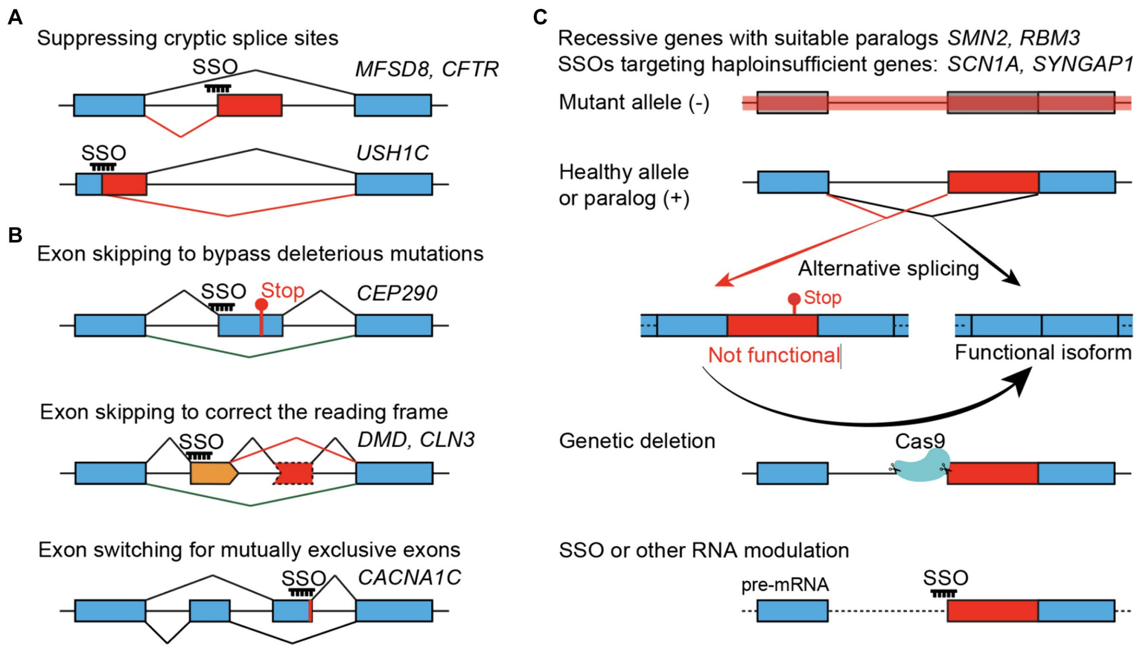


FIGURE 1
SSO-mediated therapeutic strategies. **(A)** Variant-specific SSOs suppress the gain of cryptic splice sites in the introns (top) or exons (bottom). **(B)** Exon-specific SSOs. Bypassing a non-essential exon that carries pathogenic mutations (top), skipping an additional non-essential exon (orange) to correct the translational reading frame (middle), or switching for a functional mutually exclusive exon (bottom). **(C)** Gene-specific SSOs treating recessive or haploinsufficient conditions by converting naturally occurring non-functional (or unstable) splice isoforms to functional isoforms, using *SYNGAP1* as an example. Genetic suppression of non-productive splicing, mimicking the maximal and constant effect of an SSO, can provide *in vivo* evidence about the neurological and organismal functions of the non-productive isoform, to what extent the protein level can be restored, and whether it can rescue phenotypes associated with loss-of-function alleles.

concerning congenital sensorineural deafness, vestibular dysfunction, and blindness (Verpy et al., 2000). The *USH1C* c.216G > A creates a cryptic 5′ splice site in exon 3, and an SSO covering the mutation and cryptic splice site significantly corrected the splicing error (Lentz et al., 2013). Remarkably, a single-dose SSO injection in the neonatal *Ush1c* c.216AA mice rescued abnormalities of cochlear hair cells, and vestibular and low-frequency hearing deficits, indicating strong therapeutic potential (Lentz et al., 2013).

While malfunctioning splicing can be suppressed, recessive mutations in the protein-coding regions may not be as straightforward to target with SSOs. In parallel to the nusinersen clinical trial, exon-skipping SSOs have been developed to treat Duchenne muscular dystrophy (DMD). DMD is an X-linked progressive muscle-wasting disease caused by loss-of-function mutations in the DMD/dystrophin gene. The dystrophin protein has 24 repeated spectrin-like domains, and truncated dystrophin proteins with fewer spectrin-like repeats were found in patients who showed much milder symptoms (England et al., 1990). Human genetics studies indicate that bypassing exons in the middle of dystrophin while preserving its N- and C-terminal domains can be beneficial (Matsuo, 1996). About half of DMD patients have deletion mutations in a hotspot region between exons 45–55 (Duan et al., 2021). Multiple SSOs have been successfully developed to skip exons such as 51, 53, or 45 (Exondys 51, Vyondys 53, and Amondys 45) to correct translational reading frames and produce partially functional dystrophin proteins (Roberts et al., 2023) (Figure 1B).

Exon-skipping SSOs have been explored for targeting other diseases, such as correcting the reading frame in *CLN3* Batten's disease (below), bypassing an inframe *CEP290* exon 41 that carries pathogenic mutations for Jobert syndrome (Ramsbottom et al., 2018), and suppressing a cryptic splice site in *CFTR* cystic fibrosis (Michaels et al., 2020). The Hastings group developed an exon-skipping strategy in mice to target a mutant allele that causes CLN3, a form of Batten's disease (Centa et al., 2020). Patients carrying recessive *CLN3* mutations experience disease onset in early childhood and typically decease by 20–30 years of age (IBDC, 1995). A substantial portion of patients are affected by a deletion spanning exons 7 and 8 (Δex78), leading to a shift of the translational frame. SSOs have been reported to correct the reading frame by skipping exon 5 in *cis* (Δex578, Figure 1B). The SSO has been reported to robustly induce exon 5 skipping and improve motor coordination and survival in a *Cln3* (Δex78) mouse model. The research group further created a *Cln3* (Δex578) genetic model and showed that deleting exon 5 on top of Δex78 was beneficial in mice (Centa et al., 2023). These works suggest a promising exon-skipping strategy for CLN3 (Δex78) Batten's disease.

The frontier of personalized medicine leaped forward with the N = 1 study on a child affected by CLN7, another form of Batten's disease (Kim et al., 2019). CLN7 is a late-infantile-onset lysosomal storage disorder, and affected children would experience early normal development followed by function declines of the nervous system that lead to vision loss, drug-resistant epilepsy, progressive cerebral and cerebellar atrophy, and premature death (Topcu et al., 2004). CLN7 is caused by recessive mutations in *MFSD8* (Siintola et al., 2007), but in the N = 1 case, clinical testing only identified one inherited *MFSD8* allele (Kim et al., 2019). The Yu lab performed whole genome sequencing and identified an SVA-transposon insertion in *MFSD8* intron 6, which promoted the inclusion of a cryptic 3′ splice site in

SSOs targeting predicted splicing enhancers were screened to suppress abnormal splicing of *MFSD8* (Figure 1A). The lead oligo milasen, an 18-nt SSO with 2'MOE modification and a PS backbone, was effective in patient cells and tolerated in rodents. Milasen was applied to the patient under an expanded-access protocol approved by the FDA and modeled after nusinersen. The $N = 1$ trial was shown to reduce seizure frequency and duration, suggesting a beneficial effect (Kim et al., 2019). This work paved the path for expedited genetic diagnosis and individualized drug development.

Important questions remain in SSO-mediated treatment. Given the diverse nature of pathogenic mutations, how can we identify targetable variants and design effective SSOs? A recent in-depth study of ataxia-telangiectasia (A-T) (Kim et al., 2023) began to address this question. A-T is an autosomal recessive disorder caused by the loss of the *ATM* gene required for DNA damage response and cell cycle progression (Savitsky et al., 1995). A-T patients typically show progressive cerebellar degeneration with early symptoms of ataxia, increased chance for cancer, and telangiectasias. A significant fraction of causal variants for A-T have been reported to cause abnormal splicing patterns (Teraoka et al., 1999), and SSOs (morpholino ASOs) have been developed to correct *ATM* splice variants (Du et al., 2007). A recent study reported whole-genome sequencing analyses of 235 A-T patients and classified plausible causal mutations depending on their molecular nature and potential for SSO treatment (Kim et al., 2023). Combining transcriptomic analyses and computation predictions, the authors estimated that 9 and 6% of the A-T patients carry "probable" and "possible" variants amenable to SSO targeting, respectively. The authors developed SSOs for two mutations and initiated clinical studies in A-T patients before disease onset. Thus, thorough genetics analysis estimated the SSO-targetable ratio to 9–15% in patients affected by rare diseases like A-T (Kim et al., 2023).

SSOs for dominant diseases

The variant- or exon-specific SSO strategies used in recessive disorders, such as suppressing cryptic splice sites and bypassing deleterious mutations in nonessential exons (Figures 1A,B), are also applicable to target the mutated alleles in dominant genetic disorders, especially for gain-of-function/activity alleles. Following the initial linkage analyses and cloning of inherited mutations, recent human genetics studies discovered widespread dominant mutations causal for neurodevelopmental disorders such as epilepsy and autism spectrum disorders (Helbig and Abou Tayoun, 2016, Satterstrom et al., 2020). For instance, over 1,400 *SCN1A* mutations have been reported as pathogenic in ClinVar (a public database to aggregate genetic variants and clinical findings), and a significant fraction of such mutations cause severe loss of function (frameshift, nonsense, splice site, and deletion). Furthermore, causal mutations for neurodevelopmental disorders have been reported in dozens to hundreds of genes. However, targeting such a vast number of mutated alleles using variant- or exon-specific SSOs presents a daunting task.

For haploinsufficient mutations, the healthy allele offers another layer of therapeutic potential. Increasing protein expression from the healthy allele can potentially establish a gene-specific instead of a variant- or exon-specific solution. In principle, this is achievable by boosting transcription, suppressing mRNA degradation, promoting translation, or suppressing protein degradation. Strategies suppressing naturally occurring non-productive isoforms, boosting translation by recruiting ribosomes, degrading naturally occurring antisense transcripts, and targeted de-ubiquitination have been explored to treat haploinsufficiency (Meng et al., 2015; Han et al., 2020; Kanner et al., 2020; Lim et al., 2020; Cao et al., 2023; Dawicki-McKenna et al., 2023; Yang et al., 2023).

Abnormal translation termination caused by premature codons (PTCs) triggers nonsense-mediated mRNA decay (NMD) in eukaryotes (Kurosaki et al., 2019). Interestingly, naturally occurring alternative splicing can trigger NMD (AS-NMD), and AS-NMD has been shown to autoregulate the master splicing factor SR proteins (Lewis et al., 2003; Lareau et al., 2007; Leclair et al., 2020). Recent studies have reported that AS-NMD developmentally regulates hundreds of genes in the brain (Zheng et al., 2012; Eom et al., 2013; Yan et al., 2015). Abnormally elevated AS-NMD in *SNRPB*, *FLNA*, and *SCN1A* by human mutations have been reported to cause cerebro–costo–mandibular syndrome (Lynch et al., 2014), structural brain malformation (Zhang et al., 2016), and epilepsy in humans (Carvill et al., 2018). Thus, the naturally occurring non-productive alternative splicing in disease-associated genes can be targetable switches for gene regulation.

If the gene of interest naturally expresses an alternative and non-productive isoform, converting the non-productive splice isoform to a functional form would be a promising approach to upregulate gene expression. The TANGO (targeted augmentation of nuclear gene output) method was reported in 2020, with a focus on *SCN1A* (Han et al., 2020; Lim et al., 2020). *De novo* loss-of-function mutations in *SCN1A* are leading causes of DEE, especially the Dravet syndrome, which is characterized by intractable febrile seizures. Human genetic studies showed that a fraction of *SCN1A* mRNA contains exon 20 N that triggers nonsense-mediated decay, and if the inclusion is abnormally increased by human mutations, it causes Dravet syndrome (Carvill et al., 2018). Lim et al. started by looking for non-productive alternative splicing in human disease-associated genes, screened SSOs in cultured cells, and showed the efficacy of two *SCN1A* ASOs in mice (Lim et al., 2020). Zhou et al. further showed an in-depth screening of *SCN1A* ASOs, their effect in upregulating mRNA and protein expression in mice, and their striking effects in rescuing lethality in a Dravet syndrome mouse model (Han et al., 2020). Clinical trials of the SSO in Dravet patients are ongoing and appear promising. These studies suggest that targeting the non-productive isoform can be a promising therapeutic approach.

SYNGAP1 encodes the synaptic Ras GTPase-activating protein and is required for synaptic plasticity. Haploinsufficient *SYNGAP1* mutations are the leading causes of intellectual disability, infantile epilepsy, and other neurological symptoms (Hamdan et al., 2009). Transcriptomic analysis of the developing mouse and human brains uncovered alternative 3′ splice sites of *SYNGAP1* intron10 that lead to NMD (A3SS-NMD, Figure 1C) (Yang et al., 2023). PTBP1/2 proteins directly promote the A3SS-NMD and suppress SYNGAP1 protein expression (Yang et al., 2023). Deletion of the A3SS-NMD in mice lead to upregulated Syngap1 protein. Importantly, such upregulated protein significantly alleviated the LTP deficits in the hippocampus and the neuronal excitability phenotype in cortical neurons caused by a compound *Syngap1* knockout allele (Yang et al., 2023). We further screened SSOs in human iPSCs, and the lead SSO effectively increased the functional *SYNGAP1* isoform in iPSC-derived neurons and cerebral organoids (Yang et al., 2023). Interestingly, some of the lead *SYNGAP1* SSOs identified in independent studies overlap with each other (Lim et al., 2020; Dawicki-McKenna et al., 2023; Yang et al., 2023), indicating the existence of a splicing enhancer for the A3SS-NMD.

Timothy syndrome, caused by dominant mutations in *CACNA1C*, is a multi-organ disorder characterized by congenital heart disease, lethal arrhythmias, cognitive deficits, and autism (Splawski et al., 2004). One recurrent p.G460R mutation occurs in the mutually exclusive exon 8A, promotes the exon 8A inclusion over exon 8, and leads to the loss of voltage-dependent channel inactivation (Panagiotakos et al., 2019). While *CACNA1C* exon 8 gradually replaces exon 8A during neural development, it was speculated as beneficial if the mutated exon 8A switched to exon 8 early in patients (Figure 1B). Indeed, the lead SSO was shown to increase *CACNA1C* exon 8 inclusion and rescue delayed channel inactivation and interneuron migration defects in cortical organoids (Chen et al., 2024). Furthermore, the authors transplanted cortical organoids carrying the p.G460R (exon 8A) mutation to athymic rats and showed the SSO treatment rescued molecular and functional defects (Chen et al., 2024). This study indicates that switching functionally equivalent but mutually exclusive exons can bypass deleterious effects and demonstrates the application of a human organoid-rat chimeric system.

Rodent models

Cultured cell lines, patient-derived fibroblasts, human iPSCs, and iPSC-derived neural cultures provide valuable tools for SSO screens, and the *in vivo* testing of SSO toxicity in rodents has become an integral process before clinical studies. However, identifying SSOs that work effectively *in vivo* remains a major challenge. For the N = 1 or extremely rare life-threatening variants, the limited time frame would not allow the establishment of proper genetic models or the thorough *in vivo* testing of SSO efficacy. For SSOs that target a specific gene, an exon, or a recurrent allele, the *in vivo* studies would provide valuable insights. This is exemplified by the development of nusinersen, where the SMA mouse models provide crucial tools to determine the efficacy of SSOs at the molecular and physiological levels (Monani et al., 2000; Hua et al., 2011). More recently, the Dravet mouse model (*Scn1a* knockout) was instrumental in demonstrating the efficacy of the *SCN1A* SSO in upregulating protein expression and rescuing lethality (Miller et al., 2014; Han et al., 2020). While the *SCN1A* lead SSO sequence is conserved and can be conveniently tested in mice, this would not necessarily be true for many other targets and SSOs. Mice carrying human genes of interest, through either BAC transgenic or humanized gene replacement, would be helpful tools to facilitate SSO studies. Recently, athymic rats carrying transplanted human cortical organoids have been reported as a new chimeric model to test the efficacy of SSOs (Chen et al., 2024).

In addition to testing SSOs in models of human diseases, the feasibility of SSO strategies can also be genetically tested for the desired splicing changes. This has been demonstrated by genetically deleting exon 5 in the *CLN3* (Δex78) Batten's disease model, where the *CLN3* (Δex578) allele has been shown to restore the reading frame and suppress the sensorimotor deficits (Centa et al., 2023) (Figure 1B, bottom). The heterozygous deletion of *Syngap1* A3SS-NMD has been recently shown to rescue haploinsufficiency in mice (Yang et al., 2023) (Figure 1C). These mouse genetic studies are critical to addressing questions that are otherwise hard to tackle: (1) Can the exon-skipping or NMD-suppression strategies yield the desired molecular and physiological outcome. For instance, when and how much protein upregulation can be achieved *in vivo* when the NMD exon is completely blocked. (2) Whether the splicing manipulation is deleterious for animal development. For the exon-skipping strategy, it is essential to know that the truncated protein would not gain toxicity or have more harmful effects than the otherwise loss-of-function allele. To treat haploinsufficiency by suppressing AS-NMD, it is crucial to understand the developmental function of the AS-NMD exons, which can be essential for brain development and functions. For example, deletion of the *Bak1* AS-NMD exon in mice induces abnormal neuronal loss and perinatal lethality (Lin et al., 2020). Homozygous deletion of A3SS-NMD exon in mouse Syngap1 led to deficits in long-term potentiation (Yang et al., 2023). Furthermore, CRISPR screens in cell lines have reported that AS-NMD exons can modulate cell proliferation and survival (Thomas et al., 2020). Thus, AS-NMD exons can be essential, and completely blocking AS-NMD may have undesired consequences. (3) Whether the genetic manipulation, mimicking the maximum effect of SSO treatment, can rescue or alleviate phenotypes in mouse models of human diseases.

Outlook for SSO therapy

For developmental and progressive disorders, it is important to have an early genetic diagnosis for targeted therapy. The unprecedented identification of causal variants with exome, genome, and transcriptome analyses has set the stage for precision medicine. Genetic diagnosis takes only days to weeks and saves precious time for therapeutic development. The flexible yet specific targeting by SSOs and the clinically proven chemistry make it possible to target a particular gene, an exon, or even a unique mutation. This is achieved by suppressing cryptic splice sites, skipping specific exons, or boosting gene expression by redirecting naturally occurring alternative splicing. In addition to early-onset neurological disorders, SSOs have also been designed to target models of aging and neurodegenerative disorders (Chang et al., 2018; Korecka et al., 2019; Nikom and Zheng, 2023; Preussner et al., 2023). Furthermore, splice-modulatory small molecules are rising to treat neurological disorders such as SMA and Huntington's disease (Palacino et al., 2015; Ratni et al., 2018; Bhattacharyya et al., 2021; Tang et al., 2021; Krach et al., 2022).

Naturally occurring alternative splicing events are potentially amenable to treating neurological disorders through different mechanisms: (1) Redirecting alternative splicing to promote the "healthier" allele. This has been demonstrated by nusinersen, which promotes SMN2 exon7 inclusion to make a stable protein. Most alternative exons (SE, A5SS, A3SS, MXE) are inframe, and pathogenic mutations within such exons can be potentially bypassed by enhancing alternative exon usage. (2) Treating haploinsufficiency by converting unproductive isoforms to functional forms. Suppression of *Scn1a* exon20N-NMD and *Syngap1* A3SS-NMD has been shown to alleviate haploinsufficiency in mice (Han et al., 2020; Yang et al., 2023). In *SCN1A*, *FLNA*, and *SNRPB* cases, deleterious mutations have been reported to increase AS-NMD and cause neurodevelopmental disorders (Lynch et al., 2014; Zhang et al., 2016; Carvill et al., 2018). Such human mutations may provide insights into how AS-NMD exons are regulated. In addition to AS-NMD, retained introns can be dynamically regulated and frequently prevent the host transcript from making functional proteins (Braunschweig et al., 2014; Mauger et al., 2016). Promoting intron excision may be another way to boost protein expression. Recent studies of nascent RNAs led to the estimation that ~15% of human protein-coding transcripts are degraded through AS-NMD, suggesting a large space for gene regulation (Fair et al., 2023). Deeper transcriptomic analyses and a better understanding of the splicing code will provide new insights into splice isoform regulation and enhance the discovery of SSO targets (Gandal et al., 2018; Li et al., 2018; Bao et al., 2019; Fair et al., 2023).

The gene- and exon-specific SSOs can be applied to conceivably many patients carrying mutations in the same gene or exon, and such SSOs have been going through clinical trials to determine their toxicity and efficacy. In contrast, variant-specific SSOs are enthusiastically pursued for personalized medicine or treating extremely rare cases (Kim et al., 2019; Crooke, 2022; Aartsma-Rus et al., 2023). Such N = 1 therapy presents new challenges and necessitates new guidelines for the SSO design and preclinical testing. An emerging question is what diseases, genes, and pathogenic variants are treatable by SSOs or ASOs in general. SSOs have been estimated to target 9–15% of A-T patients (Kim et al., 2023) and a higher ratio for DMD patients (Bladen et al., 2015). A much broader group of genes and about half of the pathogenic variants have been considered druggable with ASOs and other gene-regulatory mechanisms (Mittal et al., 2022). The active research and collaborative efforts in the field are drawing a promising future for SSO therapy.

Author contributions

XZ: Writing – original draft, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. XZ was supported by grants from the National Institutes of Health (DP2-GM137423 and R01-MH130594).

Acknowledgments

The author would like to thank Oriane Mauger, Clémence Bernard, and Michael Kiebler for the opportunity to contribute this review; and thank Runwei Yang and other colleagues for critically reading this manuscript.

Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

Aartsma-Rus, A., Garanto, A., van Roon-Mom, W., McConnell, E. M., Suslovitch, V., Yan, W. X., et al. (2023). Consensus guidelines for the design and in vitro preclinical efficacy testing N-of-1 exon skipping antisense oligonucleotides. *Nucleic Acid Ther.* 33, 17–25. doi: 10.1089/nat.2022.0060

Bao, S., Moakley, D. F., and Zhang, C. (2019). The splicing code Goes deep. *Cell* 176, 414–416. doi: 10.1016/j.cell.2019.01.013

Barash, Y., Calarco, J. A., Gao, W., Pan, Q., Wang, X., Shai, O., et al. (2010). Deciphering the splicing code. *Nature* 465, 53–59. doi: 10.1038/nature09000

Barbosa-Morais, N. L., Irimia, M., Pan, Q., Xiong, H. Y., Gueroussov, S., Lee, L. J., et al. (2012). The evolutionary landscape of alternative splicing in vertebrate species. *Science* 338, 1587–1593. doi: 10.1126/science.1230612

Berget, S. M., Moore, C., and Sharp, P. A. (1977). Spliced segments at the 5′ terminus of adenovirus 2 late mRNA. *Proc. Natl. Acad. Sci. USA* 74, 3171–3175. doi: 10.1073/pnas.74.8.3171

Bhattacharyya, A., Trotta, C. R., Narasimhan, J., Wiedinger, K. J., Li, W., Effenberger, K. A., et al. (2021). Small molecule splicing modifiers with systemic HTT-lowering activity. *Nat. Commun.* 12:7299. doi: 10.1038/s41467-021-27157-z

Black, D. L. (2003). Mechanisms of alternative pre-messenger RNA splicing. *Annu. Rev. Biochem.* 72, 291–336. doi: 10.1146/annurev.biochem.72.121801.161720

Bladen, C. L., Salgado, D., Monges, S., Foncuberta, M. E., Kekou, K., Kosma, K., et al. (2015). The TREAT-NMD DMD global database: analysis of more than 7,000 Duchenne muscular dystrophy mutations. *Hum. Mutat.* 36, 395–402. doi: 10.1002/humu.22758

Braunschweig, U., Barbosa-Morais, N. L., Pan, Q., Nachman, E. N., Alipanahi, B., Gonatopoulos-Pournatzis, T., et al. (2014). Widespread intron retention in mammals functionally tunes transcriptomes. *Genome Res.* 24, 1774–1786. doi: 10.1101/gr.177790.114

Calucho, M., Bernal, S., Alias, L., March, F., Vencesla, A., Rodriguez-Alvarez, F. J., et al. (2018). Correlation between SMA type and SMN2 copy number revisited: An analysis of 625 unrelated Spanish patients and a compilation of 2834 reported cases. *Neuromuscul. Disord.* 28, 208–215. doi: 10.1016/j.nmd.2018.01.003

Cao, Y., Liu, H., Lu, S. S., Jones, K. A., Govind, A. P., Jeyifous, O., et al. (2023). RNA-based translation activators for targeted gene upregulation. *Nat. Commun.* 14:6827. doi: 10.1038/s41467-023-42252-z

Carvill, G. L., Engel, K. L., Ramamurthy, A., Cochran, J. N., Roovers, J., Stamberger, H., et al. (2018). Aberrant inclusion of a poison exon causes Dravet syndrome and related SCN1A-associated genetic epilepsies. *Am. J. Hum. Genet.* 103, 1022–1029. doi: 10.1016/j.ajhg.2018.10.023

Centa, J. L., and Hastings, M. L. (2022). Targeting alternative splicing for therapeutic interventions. *Methods Mol. Biol.* 2537, 21–36. doi: 10.1007/978-1-0716-2521-7_2

Centa, J. L., Jodelka, F. M., Hinrich, A. J., Johnson, T. B., Ochaba, J., Jackson, M., et al. (2020). Therapeutic efficacy of antisense oligonucleotides in mouse models of CLN3 batten disease. *Nat. Med.* 26, 1444–1451. doi: 10.1038/s41591-020-0986-1

Centa, J. L., Stratton, M. P., Pratt, M. A., Osterlund Oltmanns, J. R., Wallace, D. G., Miller, S. A., et al. (2023). Protracted CLN3 batten disease in mice that genetically model an exon-skipping therapeutic approach. *Mol. Ther. Nucleic Acids* 33, 15–27. doi: 10.1016/j.omtn.2023.05.025

Chang, J. L., Hinrich, A. J., Roman, B., Norrbom, M., Rigo, F., Marr, R. A., et al. (2018). Targeting amyloid-beta precursor protein, APP, splicing with antisense oligonucleotides reduces toxic amyloid-beta production. *Mol. Ther.* 26, 1539–1551. doi: 10.1016/j.ymthe.2018.02.029

Chen, X., Birey, F., Li, M. Y., Revah, O., Levy, R., Thete, M. V., et al. (2024). Antisense oligonucleotide therapeutic approach for Timothy syndrome. *Nature* 628, 818–825. doi: 10.1038/s41586-024-07310-6

Chow, L. T., Gelinas, R. E., Broker, T. R., and Roberts, R. J. (1977). An amazing sequence arrangement at the 5′ ends of adenovirus 2 messenger RNA. *Cell* 12, 1–8. doi: 10.1016/0092-8674(77)90180-5

Crooke, S. T. (2022). Addressing the needs of patients with ultra-rare mutations one patient at a time: the n-lorem approach. *Nucleic Acid Ther.* 32, 95–100. doi: 10.1089/nat.2021.0046

Cummings, B. B., Marshall, J. L., Tukiainen, T., Lek, M., Donkervoort, S., Foley, A. R., et al. (2017). Improving genetic diagnosis in Mendelian disease with transcriptome sequencing. *Sci. Transl. Med.* 9:5209. doi: 10.1126/scitranslmed.aal5209

Dawicki-McKenna, J. M., Felix, A. J., Waxman, E. A., Cheng, C., Amado, D. A., Ranum, P. T., et al. (2023). Mapping PTBP2 binding in human brain identifies SYNGAP1 as a target for therapeutic splice switching. *Nat. Commun.* 14:2628. doi: 10.1038/s41467-023-38273-3

Deverman, B. E., Ravina, B. M., Bankiewicz, K. S., Paul, S. M., and Sah, D. W. Y. (2018). Gene therapy for neurological disorders: progress and prospects. *Nat. Rev. Drug Discov.* 17, 641–659. doi: 10.1038/nrd.2018.110

Du, L., Pollard, J. M., and Gatti, R. A. (2007). Correction of prototypic ATM splicing mutations and aberrant ATM function with antisense morpholino oligonucleotides. *Proc. Natl. Acad. Sci. USA* 104, 6007–6012. doi: 10.1073/pnas.0608616104

Duan, D., Goemans, N., Takeda, S., Mercuri, E., and Aartsma-Rus, A. (2021). Duchenne muscular dystrophy. *Nat. Rev. Dis. Primers* 7:13. doi: 10.1038/s41572-021-00248-3

Egli, M., and Manoharan, M. (2023). Chemistry, structure and function of approved oligonucleotide therapeutics. *Nucleic Acids Res.* 51, 2529–2573. doi: 10.1093/nar/gkad067

England, S. B., Nicholson, L. V., Johnson, M. A., Forrest, S. M., Love, D. R., Zubrzycka-Gaarn, E. E., et al. (1990). Very mild muscular dystrophy associated with the deletion of 46% of dystrophin. *Nature* 343, 180–182. doi: 10.1038/343180a0

Eom, T., Zhang, C., Wang, H., Lay, K., Fak, J., Noebels, J. L., et al. (2013). NOVA-dependent regulation of cryptic NMD exons controls synaptic protein levels after seizure. *eLife* 2:e00178. doi: 10.7554/eLife.00178

Fair, B., Najar, C. B. A., Zhao, J., Lozano, S., Reilly, A., Mossian, G., et al. (2023). Global impact of aberrant splicing on human gene expression levels. *bioRxiv*.

Feng, H., Moakley, D. F., Chen, S., McKenzie, M. G., Menon, V., and Zhang, C. (2021). Complexity and graded regulation of neuronal cell-type-specific alternative splicing revealed by single-cell RNA sequencing. *Proc. Natl. Acad. Sci. USA* 118, 1–12. doi: 10.1073/pnas.2013056118

Finkel, R. S., Mercuri, E., Darras, B. T., Connolly, A. M., Kuntz, N. L., Kirschner, J., et al. (2017). Nusinersen versus sham control in infantile-onset spinal muscular atrophy. *N. Engl. J. Med.* 377, 1723–1732. doi: 10.1056/NEJMoa1702752

Gandal, M. J., Zhang, P., Hadjimichael, E., Walker, R. L., Chen, C., Liu, S., et al. (2018). Transcriptome-wide isoform-level dysregulation in ASD, schizophrenia, and bipolar disorder. *Science* 362, 1–15. doi: 10.1126/science.aat8127

Gilbert, W. (1978). Why genes in pieces? *Nature* 271:501. doi: 10.1038/271501a0

Graveley, B. R. (2001). Alternative splicing: increasing diversity in the proteomic world. *Trends Genet.* 17, 100–107. doi: 10.1016/S0168-9525(00)02176-4

Gupta, I., Collier, P. G., Haase, B., Mahfouz, A., Joglekar, A., Floyd, T., et al. (2018). Single-cell isoform RNA sequencing characterizes isoforms in thousands of cerebellar cells. *Nat. Biotechnol.* 36, 1197–1202. doi: 10.1038/nbt.4259

Hamdan, F. F., Gauthier, J., Spiegelman, D., Noreau, A., Yang, Y., Pellerin, S., et al. (2009). Synapse to disease, mutations in SYNGAP1 in autosomal nonsyndromic mental retardation. *N. Engl. J. Med.* 360, 599–605. doi: 10.1056/NEJMoa0805392

Han, Z., Chen, C., Christiansen, A., Ji, S., Lin, Q., Anumonwo, C., et al. (2020). Antisense oligonucleotides increase Scn1a expression and reduce seizures and SUDEP incidence in a mouse model of Dravet syndrome. *Sci. Transl. Med.* 12, 1–14. doi: 10.1126/scitranslmed.aaz6100

Helbig, I., and Abou Tayoun, A. N. (2016). Understanding genotypes and phenotypes in epileptic encephalopathies. *Mol. Syndromol* 7, 172–181. doi: 10.1159/000448530

Hill, S. F., and Meisler, M. H. (2021). Antisense oligonucleotide therapy for neurodevelopmental disorders. *Dev. Neurosci.* 43, 247–252. doi: 10.1159/000517686

Hua, Y., Sahashi, K., Hung, G., Rigo, F., Passini, M. A., Bennett, C. F., et al. (2010). Antisense correction of SMN2 splicing in the CNS rescues necrosis in a type III SMA mouse model. *Genes Dev.* 24, 1634–1644. doi: 10.1101/gad.1941310

Hua, Y., Sahashi, K., Rigo, F., Hung, G., Horev, G., Bennett, C. F., et al. (2011). Peripheral SMN restoration is essential for long-term rescue of a severe spinal muscular atrophy mouse model. *Nature* 478, 123–126. doi: 10.1038/nature10485

IBDC (1995). Isolation of a novel gene underlying batten disease, CLN3. The international batten disease consortium. *Cell* 82, 949–957.

Kanner, S. A., Shuja, Z., Choudhury, P., Jain, A., and Colecraft, H. M. (2020). Targeted deubiquitination rescues distinct trafficking-deficient ion channelopathies. *Nat. Methods* 17, 1245–1253. doi: 10.1038/s41592-020-00992-6

Kim, J., Hu, C., Moufawad El Achkar, C., Black, L. E., Douville, J., Larson, A., et al. (2019). Patient-customized oligonucleotide therapy for a rare genetic disease. *N. Engl. J. Med.* 381, 1644–1652. doi: 10.1056/NEJMoa1813279

Kim, J., Woo, S., de Gusmao, C. M., Zhao, B., Chin, D. H., DiDonato, R. L., et al. (2023). A framework for individualized splice-switching oligonucleotide therapy. *Nature* 619, 828–836. doi: 10.1038/s41586-023-06277-0

Kole, R., Krainer, A. R., and Altman, S. (2012). RNA therapeutics: beyond RNA interference and antisense oligonucleotides. *Nat. Rev. Drug Discov.* 11, 125–140. doi: 10.1038/nrd3625

Korecka, J. A., Thomas, R., Christensen, D. P., Hinrich, A. J., Ferrari, E. J., Levy, S. A., et al. (2019). Mitochondrial clearance and maturation of autophagosomes are compromised in LRRK2 G2019S familial Parkinson's disease patient fibroblasts. *Hum. Mol. Genet.* 28, 3232–3243. doi: 10.1093/hmg/ddz126

Krach, F., Stemick, J., Boerstler, T., Weiss, A., Lingos, I., Reischl, S., et al. (2022). An alternative splicing modulator decreases mutant HTT and improves the molecular fingerprint in Huntington's disease patient neurons. *Nat. Commun.* 13:6797. doi: 10.1038/s41467-022-34419-x

Kurosaki, T., Popp, M. W., and Maquat, L. E. (2019). Quality and quantity control of gene expression by nonsense-mediated mRNA decay. *Nat. Rev. Mol. Cell Biol.* 20, 406–420. doi: 10.1038/s41580-019-0126-2

Lareau, L. F., Inada, M., Green, R. E., Wengrod, J. C., and Brenner, S. E. (2007). Unproductive splicing of SR genes associated with highly conserved and ultraconserved DNA elements. *Nature* 446, 926–929. doi: 10.1038/nature05676

Leclair, N. K., Brugiolo, M., Urbanski, L., Lawson, S. C., Thakar, K., Yurieva, M., et al. (2020). Poison exon splicing regulates a coordinated network of SR protein Expression during differentiation and tumorigenesis. *Mol. Cell* 80, 648–665.e9. doi: 10.1016/j.molcel.2020.10.019

Lefebvre, S., Burglen, L., Reboullet, S., Clermont, O., Burlet, P., Viollet, L., et al. (1995). Identification and characterization of a spinal muscular atrophy-determining gene. *Cell* 80, 155–165. doi: 10.1016/0092-8674(95)90460-3

Lentz, J. J., Jodelka, F. M., Hinrich, A. J., McCaffrey, K. E., Farris, H. E., Spalitta, M. J., et al. (2013). Rescue of hearing and vestibular function by antisense oligonucleotides in a mouse model of human deafness. *Nat. Med.* 19, 345–350. doi: 10.1038/nm.3106

Lewis, B. P., Green, R. E., and Brenner, S. E. (2003). Evidence for the widespread coupling of alternative splicing and nonsense-mediated mRNA decay in humans. *Proc. Natl. Acad. Sci. USA* 100, 189–192. doi: 10.1073/pnas.0136770100

Li, M., Santpere, G., Imamura Kawasawa, Y., Evgrafov, O. V., Gulden, F. O., Pochareddy, S., et al. (2018). Integrative functional genomic analysis of human brain development and neuropsychiatric risks. *Science* 362, 1–14. doi: 10.1126/science.aat7615

Lim, K. H., Han, Z., Jeon, H. Y., Kach, J., Jing, E., Weyn-Vanhentenryck, S., et al. (2020). Antisense oligonucleotide modulation of non-productive alternative splicing upregulates gene expression. *Nat. Commun.* 11:3501. doi: 10.1038/s41467-020-17093-9

Lin, L., Zhang, M., Stoilov, P., Chen, L., and Zheng, S. (2020). Developmental attenuation of neuronal apoptosis by neural-specific splicing of Bak1 microexon. *Neuron* 107, 1180–1196.e8. doi: 10.1016/j.neuron.2020.06.036

Lynch, D. C., Revil, T., Schwartzentruber, J., Bhoj, E. J., Innes, A. M., Lamont, R. E., et al. (2014). Disrupted auto-regulation of the spliceosomal gene SNRPB causes cerebro-costo-mandibular syndrome. *Nat. Commun.* 5:4483. doi: 10.1038/ncomms5483

Matsuo, M. (1996). Duchenne/Becker muscular dystrophy: from molecular diagnosis to gene therapy. *Brain and Development* 18, 167–172. doi: 10.1016/0387-7604(96)00007-1

Mauger, O., Lemoine, F., and Scheiffele, P. (2016). Targeted intron retention and excision for rapid gene regulation in response to neuronal activity. *Neuron* 92, 1266–1278. doi: 10.1016/j.neuron.2016.11.032

Meng, L., Ward, A. J., Chun, S., Bennett, C. F., Beaudet, A. L., and Rigo, F. (2015). Towards a therapy for Angelman syndrome by targeting a long non-coding RNA. *Nature* 518, 409–412. doi: 10.1038/nature13975

Mercuri, E., Darras, B. T., Chiriboga, C. A., Day, J. W., Campbell, C., Connolly, A. M., et al. (2018). Nusinersen versus sham control in later-onset spinal muscular atrophy. *N. Engl. J. Med.* 378, 625–635. doi: 10.1056/NEJMoa1710504

Merkin, J., Russell, C., Chen, P., and Burge, C. B. (2012). Evolutionary dynamics of gene and isoform regulation in mammalian tissues. *Science* 338, 1593–1599. doi: 10.1126/science.1228186

Michaels, W. E., Bridges, R. J., and Hastings, M. L. (2020). Antisense oligonucleotide-mediated correction of CFTR splicing improves chloride secretion in cystic fibrosis patient-derived bronchial epithelial cells. *Nucleic Acids Res.* 48, 7454–7467. doi: 10.1093/nar/gkaa490

Miller, A. R., Hawkins, N. A., McCollom, C. E., and Kearney, J. A. (2014). Mapping genetic modifiers of survival in a mouse model of Dravet syndrome. *Genes Brain Behav.* 13, 163–172. doi: 10.1111/gbb.12099

Mittal, S., Tang, I., and Gleeson, J. G. (2022). Evaluating human mutation databases for "treatability" using patient-customized therapy. *Med. J.* 3, 740–759. doi: 10.1016/j.medj.2022.08.006

Monani, U. R., Lorson, C. L., Parsons, D. W., Prior, T. W., Androphy, E. J., Burghes, A. H., et al. (1999). A single nucleotide difference that alters splicing patterns distinguishes the SMA gene SMN1 from the copy gene SMN2. *Hum. Mol. Genet.* 8, 1177–1183. doi: 10.1093/hmg/8.7.1177

Monani, U. R., Sendtner, M., Coovert, D. D., Parsons, D. W., Andreassi, C., Le, T. T., et al. (2000). The human centromeric survival motor neuron gene (SMN2) rescues embryonic lethality in SMN(−/−) mice and results in a mouse with spinal muscular atrophy. *Hum. Mol. Genet.* 9, 333–339. doi: 10.1093/hmg/9.3.333

Nikom, D., and Zheng, S. (2023). Alternative splicing in neurodegenerative disease and the promise of RNA therapies. *Nat. Rev. Neurosci.* 24, 457–473. doi: 10.1038/s41583-023-00717-6

Nussbacher, J. K., Tabet, R., Yeo, G. W., and Lagier-Tourenne, C. (2019). Disruption of RNA metabolism in neurological diseases and emerging therapeutic interventions. *Neuron* 102, 294–320. doi: 10.1016/j.neuron.2019.03.014

Palacino, J., Swalley, S. E., Song, C., Cheung, A. K., Shu, L., Zhang, X., et al. (2015). SMN2 splice modulators enhance U1-pre-mRNA association and rescue SMA mice. *Nat. Chem. Biol.* 11, 511–517. doi: 10.1038/nchembio.1837

Pan, Q., Shai, O., Lee, L. J., Frey, B. J., and Blencowe, B. J. (2008). Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. *Nat. Genet.* 40, 1413–1415. doi: 10.1038/ng.259

Panagiotakos, G., Haveles, C., Arjun, A., Petrova, R., Rana, A., Portmann, T., et al. (2019). Aberrant calcium channel splicing drives defects in cortical differentiation in Timothy syndrome. *eLife* 8:1037. doi: 10.7554/eLife.51037

Preussner, M., Smith, H. L., Hughes, D., Zhang, M., Emmerichs, A. K., Scalzitti, S., et al. (2023). ASO targeting RBM3 temperature-controlled poison exon splicing prevents neurodegeneration in vivo. *EMBO Mol. Med.* 15:e17157. doi: 10.15252/emmm.202217157

Raguram, A., Banskota, S., and Liu, D. R. (2022). Therapeutic in vivo delivery of gene editing agents. *Cell* 185, 2806–2827. doi: 10.1016/j.cell.2022.03.045

Ramsbottom, S. A., Molinari, E., Srivastava, S., Silberman, F., Henry, C., Alkanderi, S., et al. (2018). Targeted exon skipping of a CEP290 mutation rescues Joubert syndrome phenotypes in vitro and in a murine model. *Proc. Natl. Acad. Sci. USA* 115, 12489–12494. doi: 10.1073/pnas.1809432115

Ratni, H., Ebeling, M., Baird, J., Bendels, S., Bylund, J., Chen, K. S., et al. (2018). Discovery of Risdiplam, a selective survival of motor Neuron-2 (SMN2) gene splicing modifier for the treatment of spinal muscular atrophy (SMA). *J. Med. Chem.* 61, 6501–6517. doi: 10.1021/acs.jmedchem.8b00741

Rinaldi, C., and Wood, M. J. A. (2018). Antisense oligonucleotides: the next frontier for treatment of neurological disorders. *Nat. Rev. Neurol.* 14, 9–21. doi: 10.1038/nrneurol.2017.148

Roberts, T. C., Wood, M. J. A., and Davies, K. E. (2023). Therapeutic approaches for Duchenne muscular dystrophy. *Nat. Rev. Drug Discov.* 22, 917–934. doi: 10.1038/s41573-023-00775-6

Rochette, C. F., Gilbert, N., and Simard, L. R. (2001). SMN gene duplication and the emergence of the SMN2 gene occurred in distinct hominids: SMN2 is unique to *Homo sapiens*. *Hum. Genet.* 108, 255–266. doi: 10.1007/s004390100473

Satterstrom, F. K., Kosmicki, J. A., Wang, J., Breen, M. S., de Rubeis, S., An, J. Y., et al. (2020). Large-scale exome sequencing study implicates both developmental and functional changes in the neurobiology of autism. *Cell* 180, 568–584.e23. doi: 10.1016/j.cell.2019.12.036

Savitsky, K., Bar-Shira, A., Gilad, S., Rotman, G., Ziv, Y., Vanagaite, L., et al. (1995). A single ataxia telangiectasia gene with a product similar to PI-3 kinase. *Science* 268, 1749–1753. doi: 10.1126/science.7792600

Siintola, E., Topcu, M., Aula, N., Lohi, H., Minassian, B. A., Paterson, A. D., et al. (2007). The novel neuronal ceroid lipofuscinosis gene MFSD8 encodes a putative lysosomal transporter. *Am. J. Hum. Genet.* 81, 136–146. doi: 10.1086/518902

Soemedi, R., Cygan, K. J., Rhine, C. L., Wang, J., Bulacan, C., Yang, J., et al. (2017). Pathogenic variants that alter protein code often disrupt splicing. *Nat. Genet.* 49, 848–855. doi: 10.1038/ng.3837

Splawski, I., Timothy, K. W., Sharpe, L. M., Decher, N., Kumar, P., Bloise, R., et al. (2004). Ca(V)1.2 calcium channel dysfunction causes a multisystem disorder including arrhythmia and autism. *Cell* 119, 19–31. doi: 10.1016/j.cell.2004.09.011

Tang, Z., Zhao, J., Pearson, Z. J., Boskovic, Z. V., and Wang, J. (2021). RNA-targeting splicing modifiers: drug development and screening assays. *Molecules* 26:2263. doi: 10.3390/molecules26082263

Teraoka, S. N., Telatar, M., Becker-Catania, S., Liang, T., Onengut, S., Tolun, A., et al. (1999). Splicing defects in the ataxia-telangiectasia gene, ATM: underlying mutations and consequences. *Am. J. Hum. Genet.* 64, 1617–1631. doi: 10.1086/302418

Thomas, J. D., Polaski, J. T., Feng, Q., De Neef, E. J., Hoppe, E. R., McSharry, M. V., et al. (2020). RNA isoform screens uncover the essentiality and tumor-suppressor activity of ultraconserved poison exons. *Nat. Genet.* 52, 84–94. doi: 10.1038/s41588-019-0555-z

Topcu, M., Tan, H., Yalnizoglu, D., Usubutun, A., Saatci, I., Aynaci, M., et al. (2004). Evaluation of 36 patients from Turkey with neuronal ceroid lipofuscinosis: clinical, neurophysiological, neuroradiological and histopathologic studies. *Turk. J. Pediatr.* 46, 1–10

Van Nostrand, E. L., Freese, P., Pratt, G. A., Wang, X., Wei, X., Xiao, R., et al. (2020). A large-scale binding and functional map of human RNA-binding proteins. *Nature* 583, 711–719. doi: 10.1038/s41586-020-2077-3

Verpy, E., Leibovici, M., Zwaenepoel, I., Liu, X. Z., Gal, A., Salem, N., et al. (2000). A defect in harmonin, a PDZ domain-containing protein expressed in the inner ear sensory hair cells, underlies usher syndrome type 1C. *Nat. Genet.* 26, 51–55. doi: 10.1038/79171

Voit, T., Topaloglu, H., Straub, V., Muntoni, F., Deconinck, N., Campion, G., et al. (2014). Safety and efficacy of drisapersen for the treatment of Duchenne muscular dystrophy (DEMAND II): an exploratory, randomised, placebo-controlled phase 2 study. *Lancet Neurol.* 13, 987–996. doi: 10.1016/S1474-4422(14)70195-4

Wang, Z., and Burge, C. B. (2008). Splicing regulation: from a parts list of regulatory elements to an integrated splicing code. *RNA* 14, 802–813. doi: 10.1261/rna.876308

Wang, E. T., Sandberg, R., Luo, S., Khrebtukova, I., Zhang, L., Mayr, C., et al. (2008). Alternative isoform regulation in human tissue transcriptomes. *Nature* 456, 470–476. doi: 10.1038/nature07509

Xiong, H. Y., Alipanahi, B., Lee, L. J., Bretschneider, H., Merico, D., Yuen, R. K., et al. (2015). The human splicing code reveals new insights into the genetic determinants of disease. *Science* 347:1254806. doi: 10.1126/science.1254806

Yan, Q., Weyn-Vanhentenryck, S. M., Wu, J., Sloan, S. A., Zhang, Y., Chen, K., et al. (2015). Systematic discovery of regulated and conserved alternative exons in the mammalian brain reveals NMD modulating chromatin regulators. *Proc. Natl. Acad. Sci. USA* 112, 3445–3450. doi: 10.1073/pnas.1502849112

Yang, R., Feng, X., Arias-Cavieres, A., Mitchell, R. M., Polo, A., Hu, K., et al. (2023). Upregulation of SYNGAP1 expression in mice and human neurons by redirecting alternative splicing. *Neuron* 111, 1637–1650.e5. doi: 10.1016/j.neuron.2023.02.021

Yang, Y., Yang, R., Kang, B., Qian, S., He, X., and Zhang, X. (2023). Single-cell long-read sequencing in human cerebral organoids uncovers cell-type-specific and autism-associated exons. *Cell Rep.* 42:113335. doi: 10.1016/j.celrep.2023.113335

Zhang, Z., Bae, B., Cuddleston, W. H., and Miura, P. (2023). Coordination of alternative splicing and alternative polyadenylation revealed by targeted long read sequencing. *Nat. Commun.* 14:5506. doi: 10.1038/s41467-023-41207-8

Zhang, X., Chen, M. H., Wu, X., Kodani, A., Fan, J., Doan, R., et al. (2016). Cell-type-specific alternative splicing governs cell fate in the developing cerebral cortex. *Cell* 166, 1147–1162.e15. doi: 10.1016/j.cell.2016.07.025

Zheng, S., Gray, E. E., Chawla, G., Porse, B. T., O'Dell, T. J., and Black, D. L. (2012). PSD-95 is post-transcriptionally repressed during early neural development by PTBP1 and PTBP2. *Nat. Neurosci.* 15, 381–388, S1. doi: 10.1038/nn.3026

frontiers | Frontiers in Molecular Neuroscience

TYPE Mini Review
PUBLISHED 01 August 2024
DOI 10.3389/fnmol.2024.1426410

OPEN ACCESS

EDITED BY
Oriane Mauger,
Max Planck Institute of Psychiatry, Germany

REVIEWED BY
John Calarco,
University of Toronto, Canada
Zhiping Zhang,
UCONN Health, United States

*CORRESPONDENCE
Valérie Hilgers
✉ hilgers@ie-freiburg.mpg.de

RECEIVED 01 May 2024
ACCEPTED 22 July 2024
PUBLISHED 01 August 2024

CITATION
Ozbulut HC and Hilgers V (2024) Neuronal RNA processing: cross-talk between transcriptional regulation and RNA-binding proteins.
Front. Mol. Neurosci. 17:1426410.
doi: 10.3389/fnmol.2024.1426410

Neuronal RNA processing: cross-talk between transcriptional regulation and RNA-binding proteins

Hasan Can Ozbulut[1,2,3] and Valérie Hilgers[1]*

[1]Max-Planck-Institute of Immunobiology and Epigenetics, Freiburg, Germany, [2]Faculty of Biology, Albert Ludwig University, Freiburg, Germany, [3]International Max Planck Research School for Immunobiology, Epigenetics, and Metabolism (IMPRS-IEM), Freiburg, Germany

In the nervous system, alternative RNA processing is particularly prevalent, which results in the expression of thousands of transcript variants found in no other tissue. Neuron-specific RNA-binding proteins co-transcriptionally regulate alternative splicing, alternative polyadenylation, and RNA editing, thereby shaping the RNA identity of nervous system cells. Recent evidence suggests that interactions between RNA-binding proteins and cis-regulatory elements such as promoters and enhancers play a role in the determination of neuron-specific expression profiles. Here, we discuss possible mechanisms through which transcription and RNA processing cross-talk to generate the uniquely complex neuronal transcriptome, with a focus on alternative 3′-end formation.

KEYWORDS

neuronal RNA processing, transcription factors, RNA-binding proteins, alternative polyadenylation, RNA, nervous system

Introduction

Neurons are structurally and functionally complex cells that constantly adapt to their environment and to external stimuli. This necessitates a rapid, dynamic yet robust coordination of gene expression, a task that neurons achieve by specifically modulating transcription and RNA processing. Alternative splicing (AS) and alternative polyadenylation (APA) of mRNA precursors can generate multiple mRNA isoforms from the same transcription unit. In APA, the use of several functional polyadenylation [poly(A)] sites results in mRNA isoforms with different 3′-ends. When alternative [poly(A)] sites are located upstream of the stop codon, transcripts differ in their protein coding potential. More commonly, mRNAs with different 3′ UTRs are generated (Mitschka and Mayr, 2022). Notably, in animals from flies to humans, hundreds of genes undergo a shift toward more distal [poly(A)] sites exclusively in neurons, thus producing longer, often ultra-long, 3′ UTRs, termed "neuronal 3′ UTRs" (nUTRs) (Hilgers et al., 2011; Smibert et al., 2012; Miura et al., 2013; Carrasco et al., 2020; Wei et al., 2020). The alternative use of splice sites through AS is also particularly prevalent in neurons; the selective inclusion or exclusion of exons results in thousands of neuron-specific transcript variants (Carrasco et al., 2020; Lee et al., 2021). One particularly striking example of neural-regulated AS is the systematic inclusion of <30 nucleotide "microexons" (Irimia et al., 2014) that is mediated by the eMIC protein domain across Bilateria (Torres-Méndez et al., 2019). Interestingly, the splicing programs independently evolved in nonvertebrate and vertebrate bilaterians, but ultimately regulate neuronal excitability: in mammals, neuronal microexons encode amino acids on the surface of interaction domains of proteins involved in neurogenesis, whereas in flies, top splicing targets are enriched in ion channels (Torres-Méndez et al., 2022).

An integral and conserved feature of neurogenesis, neuronal RNA processing generates mRNA isoforms that differ in their coding or UTR sequence, thereby increasing proteome diversity and fine-tuning gene expression [reviewed in (Bhat et al., 2022, Hilgers, 2022, Wei and Lai, 2022, Lee et al., 2023)]. Neuron-specific RNA isoforms play an important role in neurogenesis (Zhang et al., 2019; Bae et al., 2020; Bae and Miura, 2020; Carrasco et al., 2020) and contribute to the versatility of neuronal cells by helping coordinate specialized processes. Although the importance of RNA-based regulation in human neurological disease has been known for decades, the underlying pathogenic mechanisms are still not well understood.

RNA-binding proteins regulate alternative RNA processing in neurons

RNA processing is regulated by a myriad of RNA-binding proteins (RBPs) that usually act in a cell-, gene-, and context-specific manner. Many RBPs are enriched or exclusively expressed in neural tissues, and consequently mediate RNA processing in a nervous-system-specific manner. Such RBPs and their molecular roles are typically well-conserved across metazoans; they include members of the protein families ELAV (Embryonic Lethal Abnormal Vision)/Hu (Human antigen) PTBP (Polypyrimidine Tract-Binding Protein), NOVA antigen (Neuro-oncological ventral), RBFOX (RNA-binding Fox-1 homolog), and CELF (CUGBP Elav-like family). The role of these protein families in neuronal RNA processing have been recently described in an excellent review (Lee et al., 2023). In this perspective article, we will maintain a focus on the well-studied protein ELAV as a representative model for neuron-specific RBPs and their interactions with transcriptional processing.

ELAV/Hu proteins highly conserved RBPs critical for neuronal differentiation, maturation and function (Mirisis and Carew, 2019; Hilgers, 2022; Wei and Lai, 2022; Mulligan and Bicknell, 2023). Typically, at least one member of the ELAV/Hu protein family is expressed specifically in neurons, and ELAV/Hu proteins serve as markers for neuronal cell types throughout the animal kingdom (Pascale et al., 2008). In Drosophila, where it was first described, ELAV regulates AS as well as APA (Koushika et al., 1996, 2000; Soller and White, 2003). Genome-wide studies in Drosophila revealed that ELAV/Hu operate on the transcriptome scale, with hundreds of genes undergoing ELAV-dependent alternative processing (Hilgers et al., 2012; Carrasco et al., 2020; Wei et al., 2020; Lee et al., 2021). Strikingly, all neuron-specific APA events were shown to depend on ELAV; the RNA signatures mediated by ELAV/Hu proteins are so manifold and distinct that the RBP is considered a "master regulator" of neuronal RNA processing in Drosophila. It remains to be seen whether ELAV/Hu proteins possess a similar monopoly in other systems; evidence from individual genes in human and mouse systems suggests that they do (Zhu et al., 2007; Dai et al., 2012; Mansfield and Keene, 2012; Dorrity et al., 2023), although the molecular intricacies remain to be solved. In Drosophila, ELAV binds nascent transcripts in the vicinity of proximal poly(A) and splice sites to inhibit their usage and foster APA and AS, respectively. Nearly all mRNAs found to be deregulated in *elav* mutants were direct binding targets of ELAV as seen by iCLIP in fly brains (Carrasco et al., 2020), suggesting that ELAV regulates its functional APA targets through direct physical interaction. In contrast, while ELAV binds to many AS targets at relevant splice sites (Carrasco et al., 2020; Lee et al., 2021), indicative of a direct effect, an indirect role was also described: ELAV mediates neuronal APA of *Srrm234*, and the resulting eMIC-containing isoform of Srrm234 in turn globally promotes the inclusion of neural microexons (Torres-Méndez et al., 2022).

A role for cis-regulatory elements in alternative RNA processing

In addition to *trans*-acting factors such as RBPs, recent findings point to a role for cis-regulatory sequences —promoters, enhancers— and their associated effectors —transcription factors— in the regulation of RNA processing. Early studies showed a physical association of RNA processing factors with the transcription machinery, as well as a positive influence of transcriptional activation on 3′-end processing (Dantonel et al., 1997; Calvo and Manley, 2003; Glover-Cutter et al., 2008; Wang et al., 2010; Nagaike et al., 2011; Yang et al., 2016; Carminati et al., 2023). Conversely, effective co-transcriptional processing is necessary for RNA Polymerase II (Pol II) processivity (Tellier et al., 2020). The mechanistic underpinnings of the regulatory coupling between transcription initiation, processing and transcription termination are not well understood. Accumulating evidence has shown that these couplings are important for context-, tissue-, and gene-specific APA and AS. Correlations between the use of distinct transcription start sites (TSSs) and 3′-end processing at different poly(A) sites have been observed, for example in different cell types (Anvar et al., 2018; Hardwick et al., 2022) and in the disease context (Demircioğlu et al., 2019). Recent studies have now established a causal link between sites of transcription initiation and sites of RNA processing: in Drosophila brains and human cerebral organoids, specific TSSs —so-called "dominant promoters"— foster the selection of distinct splice and 3′-end processing sites. Promoter dominance is highly prevalent in the nervous system, occurring in about 40–60% of genes, and broadly regulates mRNA isoform selection (Alfonso-Gonzalez et al., 2023). A role for distal gene enhancers and the relative position of the TSS relative to 3′-end sites on the DNA template have also been shown to influence 3′-end processing activity, and consequently, the expression of alternative 3′ UTR isoforms (Kwon et al., 2022; Calvo-Roitberg et al., 2024). These couplings between transcription initiation and RNA processing choices suggest a widespread coordination of events that occur during transcription; they also imply that many RNA processing events are regulated as soon as transcription initiates, many kilobases upstream.

Regulation of RNA processing by transcription factors

Transcription factors (TFs), the key effectors and regulators of transcription, likely play an important role in coordinating transcription initiation and RNA processing. While it is commonly understood that they primarily function at the chromatin level by binding directly to DNA, it is less recognized that a subset of TFs, termed DRBPs (DNA- and RNA-binding proteins), also have the capability to bind RNA.

For example, the Hox transcription factor Ultrabithorax (Ubx) binds to nascent pre-mRNAs at alternative cassette exons through its homeodomain, thereby promoting exon inclusion in the Drosophila mesoderm (Carnesecchi et al., 2022). Interestingly, Ubx interacts with chromatin in a dynamic, transcription elongation-dependent manner, indicating that Ubx may accompany Pol II from initiation to processing using different nucleic acid binding modules or assembling distinct functional complexes "on the go."

A potentially widespread function for TFs in AS arose from studies in which knockdown of TFs with C2H2-type zinc finger (ZnF) DNA-binding domains had pronounced effects on splicing events in K562 and mouse neural cells (Han et al., 2017; Ullah et al., 2023). For at least a subset of ZnF TFs, the modulation of exon inclusion/ exclusion and intron retention seemed to occur through direct binding of nascent RNA at intronic regions. One such ZnF, Zfp871, regulates hundreds of neural-differential exons in genes typically associated with neuronal morphology and function, hinting at a broad role for ZnF TFs in the regulation of neuron-specific RNA processing (Han et al., 2017).

Recent findings also reveal an involvement of the transcriptional co-activators CREB-binding protein (CBP)/p300 in alternative 3′-end site selection. In Drosophila brains, CBP was found enriched at dominant promoters as well as at their associated, usually distal, 3′-end site. Strikingly, genetic deletion of CBP resulted in a broad disruption of the 3′-end expression landscape in developing embryos (Alfonso-Gonzalez et al., 2023). How CBP connects sites of transcription initiation and alternative processing, remains unknown; given the essential role of CBP in neuronal differentiation (Lipinski et al., 2019), understanding this interaction could provide clues into the promoter-mediated establishment and maintenance of the neuron-specific 3′-end landscape.

A recent, genome-wide study found that nearly half of all TFs can bind RNA in human cells. Interactions occur through a novel, highly conserved arginine-rich motif (ARM) and were shown to enhance the TF's association with chromatin, thereby promoting target gene expression (Oksuz et al., 2023). Missense mutations in ARM motifs were associated with human diseases, including cancer and developmental syndromes; perturbations of key TF's ARMs without affecting DNA binding resulted in developmental defects in zebrafish, which suggests that RNA binding constitutes a widespread property of TFs that contribute to their function *in vivo*.

In contrast, several recent studies report that multiple chromatin proteins previously described as DRBPs, including PRC2, JARID2, and YY1, do not appear to bind RNA *in vivo*. PRC2 core subunits did not associate with RNA under stringent experimental conditions (Guo et al., 2024); the loss of PRC2 enrichment at chromatin upon RNase treatment can be explained, at least in part, by a concomitant, unspecific enrichment of non-target regions (Hall Hickman and Jenner, 2024; Healy et al., 2024). It will be important to verify the actual binding of TFs to RNA on a case-by-case basis in order to distinguish direct and indirect effects on the regulation of RNA metabolism (Nielsen and Ulitksy, 2024). Although many protein-RNA interactions remain to be functionally validated and mechanisms to be elucidated, chromatin proteins have emerged as key players in the regulation of RNA processing.

Interaction of RBPs with cis-regulatory elements

The connection between RNA processing and transcription regulation in cis is supported by the widespread occurrence of physical and genetic interactions between splicing/ polyadenylation factors and the transcription machinery [reviewed in Bentley (2014), Shenasa and Bentley (2023), and Shine et al. (2024)]. In addition to an effect of RBPs on Pol II processivity through their interaction with transcribing RNA, multiple lines of evidence also indicate that RBPs interact with chromatin to regulate promoter activity in a promoter- and gene-specific manner. Several genome-wide studies suggest that many nuclear RBPs exert their function at the chromatin level. By ChIP-seq, RBPs were found to pervasively, extensively, and specifically associate with DNA at gene promoters (Xiao et al., 2019); in another study, RBPs even constituted nearly half of all proteins obtained from the chromatome (Rafiee et al., 2021). One RBP with a demonstrated role at gene promoters is the splicing factor Rbm25, which co-associates with the TF YY1 at numerous genetic loci; the physical interaction between the two proteins is necessary for YY1 recruitment to chromatin and transcriptional output (Xiao et al., 2019), suggesting a role of RBPs in the regulation of promoter activity. Whether RBPs are recruited to the DNA template via RNA binding, through the RBP's intrinsically disordered regions (IDRs), or through chromatin-associated proteins, likely differs on a gene- and RBP-dependent basis.

The first evidence of promoter sequences in the regulation of APA was shown in the context of neuron-specific RNA processing. In the Drosophila nervous system, the RBP ELAV physically associates with the promoters of genes that undergo ELAV-dependent APA and 3′ UTR lengthening. Selection of the distal, neuron-specific 3′-end site was abrogated upon replacing the native promoter of an ELAV target gene by a generic one; moreover, ectopic ELAV expression in muscle cells induced neuronal 3′ UTRs from transgenes carrying the native, but not the generic promoter. The ELAV binding pattern coincided with the signature of Pol II promoter-proximal pausing, indicating that ELAV may be loaded onto the transcription machinery during transcription initiation (Oktaba et al., 2015). It remains unclear how ELAV then finds its way to its functional sites on the nascent RNA —proximal poly(A) sites potentially located kilobases further downstream (Hilgers, 2015; Slobodin and Agami, 2015).

The RNA and DNA-binding protein Fused in Sarcoma (FUS), linked to amyotrophic lateral sclerosis (ALS) (Kwiatkowski et al., 2009; Vance et al., 2009), functions in multiple RNA processes in neuronal cell nuclei. FUS was shown to co-transcriptionally binds to pre-mRNAs to regulate AS (Ishigaki et al., 2012; Lagier-Tourenne et al., 2012); FUS iCLIP clusters on nascent RNA positionally coincide with RNA Pol II pausing sites (Masuda et al., 2015). Moreover, *in vitro* experiments indicate that FUS mediates the physical and functional interactions between the transcription and splicing machineries (Yu and Reed, 2015). Interestingly, the histone mark H3K36me3 in actively elongating genes was recently shown to recruit FUS to chromatin and away from nascent RNA, thereby ensuring proper poly(A) site selection (Jia et al., 2024).

As more examples arise of transcription factors and cis-regulatory elements that control RBP-mediated AS and APA, it will be interesting to determine whether common mechanisms govern these interactions, or if they differ based on the gene and cellular context.

Possible mechanisms linking transcription to co-transcriptional processing

Several scenarios can be envisaged to explain how RBPs interact with gene activation and transcription processes, regulating AS and APA in a tissue-specific manner. Although our hypotheses are formulated with neuronal RBPs in mind, the proposed mechanisms are not mutually exclusive, and each of them may operate in different contexts or tissues.

Interestingly, enhancer regions help modulate 3′-end processing choices (Kwon et al., 2022; Calvo-Roitberg et al., 2024); it is conceivable that RNA processing factors are recruited to specific genes through enhancer-promoter interactions and the TFs that mediate them. One possible mechanism is exemplified by the histone acetyltransferase and chromatin remodeler CBP, which binds RNAs transcribed from enhancer regions (eRNAs) and stimulates transcription at target promoters (Bose et al., 2017). CBP binds both to neuron-specific TSSs and associated, often neuron-specific 3′-end sites (Alfonso-Gonzalez et al., 2023), thereby creating a link between the spatiotemporal regulation of gene activation and RNA processing. TFs like CBP may guide RBPs to gene promoters by first promoting recruitment to enhancer regions through direct (TF-RBP) or indirect (TF-eRNA-RBP) interactions (Figure 1A).

Transcriptional processivity and elongation speed are essential for proper RNA processing, especially in long genes; Pol II slowing, pausing, and fastening can disrupt exon selection and 3′-end patterns (Fong et al., 2014, 2015; Liu et al., 2017; Aslanzadeh et al., 2018; Muniz et al., 2021; Debès et al., 2023; Welsh and Gardini, 2023; Zukher et al., 2023). We propose that in specific contexts, RNA processing choices are modulated through recruitment of RBPs to sites of transcription initiation, allowing them to subsequently hitchhike on the elongating transcription machinery, and to be released onto nascent RNA during Pol II pausing or other changes in elongation dynamics (Figure 1B). In this process, the Pol II C-Terminal Domain (CTD) or tissue-specific TFs may provide a scaffold for interactions with RBPs.

Finally, the increasingly appreciated ability of RBPs to associate with DNA, and of TFs to bind RNA, raises the question whether the two processes —transcription initiation and RNA processing— are as separately controlled by each of the two protein groups as previously thought. Individual examples of RBPs activating transcription have been reported (Zeng et al., 2016; Bi et al., 2019; Ren et al., 2021; Xu et al., 2023), indicating that tissue-specific regulators of RNA processing may also be involved in the activation of distinct promoters. In light of promoter dominance, the RBP-mediated activation of neuron-specific promoters may constitute one mechanism by which neuronal 3′-ends and, more generally, tissue-specific mRNA isoforms are selected. In this context, it is important that nascent pre-mRNAs represent not only mere products, but also important regulators of transcription and RNA processing (Skalska et al., 2017). RBPs may bind nascent RNAs as early as transcription initiation and influence the transcription process (Figure 1C). Similarly, TFs may be recruited to sites of APA and AS by initially binding nascent RNAs in the vicinity of promoter regions.

Conclusion

Neuron-specific RNA processing is pervasive and occurs in all animals that have been studied, including humans. Variations in 3′ UTR length and sequence contribute to neurological disorders, emphasizing the importance of alternative mRNA processing in nervous system development and physiology (Mohanan et al., 2021; LaForce et al., 2022; Wilson et al., 2023). It has become more and more

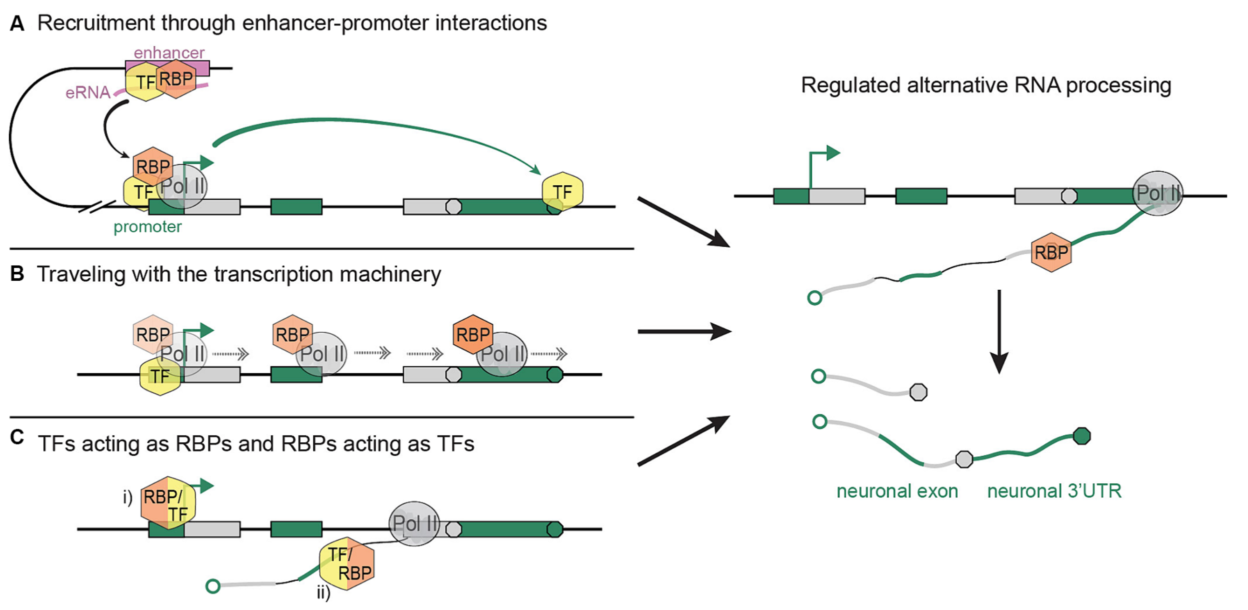


FIGURE 1
Possible mechanisms linking cis- and trans-regulation of tissue-specific RNA processing. **(A)** RBPs are recruited to tissue-specific enhancers and their target promoter through binding to TFs and/or enhancer RNAs. The activation of a dominant promoter fosters the expression of the linked, tissue-specific 3′-end. **(B)** RBPs associate with the transcription machinery at gene promoters by binding to TFs or the Pol II CTD, and accompany the elongating transcription complex to downstream sites of alternative RNA processing. **(C)** Intertwined regulation of transcription and RNA processing through (i) RBPs acting as TFs at the gene promoter, and/or (ii) TFs acting as RBPs on nascent pre-mRNAs. Resulting tissue-specific (neuronal) RNA processing events are represented on the right. Sequences that are expressed in a tissue-specific (neuronal) fashion are shown as green boxes (DNA) and lines (RNA).

evident that cis-regulatory elements and their associated biomolecules —transcription factors, coding and non-coding RNAs— contribute to the generation of neuron-specific exons and 3′ UTRs. Recent advances in long-read RNA sequencing, chromatin capture studies, protein-nucleic acid interaction analyses, and imaging of nascent mRNAs, have already provided glimpses into the coordination of co-and post-transcriptional processes. Systematically applying these approaches to nervous system tissues, in combination, will shed light on the mechanisms that link transcription and RNA processing, and help identifying and possibly targeting disease-causing mutations.

Author contributions

HO: Conceptualization, Visualization, Writing – original draft, Writing – review & editing. VH: Conceptualization, Visualization, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This work was funded by the Max Planck Society, the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) SFB 1381 (Project-ID 403222702) and under Germany's Excellence Strategy (CIBSS – EXC-2189 – Project-ID 390939984), and the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (grant agreement ERC-2018-STG-803258).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

Alfonso-Gonzalez, C., Legnini, I., Holec, S., Arrigoni, L., Ozbulut, H. C., Mateos, F., et al. (2023). Sites of transcription initiation drive mRNA isoform selection. *Cell* 186, 2438–2455.e22. doi: 10.1016/j.cell.2023.04.012

Anvar, S. Y., Allard, G., Tseng, E., Sheynkman, G. M., De Klerk, E., Vermaat, M., et al. (2018). Full-length mRNA sequencing uncovers a widespread coupling between transcription initiation and mRNA processing. *Genome Biol.* 19:46. doi: 10.1186/s13059-018-1418-0

Aslanzadeh, V., Huang, Y., Sanguinetti, G., and Beggs, J. D. (2018). Transcription rate strongly affects splicing fidelity and cotranscriptionality in budding yeast. *Genome Res.* 28, 203–213. doi: 10.1101/gr.225615.117

Bae, B., Gruner, H. N., Lynch, M., Feng, T., So, K., Oliver, D., et al. (2020). Elimination of Calm1 long 3'-UTR mRNA isoform by CRISPR-Cas9 gene editing impairs dorsal root ganglion development and hippocampal neuron activation in mice. *RNA* 26, 1414–1430. doi: 10.1261/rna.076430.120

Bae, B., and Miura, P. (2020). Emerging roles for 3' UTRs in neurons. *Int. J. Mol. Sci.* 21:3413. doi: 10.3390/ijms21103413

Bentley, D. L. (2014). Coupling mRNA processing with transcription in time and space. *Nat. Rev. Genet.* 15, 163–175. doi: 10.1038/nrg3662

Bhat, V. D., Jayaraj, J., and Babu, K. (2022). RNA and neuronal function: the importance of post-transcriptional regulation. *Oxford Open Neuroscience* 1. doi: 10.1093/oons/kvac011

Bi, X., Xu, Y., Li, T., Li, X., Li, W., Shao, W., et al. (2019). RNA targets Ribogenesis factor WDR43 to chromatin for transcription and pluripotency control. *Mol. Cell* 75:e9. doi: 10.1016/j.molcel.2019.05.007

Bose, D. A., Donahue, G., Reinberg, D., Shiekhattar, R., Bonasio, R., and Berger, S. L. (2017). RNA binding to CBP stimulates histone acetylation and transcription. *Cell* 168, 135–149.e22. doi: 10.1016/j.cell.2016.12.020

Calvo, O., and Manley, J. L. (2003). Strange bedfellows: polyadenylation factors at the promoter. *Genes Dev.* 17, 1321–1327. doi: 10.1101/gad.1093603

Calvo-Roitberg, E., Carroll, C. L., Venev, S. V., Kim, G., Mick, S. T., Dekker, J., et al. (2024). mRNA initiation and termination are spatially coordinated. *bioRxiv*. [Preprint]. doi: 10.1101/2024.01.05.574404

Carminati, M., Rodriguez-Molina, J. B., Manav, M. C., Bellini, D., and Passmore, L. A. (2023). A direct interaction between CPF and RNA pol II links RNA 3′ end processing to transcription. *Mol. Cell* 83, 4461–4478.e13. doi: 10.1016/j.molcel.2023.11.004

Carnesecchi, J., Boumpas, P., Van Nierop, Y. S. P., Domsch, K., Pinto, H. D., Borges Pinto, P., et al. (2022). The Hox transcription factor Ultrabithorax binds RNA and regulates co-transcriptional splicing through an interplay with RNA polymerase II. *Nucleic Acids Res.* 50, 763–783. doi: 10.1093/nar/gkab1250

Carrasco, J., Rauer, M., Hummel, B., Grzejda, D., Alfonso-Gonzalez, C., Lee, Y., et al. (2020). ELAV and FNE determine neuronal transcript signatures through EXon-activated rescue. *Mol. Cell* 80, 156–163.e6. doi: 10.1016/j.molcel.2020.09.011

Dai, W., Zhang, G., and Makeyev, E. V. (2012). RNA-binding protein HuR autoregulates its expression by promoting alternative polyadenylation site usage. *Nucleic Acids Res.* 40, 787–800. doi: 10.1093/nar/gkr783

Dantonel, J.-C., Murthy, K. G. K., Manley, J. L., and Tora, L. (1997). Transcription factor TFIID recruits factor CPSF for formation of 3′ end of mRNA. *Nature* 389, 399–402. doi: 10.1038/38763

Debès, C., Papadakis, A., Grönke, S., Karalay, Ö., Tain, L. S., Mizi, A., et al. (2023). Ageing-associated changes in transcriptional elongation influence longevity. *Nature* 616, 814–821. doi: 10.1038/s41586-023-05922-y

Demircioğlu, D., Cukuroglu, E., Kindermans, M., Nandi, T., Calabrese, C., Fonseca, N. A., et al. (2019). A Pan-cancer transcriptome analysis reveals pervasive regulation through alternative promoters. *Cell* 178, 1465–1477.e17. doi: 10.1016/j.cell.2019.08.018

Dorrity, T. J., Shin, H., Wiegand, K. A., Aruda, J., Closser, M., Jung, E., et al. (2023). Long 3'UTRs predispose neurons to inflammation by promoting immunostimulatory double-stranded RNA formation. *Sci. Immunol.* 8:eadg2979. doi: 10.1126/sciimmunol.adg2979

Fong, N., Brannan, K., Erickson, B., Kim, H., Cortazar, M. A., Sheridan, R. M., et al. (2015). Effects of transcription elongation rate and Xrn2 exonuclease activity on RNA polymerase II termination suggest widespread kinetic competition. *Mol. Cell* 60, 256–267. doi: 10.1016/j.molcel.2015.09.026

Fong, N., Kim, H., Zhou, Y., Ji, X., Qiu, J., Saldi, T., et al. (2014). Pre-mRNA splicing is facilitated by an optimal RNA polymerase II elongation rate. *Genes Dev.* 28, 2663–2676. doi: 10.1101/gad.252106.114

Glover-Cutter, K., Kim, S., Espinosa, J., and Bentley, D. L. (2008). RNA polymerase II pauses and associates with pre-mRNA processing factors at both ends of genes. *Nat. Struct. Mol. Biol.* 15, 71–78. doi: 10.1038/nsmb1352

Guo, J. K., Blanco, M. R., Walkup, W. G. T., Bonesteele, G., Urbinati, C. R., Banerjee, A. K., et al. (2024). Denaturing purifications demonstrate that PRC2 and other widely reported chromatin proteins do not appear to bind directly to RNA in vivo. *Mol. Cell* 84, 1271–1289.e12. doi: 10.1016/j.molcel.2024.01.026

Hall Hickman, A., and Jenner, R. G. (2024). Apparent RNA bridging between PRC2 and chromatin is an artifact of non-specific chromatin precipitation upon RNA degradation. *Cell Rep.* 43:113856. doi: 10.1016/j.celrep.2024.113856

Han, H., Braunschweig, U., Gonatopoulos-Pournatzis, T., Weatheritt, R. J., Hirsch, C. L., Ha, K. C. H., et al. (2017). Multilayered control of alternative splicing regulatory networks by transcription factors. *Mol. Cell* 65:e7. doi: 10.1016/j.molcel.2017.01.011

Hardwick, S. A., Hu, W., Joglekar, A., Fan, L., Collier, P. G., Foord, C., et al. (2022). Single-nuclei isoform RNA sequencing unlocks barcoded exon connectivity in frozen brain tissue. *Nat. Biotechnol.* 40, 1082–1092. doi: 10.1038/s41587-022-01231-3

Healy, E., Zhang, Q., Gail, E. H., Agius, S. C., Sun, G., Bullen, M., et al. (2024). The apparent loss of PRC2 chromatin occupancy as an artifact of RNA depletion. *Cell Rep.* 43:113858. doi: 10.1016/j.celrep.2024.113858

Hilgers, V. (2015). Alternative polyadenylation coupled to transcription initiation: insights from ELAV-mediated 3 ' UTR extension. *RNA Biol.* 12, 918–921. doi: 10.1080/15476286.2015.1060393

Hilgers, V. (2022). Regulation of neuronal RNA signatures by ELAV/Hu proteins. Wiley Interdiscip rev. *RNA*. 14:e1733. doi: 10.1002/wrna.1733

Hilgers, V., Lemke, S. B., and Levine, M. (2012). ELAV mediates 3 ' UTR extension in the Drosophila nervous system. *Genes Dev.* 26, 2259–2264. doi: 10.1101/gad.199653.112

Hilgers, V., Perry, M. W., Hendrix, D., Stark, A., Levine, M., and Haley, B. (2011). Neural-specific elongation of 3 ' UTRs during Drosophila development. *Proc. Natl. Acad. Sci. USA* 108, 15864–15869. doi: 10.1073/pnas.1112672108

Irimia, M., Weatheritt, R. J., Ellis, J. D., Parikshak, N. N., Gonatopoulos-Pournatzis, T., Babor, M., et al. (2014). A highly conserved program of neuronal microexons is misregulated in autistic brains. *Cell* 159, 1511–1523. doi: 10.1016/j.cell.2014.11.035

Ishigaki, S., Masuda, A., Fujioka, Y., Iguchi, Y., Katsuno, M., Shibata, A., et al. (2012). Position-dependent FUS-RNA interactions regulate alternative splicing events and transcriptions. *Sci. Rep.* 2:529. doi: 10.1038/srep00529

Jia, J., Fan, H., Wan, X., Fang, Y., Li, Z., Tang, Y., et al. (2024). FUS reads histone H3K36me3 to regulate alternative polyadenylation. *Nucleic Acids Res.* 52, 5549–5571. doi: 10.1093/nar/gkae184

Koushika, S. P., Lisbin, M. J., and White, K. (1996). ELAV, a Drosophila neuron-specific protein, mediates the generation of an alternatively spliced neural protein isoform. *Curr. Biol.* 6, 1634–1641. doi: 10.1016/S0960-9822(02)70787-2

Koushika, S. P., Soller, M., and White, K. (2000). The neuron-enriched splicing pattern of Drosophila erect wing is dependent on the presence of ELAV protein. *Mol. Cell. Biol.* 20, 1836–1845. doi: 10.1128/MCB.20.5.1836-1845.2000

Kwiatkowski, T. J. Jr., Bosco, D. A., Leclerc, A. L., Tamrazian, E., Vanderburg, C. R., Russ, C., et al. (2009). Mutations in the FUS/TLS gene on chromosome 16 cause familial amyotrophic lateral sclerosis. *Science* 323, 1205–1208. doi: 10.1126/science.1166066

Kwon, B., Fansler, M. M., Patel, N. D., Lee, J., Ma, W., and Mayr, C. (2022). Enhancers regulate 3′ end processing activity to control expression of alternative 3′UTR isoforms. *Nat. Commun.* 13:2709. doi: 10.1038/s41467-022-30525-y

Laforce, G. R., Philippidou, P., and Schaffer, A. E. (2022). mRNA isoform balance in neuronal development and disease. *Wiley Interdiscip. Rev. RNA*. 14:e1762. doi: 10.1002/wrna.1762

Lagier-Tourenne, C., Polymenidou, M., Hutt, K. R., Vu, A. Q., Baughn, M., Huelga, S. C., et al. (2012). Divergent roles of ALS-linked proteins FUS/TLS and TDP-43 intersect in processing long pre-mRNAs. *Nat. Neurosci.* 15, 1488–1497. doi: 10.1038/nn.3230

Lee, S., Aubee, J. I., and Lai, E. C. (2023). Regulation of alternative splicing and polyadenylation in neurons. *Life Sci. Alliance* 6:e202302000. doi: 10.26508/lsa.202302000

Lee, S., Wei, L., Zhang, B., Goering, R., Majumdar, S., Wen, J., et al. (2021). ELAV/Hu RNA binding proteins determine multiple programs of neural alternative splicing. *PLoS Genet.* 17:e1009439. doi: 10.1371/journal.pgen.1009439

Lipinski, M., Del Blanco, B., and Barco, A. (2019). CBP/p300 in brain development and plasticity: disentangling the KAT's cradle. *Curr. Opin. Neurobiol.* 59, 1–8. doi: 10.1016/j.conb.2019.01.023

Liu, X., Freitas, J., Zheng, D., Oliveira, M. S., Hoque, M., Martins, T., et al. (2017). Transcription elongation rate has a tissue-specific impact on alternative cleavage and polyadenylation in Drosophila melanogaster. *RNA* 23, 1807–1816. doi: 10.1261/rna.062661.117

Mansfield, K. D., and Keene, J. D. (2012). Neuron-specific ELAV/Hu proteins suppress HuR mRNA during neuronal differentiation by alternative polyadenylation. *Nucleic Acids Res.* 40, 2734–2746. doi: 10.1093/nar/gkr1114

Masuda, A., Takeda, J., Okuno, T., Okamoto, T., Ohkawara, B., Ito, M., et al. (2015). Position-specific binding of FUS to nascent RNA regulates mRNA length. *Genes Dev.* 29, 1045–1057. doi: 10.1101/gad.255737.114

Mirisis, A. A., and Carew, T. J. (2019). The ELAV family of RNA-binding proteins in synaptic plasticity and long-term memory. *Neurobiol. Learn. Mem.* 161, 143–148. doi: 10.1016/j.nlm.2019.04.007

Mitschka, S., and Mayr, C. (2022). Context-specific regulation and function of mRNA alternative polyadenylation. *Nat. Rev. Mol. Cell Biol.* 23, 779–796. doi: 10.1038/s41580-022-00507-5

Miura, P., Shenker, S., Andreu-Agullo, C., Westholm, J. O., and Lai, E. C. (2013). Widespread and extensive lengthening of 3' UTRs in the mammalian brain. *Genome Res.* 23, 812–825. doi: 10.1101/gr.146886.112

Mohanan, N. K., Shaji, F., Koshre, G. R., and Laishram, R. S. (2021). Alternative polyadenylation: an enigma of transcript length variation in health and disease. *Wiley Interdiscip. Rev. RNA*. 13:e1692. doi: 10.1002/wrna.1692

Mulligan, M. R., and Bicknell, L. S. (2023). The molecular genetics of nELAVL in brain development and disease. *Eur. J. Hum. Genet.* 31, 1209–1217. doi: 10.1038/s41431-023-01456-z

Muniz, L., Nicolas, E., and Trouche, D. (2021). RNA polymerase II speed: a key player in controlling and adapting transcriptome composition. *EMBO J.* 40:e105740. doi: 10.15252/embj.2020105740

Nagaike, T., Logan, C., Hotta, I., Rozenblatt-Rosen, O., Meyerson, M., and Manley, J. L. (2011). Transcriptional activators enhance polyadenylation of mRNA precursors. *Mol. Cell* 41, 409–418. doi: 10.1016/j.molcel.2011.01.022

Nielsen, M., and Ulitksy, I. (2024). The links are still missing: revisiting the role of RNA as a guide for chromatin-associated proteins. *Mol. Cell* 84, 1178–1179. doi: 10.1016/j.molcel.2024.03.005

Oksuz, O., Henninger, J. E., Warneford-Thomson, R., Zheng, M. M., Erb, H., Vancura, A., et al. (2023). Transcription factors interact with RNA to regulate genes. *Mol. Cell* 83, 2449–2463.e13. doi: 10.1016/j.molcel.2023.06.012

Oktaba, K., Zhang, W., Lotz, T. S., Jun, D. J., Lemke, S. B., Ng, S. P., et al. (2015). ELAV links paused pol II to alternative polyadenylation in the Drosophila nervous system. *Mol. Cell* 57, 341–348. doi: 10.1016/j.molcel.2014.11.024

Pascale, A., Amadio, M., and Quattrone, A. (2008). Defining a neuron: neuronal ELAV proteins. *Cell. Mol. Life Sci.* 65, 128–140. doi: 10.1007/s00018-007-7017-y

Rafiee, M. R., Zagalak, J. A., Sidorov, S., Steinhauser, S., Davey, K., Ule, J., et al. (2021). Chromatin-contact atlas reveals disorder-mediated protein interactions and moonlighting chromatin-associated RBPs. *Nucleic Acids Res.* 49, 13092–13107. doi: 10.1093/nar/gkab1180

Ren, Y., Huo, Y., Li, W., He, M., Liu, S., Yang, J., et al. (2021). A global screening identifies chromatin-enriched RNA-binding proteins and the transcriptional regulatory activity of QKI5 during monocytic differentiation. *Genome Biol.* 22:290. doi: 10.1186/s13059-021-02508-7

Shenasa, H., and Bentley, D. L. (2023). Pre-mRNA splicing and its cotranscriptional connections. *Trends Genet.* 39, 672–685. doi: 10.1016/j.tig.2023.04.008

Shine, M., Gordon, J., Scharfen, L., Zigackova, D., Herzel, L., and Neugebauer, K. M. (2024). Co-transcriptional gene regulation in eukaryotes and prokaryotes. *Nat. Rev. Mol. Cell Biol.* 25, 534–554. doi: 10.1038/s41580-024-00706-2

Skalska, L., Beltran-Nebot, M., Ule, J., and Jenner, R. G. (2017). Regulatory feedback from nascent RNA to chromatin and transcription. *Nat. Rev. Mol. Cell Biol.* 18, 331–337. doi: 10.1038/nrm.2017.12

Slobodin, B., and Agami, R. (2015). Transcription initiation determines its end. *Mol. Cell* 57, 205–206. doi: 10.1016/j.molcel.2015.01.006

Smibert, P., Miura, P., Westholm, J. O., Shenker, S., May, G., Duff, M. O., et al. (2012). Global patterns of tissue-specific alternative polyadenylation in Drosophila. *Cell Rep.* 1, 277–289. doi: 10.1016/j.celrep.2012.01.001

Soller, M., and White, K. (2003). ELAV inhibits 3′-end processing to promote neural splicing of ewg pre-mRNA. *Genes Dev.* 17, 2526–2538. doi: 10.1101/gad.1106703

Tellier, M., Maudlin, I., and Murphy, S. (2020). Transcription and splicing: A two-way street. *Wiley Interdiscip. Rev.* 11, –e1593. doi: 10.1002/wrna.1593

Torres-Méndez, A., Bonnal, S., Marquez, Y., Roth, J., Iglesias, M., Permanyer, J., et al. (2019). A novel protein domain in an ancestral splicing factor drove the evolution of neural microexons. *Nat. Ecol. Evol.* 3, 691–701. doi: 10.1038/s41559-019-0813-6

Torres-Méndez, A., Pop, S., Bonnal, S., Almudi, I., Avola, A., Roberts, R. J. V., et al. (2022). Parallel evolution of a splicing program controlling neuronal excitability in flies and mammals. *Sci. Adv.* 8:eabk0445. doi: 10.1126/sciadv.abk0445

Ullah, F., Jabeen, S., Salton, M., Reddy, A. S. N., and Ben-Hur, A. (2023). Evidence for the role of transcription factors in the co-transcriptional regulation of intron retention. *Genome Biol.* 24:53. doi: 10.1186/s13059-023-02885-1

Vance, C., Rogelj, B., Hortobágyi, T., De Vos, K. J., Nishimura, A. L., Sreedharan, J., et al. (2009). Mutations in FUS, an RNA processing protein, cause familial amyotrophic lateral sclerosis type 6. *Science* 323, 1208–1211. doi: 10.1126/science.1165942

Wang, Y., Fairley, J. A., and Roberts, S. G. (2010). Phosphorylation of TFIIB links transcription initiation and termination. *Curr. Biol.* 20, 548–553. doi: 10.1016/j.cub.2010.01.052

Wei, L., and Lai, E. C. (2022). Regulation of the alternative neural transcriptome by ELAV/Hu RNA binding proteins. *Front. Genet.* 13:848626. doi: 10.3389/fgene.2022.848626

Wei, L., Lee, S., Majumdar, S., Zhang, B., Sanfilippo, P., Joseph, B., et al. (2020). Overlapping activities of ELAV/Hu family RNA binding proteins specify the extended neuronal 3' UTR landscape in Drosophila. *Mol. Cell* 80, 140–155.e6. doi: 10.1016/j.molcel.2020.09.007

Welsh, S. A., and Gardini, A. (2023). Genomic regulation of transcription and RNA processing by the multitasking integrator complex. *Nat. Rev. Mol. Cell Biol.* 24, 204–220. doi: 10.1038/s41580-022-00534-2

Wilson, D. M. III, Cookson, M. R., Van Den Bosch, L., Zetterberg, H., Holtzman, D. M., and Dewachter, I. (2023). Hallmarks of neurodegenerative diseases. *Cell* 186, 693–714. doi: 10.1016/j.cell.2022.12.032

Xiao, R., Chen, J.-Y., Liang, Z., Luo, D., Chen, G., Lu, Z. J., et al. (2019). Pervasive chromatin-RNA binding protein interactions enable RNA-based regulation of transcription. *Cell* 178, 107–121.e18. doi: 10.1016/j.cell.2019.06.001

Xu, F., Li, R., Von Gromoff, E. D., Drepper, F., Knapp, B., Warscheid, B., et al. (2023). Reprogramming of the transcriptome after heat stress mediates heat hormesis in Caenorhabditis elegans. *Nat. Commun.* 14:4176. doi: 10.1038/s41467-023-39882-8

Yang, Y., Li, W., Hoque, M., Hou, L., Shen, S., Tian, B., et al. (2016). PAF complex plays novel subunit-specific roles in alternative cleavage and polyadenylation. *PLoS Genet.* 12:e1005794. doi: 10.1371/journal.pgen.1005794

Yu, Y., and Reed, R. (2015). FUS functions in coupling transcription to splicing by mediating an interaction between RNAP II and U1 snRNP. *Proc. Natl. Acad. Sci. USA* 112, 8608–8613. doi: 10.1073/pnas.1506282112

Zeng, Y., Yao, B., Shin, J., Lin, L., Kim, N., Song, Q., et al. (2016). Lin28A binds active promoters and recruits Tet1 to regulate gene expression. *Mol. Cell* 61, 153–160. doi: 10.1016/j.molcel.2015.11.020

Zhang, Z., So, K., Peterson, R., Bauer, M., Ng, H., Zhang, Y., et al. (2019). Elav-mediated exon skipping and alternative polyadenylation of the Dscam1 gene are required for axon outgrowth. *Cell Rep.* 27, 3808–3817.e7. doi: 10.1016/j.celrep.2019.05.083

Zhu, H., Zhou, H. L., Hasman, R. A., and Lou, H. (2007). Hu proteins regulate polyadenylation by blocking sites containing U-rich sequences. *J. Biol. Chem.* 282, 2203–2210. doi: 10.1074/jbc.M609349200

Zukher, I., Dujardin, G., Sousa-Luís, R., and Proudfoot, N. J. (2023). Elongation roadblocks mediated by dCas9 across human genes modulate transcription and nascent RNA processing. *Nat. Struct. Mol. Biol.* 30, 1536–1548. doi: 10.1038/s41594-023-01090-9

frontiers | Frontiers in Molecular Neuroscience

TYPE Review
PUBLISHED 01 August 2024
DOI 10.3389/fnmol.2024.1414949

OPEN ACCESS

EDITED BY
Oriane Mauger,
Max Planck Institute of Psychiatry, Germany

REVIEWED BY
Karl Emory Bauer,
Ludwig Maximilian University of Munich, Germany
Stephanie Ceman,
University of Illinois at Urbana-Champaign, United States

*CORRESPONDENCE
Davor Lessel
✉ d.lessel@salk.at
Hans-Jürgen Kreienkamp
✉ Kreienkamp@uke.de

RECEIVED 09 April 2024
ACCEPTED 22 July 2024
PUBLISHED 01 August 2024

CITATION
Lederbauer J, Das S, Piton A, Lessel D and Kreienkamp H-J (2024) The role of DEAD- and DExH-box RNA helicases in neurodevelopmental disorders.
Front. Mol. Neurosci. 17:1414949.
doi: 10.3389/fnmol.2024.1414949

The role of DEAD- and DExH-box RNA helicases in neurodevelopmental disorders

Johannes Lederbauer[1,2], Sarada Das[2], Amelie Piton[3], Davor Lessel[1,2]* and Hans-Jürgen Kreienkamp[2]*

[1]Institute of Human Genetics, University Hospital Salzburg, Paracelsus Medical University, Salzburg, Austria, [2]Institute of Human Genetics, University Medical Center Hamburg-Eppendorf, Hamburg, Germany, [3]Department of Translational Medicine and Neurogenetics, Institute of Genetics and Molecular and Cellular Biology, Strasbourg University, CNRS UMR7104, INSERM U1258, Illkirch, France

Neurodevelopmental disorders (NDDs) represent a large group of disorders with an onset in the neonatal or early childhood period; NDDs include intellectual disability (ID), autism spectrum disorders (ASD), attention deficit hyperactivity disorders (ADHD), seizures, various motor disabilities and abnormal muscle tone. Among the many underlying Mendelian genetic causes for these conditions, genes coding for proteins involved in all aspects of the gene expression pathway, ranging from transcription, splicing, translation to the eventual RNA decay, feature rather prominently. Here we focus on two large families of RNA helicases (DEAD- and DExH-box helicases). Genetic variants in the coding genes for several helicases have recently been shown to be associated with NDD. We address genetic constraints for helicases, types of pathological variants which have been discovered and discuss the biological pathways in which the affected helicase proteins are involved.

KEYWORDS

stress granules, P-bodies, miRNA, translation, R-loop

Introduction

RNA helicases use energy from the hydrolysis of ATP to unwind double stranded sections of RNA/RNA or RNA/DNA hybrids, and may also assist in the restructuring of RNA/protein complexes (ribonucleoproteins, RNPs). They carry out essential cellular functions, many of which are conserved from yeast to humans (Linder and Jankowsky, 2011). Accordingly, the distinct families of these helicases are also highly conserved throughout eukaryotic evolution (Pyle, 2008). Six major helicase superfamilies have been identified (SF1-SF6) which have distinct functions in several aspects of DNA and RNA metabolism (Fairman-Williams et al., 2010). While members of SF3-6 superfamilies are active in a "toroidal," hexameric form, SF1 and SF2 are active as monomers. SF1 and SF2 helicases are rather similar to each other, but individual differences in conserved helicase core motifs allow for a clear differentiation between the two families (Gorbalenya and Koonin, 1993; Tanner and Linder, 2001; Fairman-Williams et al., 2010). We will focus here on the DEAD-and DExH-box helicase families which are part of the SF2 superfamily, and which in humans consist of 54 different members. While it appears surprising that our transcriptome (and genome) requires such a diversity of different helicase activities, it should be noted that every single aspect, e.g., of the life cycle of an RNA requires in most cases not only one, but several different helicases. Quite often a complete knockout of one of the RNA helicase coding genes is lethal early in mouse development (Li et al., 2014; Zheng et al., 2015; Kim et al., 2022). On the other hand, heterozygous, loss-of-function or missense variants in helicase genes are often associated with a neuronal, or neurodevelopmental phenotype in humans (Snijders Blok et al., 2015; Lessel et al., 2017; Balak et al., 2019). This may reflect the specific requirements of the developing nervous system for precise regulation of gene expression and RNA metabolism.

Neurodevelopmental disorders (NDDs)

NDDs represent a large, clinically and genetically, heterogeneous group of human disorders with an onset in the neonatal or early childhood period. NDDs include intellectual disability (ID), autism spectrum disorders (ASD), attention deficit hyperactivity disorders (ADHD), seizures, various motor disabilities and abnormal muscle tone (Morris-Rosendahl and Crocq, 2020). NDDs are usually characterized by impairments in cognition, communication, adaptive behavior and psychomotor skills.

NDDs are often associated with Mendelian, single genetic events such as chromosomal rearrangements, copy number variations, small insertions/deletions, nonsense or missense variants. NDDs have been estimated to affect 3% of the general population (Gilissen et al., 2014), with 0.5% of all newborn affected by severe ID (Parenti et al., 2020). Although each of the underlying genetic causes of NDD is rare, their accumulated number is high enough worldwide to cause a serious socio-economic problem for health care systems. The genetic testing now routinely relies on next generation sequencing (NGS) techniques, i.e., whole exome/whole genome sequencing, or a panel based approach focused on known NDD genes. Identification of novel Mendelian, genetic causes requires a complex process, which includes evaluation of databases of human genetic variations such as the gnomAD database (Chen S. et al., 2024), identification of similarly affected individuals harboring similar genetic variants and in most instances, confirmatory functional analyses. Due to the increased use of NGS, the last decade has seen a tremendous increase in identification of novel genetic causes for NDDs. However, for the majority of NDDs two main challenges remain: reliable assessment of the pathogenicity of identified variants and meaningful clinical interventions. Thus, there is currently an urgent need for improved understanding of NDD pathology (Gilissen et al., 2014; Niemi et al., 2018; Parenti et al., 2020).

Which genes are affected in NDD patients?

One might have thought that genes involved in neuron-specific functions would contribute to the prevalence of NDDs. Indeed, pathogenic variants in genes coding for synaptic proteins have been implicated in autism spectrum disorders and in ID (Bourgeron, 2009). However, a more quantitative analysis points to genes coding for proteins involved in control of the different steps of the gene expression pathway, such as transcriptional regulators, splicing factors, translational regulators or aspects of miRNA pathways (Greene et al., 2023). In fact, the most prevalent cause for ID now appears to be a pathogenic variant affecting the non-coding RNA RNU4-2, which is a component of the spliceosome (Chen Y. et al., 2024; Greene et al., 2024). It should be noted that in contrast to other tissues, the nervous system consists of an amazingly large number of different cell types. These can be differentiated based on single cell transcriptomics. Thus, one recent study identified 461 clusters of different cell types in the human brain, with 3,313 individual subclusters of cell types defined by a specific pattern of transcripts (Siletti et al., 2023). Due to this extreme transcriptomic diversity, the developing brain may require the precise regulation of gene expression pathways much more than other tissues. Furthermore, neurons engage in localized protein synthesis both in dendrites and in axons (Steward and Schuman, 2001; Cajigas et al., 2012). Local protein synthesis in dendrites, close to postsynaptic sites, is believed to contribute to synaptic plasticity, i.e., to activity dependent changes in synaptic strength which should be specific to those synapses which have been activated (Kindler and Kreienkamp, 2012; Sun et al., 2021). Local protein synthesis in axons is necessary due to the long distance of axon terminals from the cell body, making somatic protein synthesis followed by protein transport to synaptic terminals too slow for replenishment of and adaptation of protein levels. Many mRNAs have been shown to be not only present, but also translated locally near pre-or postsynaptic sites (Hafner et al., 2019; Glock et al., 2021). This concept entails transport of ribosomes, tRNAs and mRNAs to dendrites or axons, localized control of translation as well as the eventual degradation of the localized mRNAs. Indeed, specific structures involved in the regulation of mRNA translation such as stress granule components and P-bodies have been detected in neuronal axons and dendrites (Shiina et al., 2005; Cougot et al., 2008; Zeitelhofer et al., 2008; Sahoo et al., 2018; Lessel et al., 2020). Again, the requirements for proteins involved in RNA metabolism appear to be more complex in neurons when compared to non-neuronal cells. RNA helicases of the DExH/DEAD-box families constitute a large group of proteins which may be present in dendrites (Kanai et al., 2004), which contribute to these processes, and which may cause neurological problems upon the occurrence of damaging alterations in their coding genes.

Structure of DExH/DEAD-box helicases

The core functions of DexH/DEAD-box RNA helicases (ATP binding and hydrolysis, nucleic acid binding and unwinding) are carried out by two adjacent core domains which show structural similarity to the recombination protein RecA. Within these helicase core domains, up to 14 conserved helicase core motifs (HCMs) can be identified (Fairman-Williams et al., 2010). Out of these, Ia – Ib, IV, IVa, V and Vb bind nucleic acid substrates. HCMs Q, I, II, IIIa and VI are involved in ATP binding and hydrolysis. Interestingly, not all HCMs are present in all members of these helicase families (Fairman-Williams et al., 2010). Thus, the Q-HCM, a 9 amino acid (aa) sequence containing an invariant glutamine residue along with a conserved phenylalanine residue 17 aa further upstream, is found only in DEAD-box RNA helicases. The namesake DEAD or DExH sequence motifs constitute motif II. In motif II, specifically the Asp-Glu part is involved in coordinating the ATP associated Mg^{2+} ion, and in positioning the water molecule which performs the ATP hydrolysis.

In 3D structures of both DEAD-box as well as DExH box proteins, the side chains of the C-terminal Asp/His residues of motif III are in direct contact to the Ser and Thr side chains of motif III (sequence SAT), which couples NTP binding and hydrolysis to nucleic acid binding and unwinding (Tanner et al., 2003; Cordin et al., 2006; Figure 1; see Supplementary Figure S1 for a complete view on human DEAD box proteins).

DExH helicases are processive helicases which move along a dsRNA substrate, capable of performing several unwinding steps along the way. In contrast, DEAD-box proteins dissociate from their RNA substrates after a single unwinding step (Bohnsack et al., 2023). The unique C-terminal domains of DExH helicases, i.e., the winged-helix (WH) and ratchet-like domains (often described together as helicase associated 2 or HA2 domain), as well as the oligonucleotide/oligosaccharide-binding (OB)-fold domain are relevant here (Murakami et al., 2017; Jagtap et al., 2023; Figure 1; see Supplementary Figure S2 for a complete view on human DExH box proteins). For these helicases, HA2 and OB motifs are integral parts of the helicase function as they contribute to a tunnel for single stranded RNA. In the case of the *Drosophila maleless* (MLE) helicase, a member of the DExH family which has been studied in much detail, the core RecA domains bind the RNA substrate via non sequence specific interaction with the sugar/phosphate backbone. In contrast, the OB domain is involved in base-specific contacts at the 5′ end of the tunnel. Here, also the second of the two N-terminal dsRBD domains (dsRBD2) is essential for activity as it is involved in regulating helicase activity, and also provides for proper positioning of dsRNA substrates at the entrance of the RNA tunnel (Prabu et al., 2015; Jagtap et al., 2023).

Other accessory domains, including additional dsRNA binding domains or RNA recognition motifs (RRMs), are typically not conserved within a given family of these RNA helicases. Both, N-and C-terminal domains frequently determine the integration of helicase proteins into larger functional complexes and are therefore highly relevant for the physiological function.

Multiple functions of RNA helicases during the RNA life cycle

Different stages of the RNA life cycle are shown in Figure 2. Transcription produces the crude RNA that will go through several interactions with various proteins or different kinds of RNA in its path that shapes its journey. Transcription is coordinated by several RNA helicases which act as coactivators or corepressors by binding to key transcriptional machinery (Rajendran et al., 2003; Rossow and Janknecht, 2003; Yan et al., 2003). This is relevant for both Pol1 and Pol2-transcribed genes, as has been shown for DDX21 which associates with genes coding for ribosomal RNA, as well as ribosomal proteins and positively regulates their transcription (Calo et al., 2015). In addition, several RNA helicases (e.g., DDX1, DDX17 and DHX9) have been suggested to contribute to the formation and resolving of so-called R-loops. These three-stranded nucleic acids, consisting of an RNA–DNA hybrid and a displaced single-stranded DNA, occur during transcription and DNA replication, and must be resolved to avoid epigenetic misregulation (Al-Hadid and Yang, 2016) and genome instability (Yang et al., 2023).

Ribosome biogenesis starts in the nucleolus, with a few final steps occurring in the cytoplasm. As it involves the dynamic rearrangement of ribonucleoprotein complexes, it requires a multitude of RNA helicases to help avoid undesirable configuration of RNA and its interactions. In the yeast model system, at least 19 RNA helicases are required for the maturation of ribosomes, and even more helicases are involved in this process in human cells (Martin et al., 2013). A single large precursor rRNA molecule undergoes systematic cleavage by endo-and exonucleases along with several other hundreds of transacting factors to produce the mature rRNAs. The main roles of the DExH/DEAD-box helicases during this process are to mechanistically fold the precursor rRNA for formation of several RNP complexes, careful removal of specific small nuclear RNAs guiding rRNA folding, and mediating structural alteration in ribosomal subunits during the ribosome assembly (Jalal et al., 2007; Martin et al., 2014; Kellner et al., 2015; Xing et al., 2019).

Splicing of newly transcribed pre-mRNAs to form the mature mRNA involves structural rearrangements and folding which requires assistance by several RNA helicases. Studies of yeast as well as human spliceosomes have shown that at least eight RNA helicases act in a sequential manner, with seven of them belonging to DExH/DEAD-box families (Fleckner et al., 1997; Gencheva et al., 2010; English et al., 2012; Zanini et al., 2017; Hug et al., 2022; Obuća et al., 2022). Often these helicases share more than one role in the cellular system. For example, RNA helicase DDX39B is part of the spliceosome affecting splicing and also regulates nuclear export of mRNAs by being part of the TREX mRNA export complex (Li et al., 2005; Kota et al., 2008; Hautbergue et al., 2009; Shen, 2009). On the other hand, DDX39B also contributes to translation by regulating pre-ribosomal RNA levels (Awasthi et al., 2018).

The translation machinery associates with most of the major kinds of RNA in the cell. This is one process that involves the three major types of RNA in the cell: the mRNA, rRNA, and tRNA. So naturally, the helicases involved here inter-share their roles to maintain a coherent system. The eIF4A RNA helicase (also known as DDX2) constitutes one of the smallest DEAD-box helicases. Its evolutionary conservation is due to its indispensable role for translation initiation, to unwind the 5’UTR of mRNA, thus facilitating scanning of the 5’UTR by small ribosomal subunits to identify the start codon (Pause and Sonenberg, 1992). Other RNA helicases such as DDX3, DHX9 and DHX36 are needed to overcome highly structured 5’UTRs on large transcripts during the scanning process (Sheng et al., 2006; Lee et al., 2008; Calviello et al., 2021). Another RNA helicase recently found crucial for protein biosynthesis is DHX19, which assists in formation of the termination complex and release of the newly formed protein from the ribosomal complex (Mikhailova et al., 2017).

Several regulatory mechanisms act on mRNAs to control translation. Translational shutdown upon various cellular stresses leads to sequestration of mRNAs in large protein/RNA complexes within the cytoplasm called stress granules (SGs). Several RNA helicases such as DDX3X, DHX30, DHX36 act in the assembly, and possibly also in the disassembly or clearance of SGs (Chalupnikova et al., 2008; Valentin-Vega et al., 2016; Sauer et al., 2019; Mannucci et al., 2021). In addition, mRNAs may be degraded in processing (P-) bodies, which also exist under basal (non-stressed) conditions and are associated with miRNA dependent silencing of mRNAs. The helicase DDX6 plays major role in P-bodies and is involved in several aspects

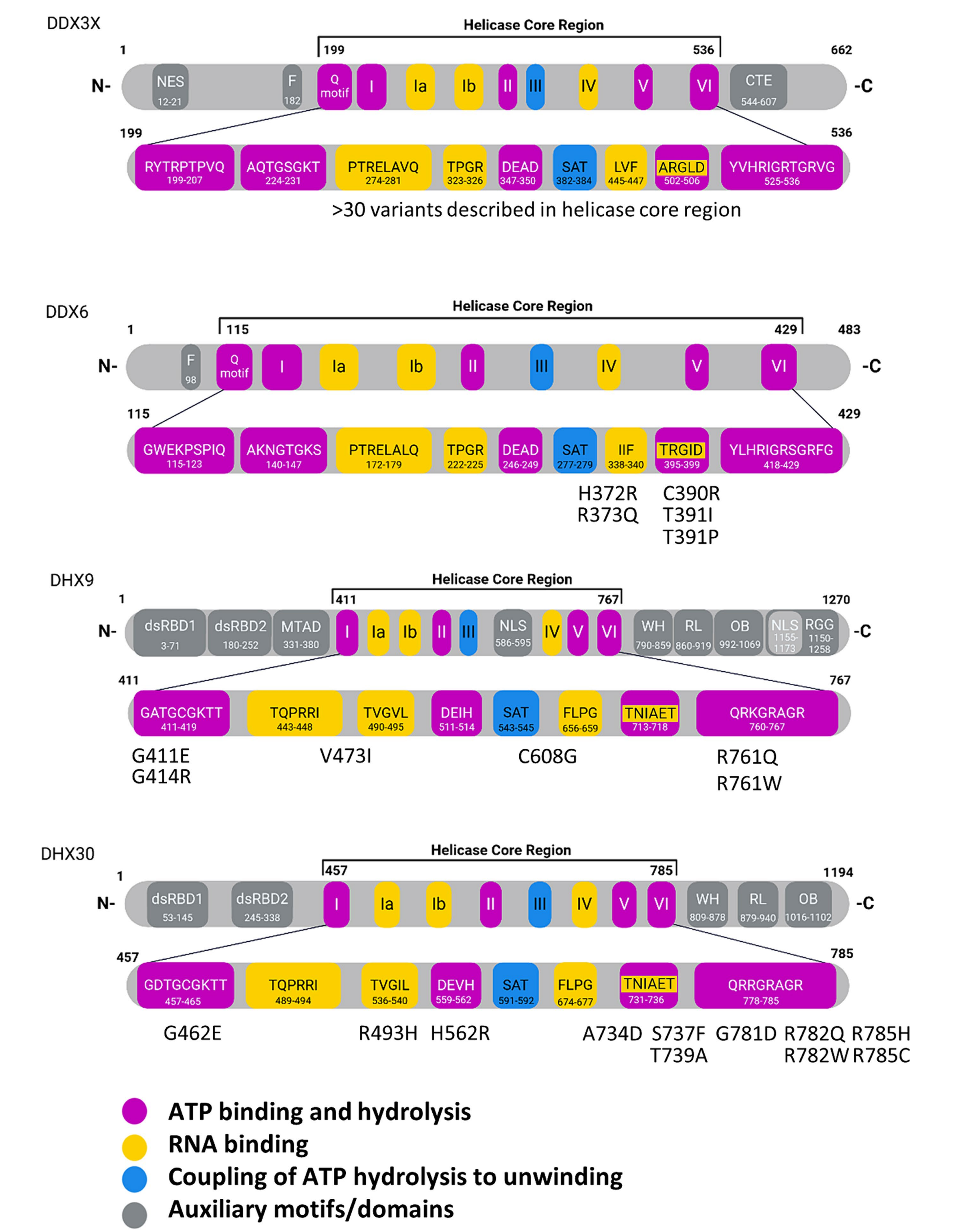


FIGURE 1
Domain structures of individual RNA helicases involved in NDDs. In the enlargement of the helicase core regions, individual core motifs are indicated with amino acid sequences and positions, based on Uniprot entries for the human proteins. Missense variants identified in NDD patients are indicated for DDX6, DHX9 and DHX30. For DDX3X, more than 30 variants have been described in the helicase core region. Motif V has been implicated in RNA and ATP binding. WH, Winged-helix; RL, Rachet-like; OB, Oligosaccharide binding; Znf, Zinc finger; dsRBD, double stranded RNA binding domain; mtad, minimal transactivation domain; NLS, nuclear localization signal; NES, nuclear export signal; NTD, N-terminal domain; RGG, arginine-glycine–glycine domain. Created with Biorender.com.

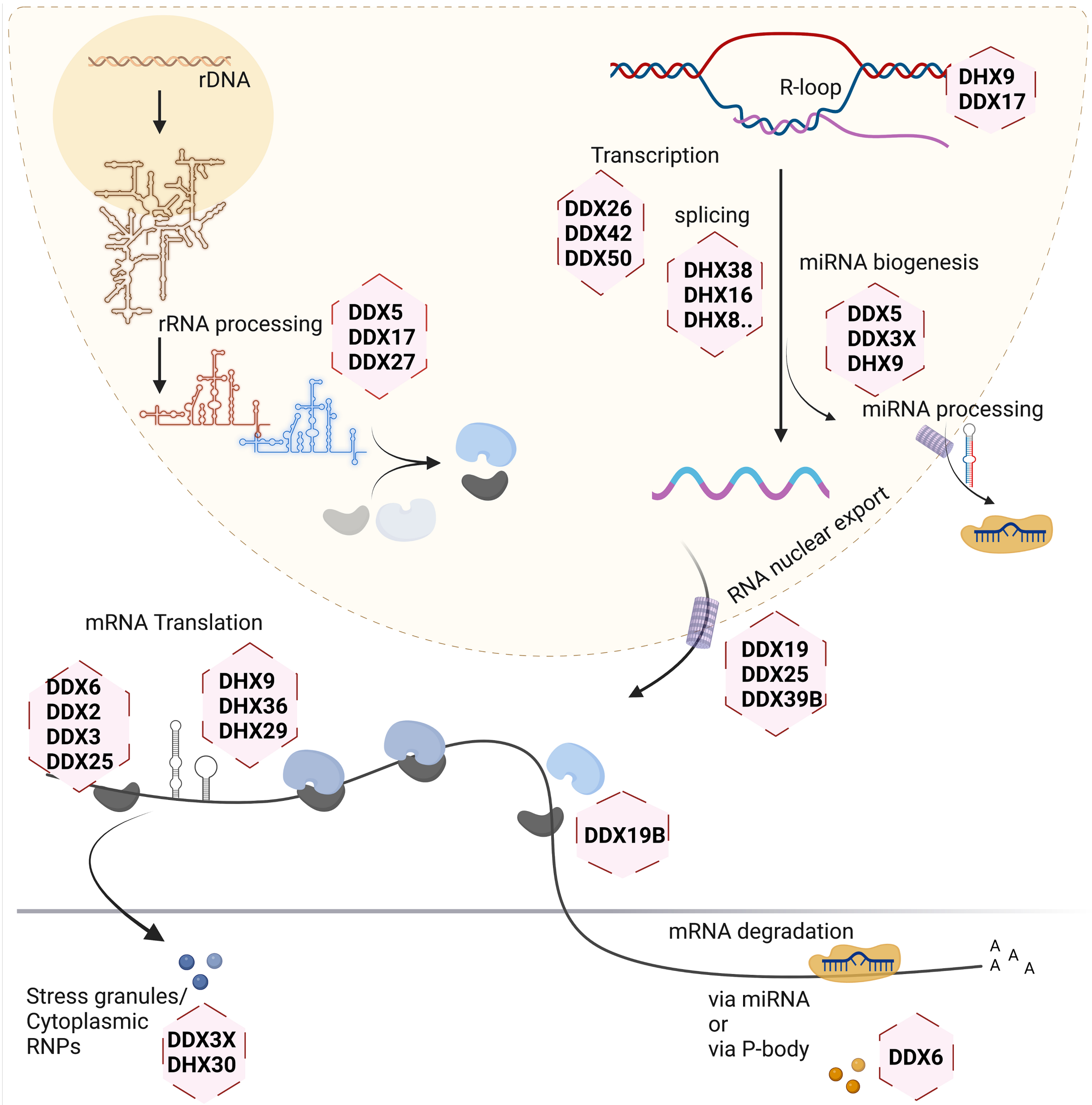


FIGURE 2
Involvement of RNA helicases in different stages of eukaryotic RNA metabolism. Created with Biorender.com.

of mRNA degradation (Nissan et al., 2010; Ostareck et al., 2014; Rouya et al., 2014; Weber et al., 2024).

Both SGs and P-bodies, together with nucleoli and nuclear speckles, constitute membrane-less organelles which perform a large portion of cellular RNA processing. These cellular bodies are held together by a multitude of interactions of RNAs with RNA binding proteins, and frequently involve a biomolecular condensation process termed liquid-/liquid phase separation (LLPS) (Dorner and Hondele, 2024). Proteins can contribute to condensate formation through their various interaction domains, but also through larger segments of intrinsically disordered regions (Rosa et al., 2024). Most if not all DEAD-and DExH helicases are present in such condensates at some point of the RNA life cycle, and they are probably needed for both the assembly as well as the disassembly of such organelles (Hondele et al., 2019). This is exemplified by the two sexually dimorphic DDX3 variants, DDX3x and DDX3y. Here, it was recently shown that the disordered region in DDX3y promoted LLPS more strongly, while its weaker ATPase activity was less active in SG dsassembly. This leads to increased LLPS, reduced translation and increased mRNA aggregation, e.g., under stress in a sex-specific manner (Shen et al., 2022). Aberrant formation of stress granules is an important feature of NDD associated pathogenic variants in DHX30 and DDX3x (Lessel et al., 2017; Lennox et al., 2020; Mannucci et al., 2021).

Involvement of DExH/DEAD-box RNA helicases in NDDs

In order to provide a detailed overview of the DExH/DEAD-box RNA helicases involved in NDDs we first queried the Online

TABLE 1 DExH/DEAD-box RNA helicases associated with NDDs.

Gene name	Inheritance	OMIM	Evidence (SysNDD)
DDX1	AR	No	Single affected individual
EIF4A2 (DDX2B)	AD, AR?	Neurodevelopmental disorder with hypotonia and speech delay, with or without seizures (NEDHSS, #620455)	*De novo* variants, 1 individual with a homozygous variant
DDX3X	XLD, XLR	Syndromic X-linked intellectual developmental disorder of the Snijders Blok type (MRXSSB, #300958)	*De novo* variants in females, inherited missense variants in affected males
DDX6	AD	Intellectual developmental disorder with impaired language and dysmorphic facies (IDDILF, #618653)	*De novo* missense variants
DDX11	AR	Warsaw breakage syndrome (#601150)	Homozygous and compound heterozygous variants
DDX17	AD?	No	*De novo* truncating variants in 2 individuals
DDX23	AD?	No	*De novo* missense variants
DDX24	AD?	No	Balanced translocation in a single individual
DDX47	AD?	No	One individual with comp het missense variants
EIF4A3 (DDX48)	AR	Robin sequence with cleft mandible and limb anomalies (#268305)	Homozygous and compound heterozygous variants
DDX50	AD?	No	Inframe deletion in a single individual
DDX54	AR?	No	Two individuals with hom or comp het missense variants
DDX59	AR	Orofaciodigital syndrome V (OFD5, #174300)	Homozygous and compound heterozygous variants
DHX9	AD?	No	*De novo* variants
DHX16	AD	Neuromuscular oculoauditory syndrome (NMOAS, #618733)	*De novo* variants
DHX30	AD, AR?	Neurodevelopmental disorder with variable motor and language impairment (NEDMIAL, #617804)	*De novo* variants, single individual with homozygous variant
DHX34	AD?, AR?	No	Single individual with a *de novo* variant, two individuals with homozygous variants
DHX37	AR, AD?	Neurodevelopmental disorder with brain anomalies and with or without vertebral or cardiac anomalies (NEDBAVC, #618731)	Five individuals with AR, two individuals with *de novo* variants
DHX58	AR?	No	One individual with homozygous missense variant

Definitely established genes are marked in black, candidate genes are marked in purple. AD, autosomal dominant; AR, autosomal recessive; XLD, X-linked dominant, XLR, X-linked recessive; ?, not established. OMIM, Online Mendelian Inheritance in Man (www.omim.org) database. SysNDD database (sysndd.dbmr.unibe.ch), last accessed July 2024.

Mendelian Inheritance in Man (OMIM)[1] database. OMIM is a comprehensive compendium that continuously updates human genes and their association to human genetic disorders according to the Human Genome Organization (HUGO) Gene Nomenclature Committee (HGNC).[2] This search revealed nine RNA helicase genes, that can be regarded as definitely associated with a human disorders. Out of these six genes were associated with a primary neurodevelopmental disorder. In more detail, missense and loss-of-function variants in *EIF4A2* (*DDX2B*) are associated with the Neurodevelopmental disorder with hypotonia and speech delay, with or without seizures (NEDHSS, #620455) (Paul et al., 2023). Similarly, a broad spectrum of variants in *DDX3X* is associated with Syndromic X-linked intellectual developmental disorder of the Snijders Blok type [MRXSSB, #300958 (Snijders Blok et al., 2015)]. Missense variants in *DDX6* and *DHX16* are associated with Intellectual developmental disorder with impaired language and dysmorphic facies [IDDILF, #618653 (Balak et al., 2019)] and Neuromuscular oculoauditory syndrome [NMOAS, #618733 (Paine et al., 2019)], respectively. Missense variants in *DHX30* are associated with Neurodevelopmental disorder with variable motor and language impairment (NEDMIAL, #617804), with loss-of-function variants causing a milder phenotype (Lessel et al., 2017; Mannucci et al., 2021). Missense variants in *DHX37* are associated with Neurodevelopmental disorder with brain anomalies and with or without vertebral or cardiac anomalies [NEDBAVC, #618731 (Paine et al., 2019)]. In addition, *DDX11*, *EIF4A3* and *DDX59* have been associated with complex human disorders that involve a variable degree of neurodevelopmental delay. These genes have bene associated with Warsaw breakage syndrome [#601150 (van der Lelij et al., 2010)], Robin sequence with cleft mandible and limb anomalies [#268305 (Favaro et al., 2014)] and Orofaciodigital syndrome V [OFD5, #174300 (Shamseldin et al., 2013)], respectively (Table 1).

To search for further candidate genes, we next enquired the SysNDD database (sysndd.dbmr.unibe.ch) that curates gene disease relationships in NDDs. This search revealed 10 potential candidate NDD genes, variants in which have been identified in at least one affected individual (Table 1). Out of these, an association of *DHX9*

1 www.omim.org
2 www.genenames.org

(Calame et al., 2023; Yamada et al., 2023) and *DDX23* (Burns et al., 2021) with a NDD has been published only recently.

The gross majority of genes involved in human NDDs are highly intolerant to genetic variation, both to missense and loss-of-function variants (Samocha et al., 2014). This is nicely exemplified by the *DHX30* gene, being one of the most variation-intolerant genes in the human genome (Lessel et al., 2017). We have therefore utilized the large sequencing data from the Genome Aggregation Database (gnomAD V2.1)[3] (Karczewski et al., 2020) to document constraint metrics of the 54 human DExH/DEAD-box RNA helicases (Table 2). We were interested in the tolerance to missense variants (missense Z-scores), where a score of >3 is regarded as intolerant, and tolerance to loss-of-function variants [probability of being loss-of-function intolerant (pLI)], where a score of >0.90 is regarded as intolerant (Samocha et al., 2014; Lek et al., 2016). Genes with either high Z score or pLI values, or both, are regarded as strong candidates for disorders caused by heterozygous, *de novo* variants (Samocha et al., 2014). Indeed, out of the already established NDD-related DExH/DEAD-box RNA helicase genes, primarily caused by *de novo* variants, all bear high scores (*DDX3X*, *DDX6*, *DHX16* and *DHX30*). Out of the candidate genes listed in Table 1, *DDX17*, *DDX23* and *DHX9* show high missense Z scores and pLI's.

Neurodevelopmental disorders arise due to various perturbations during brain development (Khodosevich and Sellgren, 2023). Thus, we were also interested in the expression profiles of the DExH/DEAD-box RNA helicases in various brain regions during brain development. For this, we utilized the Human Brain Transcriptome (HBT) database,[4] which provides transcriptome data for the developing and adult human brain (Kang et al., 2011) in six brain regions (neocortex, hippocampus, amygdala, striatum, mediodorsal nucleus of the thalamus and cerebellar cortex). We documented the mean expression levels at four different time points during development, namely during the embryonic period (TP1; 4 PCW ≤ Age < 8 PCW), late fetal period (TP7, 24 PCW ≤ Age < 38 PCW), neonatal and early infancy period (TP8, 0 M (birth) ≤ Age < 6 M) and early childhood (TP10, 1 Y ≤ Age < 6 Y) (Table 3). Out of the already established NDD-associated DExH/DEAD-box RNA helicase genes, *EIF4A2* (DDX2B), *DDX3X*, *DDX6* and *DHX30*, showed a strong expression during all developmental periods. Genes associated with complex human disorders, *DDX11* and *EIF4A3,* displayed strong expression only at certain periods. *DDX59*, *DHX16* and *DHX37* are not highly expressed during brain development. Out of the candidate genes, *DDX1*, *DDX17*, *DDX24*, *DDX47* and *DHX9* display a strong expression during all developmental periods, whereas DDX23 displays a somewhat lower expression only at TP7.

Taken together the constraint metrics and brain expression data provide evidence for heterozygous, *de novo* variants in *DDX17*, *DDX23* and *DHX9* as being associated with NDDs. Based on both datasets we suggest that heterozygous, *de novo* variants in *DDX2A*, *DDX42*, *DDX46*, *DHX8*, *DHX15* and *DHX40* might represent additional candidates for this group of disorders. Finally, based on brain expression levels, *DDX1*, *DDX5*, *DDX24*, *DDX47* and *DHX36* might constitute NDD associated genes following an autosomal recessive mode of inheritance. Clearly, further high-throughput sequencing studies are needed to confirm these hypotheses.

Below we provide a brief overview of the four well-studied NDDs associated with pathogenic variants in DExH/DEAD-box RNA helicases.

DHX30

This DExH helicase has received little attention until recently; early studies in mice revealed that the *Dhx30* gene is essential for survival, as complete *Dhx30* ko mice die early in embryonic development (Zheng et al., 2015). Several transcript variants arise due to the use of alternative promoters, and possibly also alternative splicing. Variations in the N-termini allow for either import into mitochondria or targeting to the cytosol of the expressed protein (Lessel et al., 2017; Bosco et al., 2021). As a consequence, a substantial portion of the protein resides in mitochondrial RNA granules which play a role in RNA processing and biogenesis of mitochondrial ribosomes (Antonicka and Shoubridge, 2015). Like several other DExH-type helicases, DHX30 carries additional domains besides the two RecA domains which constitute the helicase core. There are two dsRBDs in the N-terminal part of the protein, and the winged helix, ratchet like and OB fold domains in the C-terminus which are typical for DExH helicases.

We have previously established *de novo*, heterozygous, *DHX30* missense variants, affecting highly conserved residues within its HCMs, as a cause of a severe neurodevelopmental disorder, Neurodevelopmental disorder with variable motor and language impairment (NEDMIAL; #OMIM 617804) (Lessel et al., 2017). This condition is primarily characterized by severe global developmental delay (GDD), intellectual disability (ID), absent speech or speech limited to single words along with severe gait abnormalities (if walking is acquired at all). In contrast, individuals harboring a loss-of-function (frameshift or nonsense) variant develop a milder clinical course (Mannucci et al., 2021). The latter individuals have a mild GDD and ID, learn to speak full sentences and learn to walk in the second year of life. Two other missense variants outside HCMs have been described which are associated with a different clinical course. However, their causality still remains to be fully confirmed.

By performing *in-depth* functional analyses we were able to provide a molecular understanding for this genotype–phenotype correlation. Missense variants within the helicase core motifs (HCMs) of DHX30 impair either its ATPase activity or RNA binding capacity, and thereby its RNA helicase activity (Lessel et al., 2017; Mannucci et al., 2021). However, in addition to this clear loss-of-function, these missense variants additionally lead to a detrimental gain-of-function by inducing stress granule (SG) formation with concomitant global translation impairment. Utilizing CRISPR/Cas9 based technology, analyses of two DHX30 knockdown/knockout models, HEK293T cells and zebrafish model, revealed an impairment of SG formation (Mannucci et al., 2021). These data strongly suggest that the severe DHX30-associated phenotype (NEDMIAL) is due to the selective gain-of-function by triggering SG formation.

3 gnomad.broadinstitute.org

4 hbatlas.org

TABLE 2 Constraint metrics of DExH/DEAD-box RNA helicases according to gnomAD V2.1.

Gene	Z score	pLI	Gene	Z score	pLI
DDX1	1.82	0.99	*DDX47*	0.39	0
DDX2A	3.93	1	*DDX48*	4.02	1
DDX2B	3.89	1	*DDX49*	0.96	0
DDX3Y	2.1	0.96	*DDX50*	2.07	0
DDX3X	4.33	1	*DDX51*	−1.41	0
DDX4	2.05	1	*DDX52*	0.18	0
DDX5	2.76	1	*DDX53*	0.01	0.66
DDX6	3.78	1	*DDX54*	0.68	0
DDX10	0.3	0	*DDX55*	0.65	0
DDX11	−0.22	0	*DDX56*	−0.23	0
DDX17	3.87	1	*DDX58*	0.82	0
DDX18	0.47	0.14	*DDX59*	0.78	0
DDX19A	2.37	0.84	*DHX8*	5.03	0
DDX19B	2.22	0	*DHX9*	5.84	1
DDX20	0.08	0	*DHX15*	5.63	1
DDX21	2.45	1	*DHX16*	3.08	0
DDX23	4.62	0.54	*DHX29*	1.93	0
DDX24	0.09	0.68	*DHX30*	5.3	1
DDX25	1.21	0	*DHX33*	0.46	0
DDX27	1.73	0.58	*DHX34*	−0.08	0
DDX28	−0.75	0	*DHX35*	0.48	0
DDX31	0.03	0	*DHX36*	1.84	0.47
DDX39	3.55	1	*DHX37*	1.83	0.99
DDX41	2.28	0	*DHX38*	2.67	0
DDX42	3.29	1	*DHX40*	3.02	0.76
DDX43	1.27	0	*DHX57*	−0.96	0
DDX46	5.48	1	*DHX58*	0.52	0

Indicated in yellow are genes already associated with a NDD, in red are the high missense Z scores (>3) and pLIs (Probability of being loss-of-function intolerant; >0.9) indicated.

DDX3X

The gene coding for the DEAD-box helicase DDX3X is localized on the X-chromosome and is one of the few genes known to escape X inactivation in females; a second gene on the Y-chromosome codes for the almost identical DDX3Y protein, apparently ensuring equal gene dosage for this type of helicase in males (Lahn and Page, 1997). Germline variants in *DDX3X* are one of the most common causes for intellectual disability in females; indeed more than a hundred affected individuals have been reported (Snijders Blok et al., 2015; Lennox et al., 2020; Greene et al., 2023). Variants in *DDX3X* are associated with a wide spectrum of neuronal phenotypes, ranging from ID and loss of speech to severe failures of cortical development including microcephaly and polymicrogyria (a condition characterized by too many, but too small folds in the surface of the cortex). In most cases, females carry *de novo*, heterozygous loss-of-function or missense variants. In addition, hemizygous variants inherited from unaffected mothers have also been identified in rare male patients. Missense variants lead to a significantly more severe outcome, suggesting a dominant effect of these variants (Lennox et al., 2020). Similar to the situation in *DHX30*, several missense variants alter residues in one of the conserved HCMs; thus, there are four cases with a severe phenotype carrying the T532M variant in motif VI. In addition, there is a number of cases with variants outside of the HCMs but within the two RecA helicase core domains. Functional analysis shows that lack of ATPase and RNA helicase activity in missense variants correlates with severity of disease (Lennox et al., 2020).

Studies of the mammalian DDX3X protein, as well as the yeast homolog Ded1p, suggest that this helicase is involved in translation and may play a role in unwinding complex 5′ untranslated regions (5'UTRs) during scanning of the preinitiation complex (Chuang et al., 1997; Hilliker et al., 2011). Work by Soto-Rifo et al. (2012) suggested that DDX3X needs to unwind secondary structures close to the 5′ end which occlude the 5'cap structure, thereby preventing access of the cap binding protein eIF4F. An additional role in the formation of stress granules was also discussed which might be directly related to the role of DDX3X in translation (Hilliker et al., 2011). Interestingly, somatic missense variants found in medulloblastoma, as well as germline variants in NDD patients, lead to excessive formation of stress granules even in the absence of cellular stressors such as heat or oxidative stress (Valentin-Vega et al., 2016; Lennox et al., 2020). In this respect, pathogenic variants in *DDX3X* to some extent mimic missense variants in *DHX30* which also lead to enhanced stress granule formation [see above (Lessel et al., 2017; Mannucci et al., 2021)]. However, a recent analysis in neuronal progenitor cells suggested that some of the NDD-associated missense variants trigger formation of ribonucleoprotein (RNP) granules that may not be stress granules (Lennox et al., 2020).

In the mammalian brain, lack of DDX3X leads to reduced neurogenesis during embryonic development, likely explaining the polymicrogyria (Lennox et al., 2020). A conditional knockout line in mice with deletion of Ddx3x expression in neural progenitor cells in early embryonic development showed that the encoded protein is necessary for cell cycle control and for the generation of a sufficient number of neuronal cells. DDX3x does so by promoting translation of a small set of mRNAs relevant for neurogenesis (Calviello et al., 2021; Hoye et al., 2022). Similar defects in cortical neurogenesis were observed in mice lacking another helicase gene, EIF43/DDX48, suggesting a common pathological mechanism (Lupan et al., 2023). In zebrafish, mutant *Ddx3x* causes a deficit in the Wnt signaling pathway (Snijders Blok et al., 2015). A previous study had shown that the DDX3X protein performs some "moonlighting" in this pathway as an essential positive regulator of casein kinase 1 (CK1ε), which is required for phosphorylation of Disheveled and activation of the transcriptional role of β-catenin (Cruciat et al., 2013). This raises the question, whether the helicase activity or the CK1ε-dependent activity of DDX3 is relevant for human pathologies. Activation of CK1ε by DDX3 does not require RNA binding or helicase activity of the DDX3 protein, as many of the conserved motifs involved in RNA or ATP binding can be deleted without affecting CK1ε binding or activation of the signaling pathway. Instead, the DDX3X function in this pathway depends on its ability to interact with CK1ε though a sequence element in its C-terminus (Cruciat et al., 2013). Thus, current evidence indicates that a disrupted RNA helicase function of DDX3X, primarily

TABLE 3 Gene expression data of DExH/DEAD-box RNA helicases in 6 brain areas during human development.

	TP 1	TP 7	TP 8	TP 10		TP 1	TP 7	TP 8	TP 10		TP 1	TP 7	TP 8	TP 10
DDX1	10.5	8.5	9	9	DDX28	5.5	4.8	4.8	5	DHX8	8.5	7	7	7
DDX2A	12	11	10	10	DDX31	5.9	5.7	5.3	5.3	DHX9	10.8	8	8	8
DDX2B	10.3	11	11	12	DDX39	7.5	6	5.8	5.8	DHX15	10.5	8.3	8.3	8.3
DDX3X	9.5	8.3	8.5	8.5	DDX41	-	-	-	-	DHX16	7	5.8	5.8	5.8
DDX3Y	8.2	8	7.8	7.8	DDX42	8.5	7.8	7.8	8	DHX29	9	8	9	10
DDX4	3.7	4	4	4	DDX43	3.9	4.1	4.1	4.1	DHX30	9.5	8	9	9
DDX5	11.5	10.2	10.2	10.2	DDX46	9	7	7	7	DHX32	8.7	6.5	6.5	6.5
DDX6	11.8	11.5	11	11	DDX47	9	8	8	8	DHX33	7.5	6.3	6.3	6.3
DDX10	9.2	8	8	8.5	DDX48	10	7.5	8	7.5	DHX34	6	5.8	5.6	5.5
DDX11	7	11	11	10	DDX49	7.2	7	6.9	6.5	DHX35	8	5.7	5.8	5.8
DDX17	12	11	11	10.8	DDX50	8.3	7	7	7	DHX36	9	8	8	8
DDX18	9	6.8	7	6.5	DDX51	6	6	6	6	DHX37	6.3	6	6	6
DDX19A	-	-	-	-	DDX52	10	7.2	7	7	DHX38	7	6.6	6.6	6.6
DDX19B	8.2	7.5	7	7	DDX53	3.7	3.7	3.7	3.7	DHX40	9	6.5	6.3	6.3
DDX20	7.5	5.5	5.8	5.5	DDX54	7.8	6.8	6.8	6.8	DHX57	8.5	6.8	6.3	6.3
DDX21	8.8	6.9	6.8	6.8	DDX55	6.2	6	5.7	5.7	DHX58	4.5	5	5.3	5.5
DDX23	9.8	7.1	8	8	DDX56	8.7	7	7.4	7.3					
DDX24	10	9.5	10	10.4	DDX58	5.7	5.7	5.7	5.7					
DDX25	6.3	7	7.5	7.7	DDX59	6.5	6.1	5.8	5.8					
DDX27	6.5	6.3	6.3	6										

Expression levels of all DExH/DEAD-box RNA-helicases during human development was taken from Human Brain Transcriptome (HBT) database. TP1 – embryonic; TP7 – Late fetal; TP8 – Neonatal and early infancy; TP10 – early childhood; indicated in pink are values ≥8.

caused by pathogenic variants in the helicase core region, is the main cause of NDD in patients.

DDX6

The *DDX6* gene (MIM: 600326) encodes the DEAD-box helicase 6, involved in the regulation of mRNA decay and translation. DDX6 is an essential component of P-bodies, cytoplasmic granules containing enzymes necessary for the post-transcriptional regulation of mRNA. In fact, DDX6 is one of the very few proteins which are actually essential for P-body formation (Weston and Sommerville, 2006; Ayache et al., 2015). An initial publication from 2019 reported five *de novo* missense variants in DDX6 in individuals with neurodevelopmental disorders (Balak et al., 2019). All the variants were located in the same exon of the gene and affected four amino acids from two conserved motifs (amino acids 372–373 and 390–391) of the second RecA domain of the protein, namely the QxxR domain and the motif V. These variants affect the ability of DDX6 to form P-bodies and to interact with several of its partners involved in translation control. All individuals present with global developmental delay, intellectual disability, hypotonia, gait instability with a delay in walk acquisition, and similar dysmorphic features including a high-bossing forehead, bulbous nasal tip, hypertelorism, epicanthus, arched eyebrows and low-set ears, associated with a small head circumference. They also present with additional non-neurological symptoms, such as cardiac, hand/foot, and urogenital anomalies. The identification of additional nonsynonymous variants in individuals with NDD will be necessary to refine the syndrome associated with *DDX6* variants and to establish if missense changes located outside the QxxR and the motif V domains and truncating variants might also be pathogenic. So far, little is known about the role of DDX6 in the brain and how its dysfunction could alter normal processes of brain development, but few studies have reported its involvement in neuronal differentiation and synaptic plasticity. In mouse neural stem cells, DDX6 is needed for neuronal differentiation by regulating let7a activity through cooperation with TRIM32 (Nicklas et al., 2015). DDX6 also regulates the retinoic acid-induced neuronal differentiation of human neuroblastoma cell lines SH-SY5Y and SK-N-SH (Shih et al., 2023). Finally, a role of DDX6 in mediating NMDAR-dependent spine shrinkage via the Ago2 dependent silencing of Limk1 has been recently described in rat neurons (Perooli et al., 2024).

DHX9

DHX9 is involved in transcription, in the regulation of R-loops and in the repair of DNA double strand breaks by BRCA1 (Chakraborty et al., 2018; Cristini et al., 2018). In addition DHX9 represses the effects of Alu elements in the human genome on RNA processing (Aktas et al., 2017). For these purposes, DHX9 needs to be targeted to the nucleus by a nuclear localization sequence (NLS) in the C-terminal part of the protein. However, recent work in hippocampal neurons has shown that a substantial part of the protein may be present in the cytosol, specifically associated with the dendritically localized mRNA coding for Dendrin (Yang et al., 2024). Dhx9 deficient mice are viable but display distinct behavioral and neurological abnormalities (Calame et al., 2023). Although, OMIM still does not list *DHX9* as associated with human disease, two very recent studies identified pathogenic variants in *DHX9* in human patients. Thus, actually already formally establishing the link to NDD. Again, some variants associated with a moderate NDD phenotype alter conserved residues in motif I or motif VI and interfere with ATPase activity (Calame et al., 2023; Yamada et al., 2023). More severe NDD is observed with variants which alter the NLS and interfere with nuclear localization of DHX9; these lead to a higher number of R-loops and double strand breaks, indicating that the function of DHX9 in these processes is indeed required for neuronal homeostasis and function. Adding to phenotypic complexity, Calame et al. (2023) also identified missense variants in the winged helix and the C-terminal RGG domains of DHX9 which associate with hereditary motor and sensory neuropathy (also known as Charcot–Marie Tooth type 2 disease).

Conclusion

The recent identification of pathogenic variants in several genes coding for DExH/DEAD-box RNA helicases has raised a strong interest in the function of this group of enzymes. Due to extensive studies in model organisms such as yeast, the functional relevance of the conserved helicase core domains has been elucidated in much detail (Linder and Jankowsky, 2011). These data from basic science strongly aided the interpretation of individual missense variants found in patients. Thus, it became clear that quite often the genetic variants affected key residues in HCMs, thereby interfering with the RNA binding, ATPase and eventually helicase activity of the encoded proteins. Nevertheless, in many cases it is still unclear whether a specific variant identified in a patient is pathogenic, or a harmless polymorphism. Inexpensive, non-complicated assays are needed to assess molecular relevance. Furthermore, there is still a large gap between understanding the molecular relevance of individual genetic variants, and understanding the relevance of these variants on a neuronal or systems level. Quite often, the particular cellular process which is relevant for disease is unknown. Thus, while DHX30 is partially present in mitochondria (Antonicka and Shoubridge, 2015; Bosco et al., 2021), the phenotypes of patients carrying variants in helicase core motifs of DHX30 are not typical for a mitochondrial disorder (Lessel et al., 2017). As the function of non-mitochondrial DHX30 is currently unknown, it is difficult to determine why variants in this gene lead to such a severe phenotype. In the case of DDX3X, most data now point to the relevance of translational control, as this helicase is needed for efficient translation of mRNAs with longer, structured 5'UTRs (Hoye et al., 2022). Nevertheless, it is unclear how the role of DDX3X in Wnt signaling may contribute to the development of intellectual disability in carriers of pathogenic variants. Further work both in mouse models as well as in induced pluripotent stem cell models derived from patient cells will be necessary to determine which particular aspects of neuronal RNA metabolism are affected by particular variants in DEAD-and DExH-box helicases.

Finally, it is mostly unclear how treatment can be achieved for these very rare disorders. Small molecule inhibitors have been identified for some helicases (reviewed by Naineni et al., 2023), which may be helpful in cases where the pathogenic variants cause a clear gain-of-function. Alternatively, specific antisense oligonucleotides may be considered in cases where a missense variant causes a more severe phenotype than the loss-of-function variants (as observed in DHX30; Mannucci et al., 2021).

Author contributions

JL: Writing – original draft, Data curation, Formal analysis, Visualization. SD: Formal analysis, Visualization, Writing – original draft. AP: Writing – original draft, Conceptualization, Writing – review & editing. DL: Conceptualization, Writing – original draft, Writing – review & editing, Data curation, Funding acquisition, Supervision. H-JK: Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. Work in the author's laboratories is funded by grants from Deutsche Forschungsgemeinschaft (Kr1321/11-1 to H-JK; Le 4223/3-1 to DL) and The Austrian Science Fund (FWF; I 6657-B to DL).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2024.1414949/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Helicase core motifs of human DEAD box helicases.

SUPPLEMENTARY FIGURE S2
Helicase core motifs of human DExH helicases.

References

Aktas, T., Avsar Ilik, I., Maticzka, D., Bhardwaj, V., Pessoa Rodrigues, C., Mittler, G., et al. (2017). DHX9 suppresses RNA processing defects originating from the Alu invasion of the human genome. *Nature* 544, 115–119. doi: 10.1038/nature21715

Al-Hadid, Q., and Yang, Y. (2016). R-loop: an emerging regulator of chromatin dynamics. *Acta Biochim. Biophys. Sin. Shanghai* 48, 623–631. doi: 10.1093/abbs/gmw052

Antonicka, H., and Shoubridge, E. A. (2015). Mitochondrial RNA granules are centers for posttranscriptional RNA processing and ribosome biogenesis. *Cell Rep.* 10, 920–932. doi: 10.1016/j.celrep.2015.01.030

Awasthi, S., Chakrapani, B., Mahesh, A., Chavali, P. L., Chavali, S., and Dhayalan, A. (2018). DDX39B promotes translation through regulation of pre-ribosomal RNA levels. *RNA Biol.* 15, 1157–1166. doi: 10.1080/15476286.2018.1517011

Ayache, J., Benard, M., Ernoult-Lange, M., Minshall, N., Standart, N., Kress, M., et al. (2015). P-body assembly requires DDX6 repression complexes rather than decay or Ataxin2/2L complexes. *Mol. Biol. Cell* 26, 2579–2595. doi: 10.1091/mbc.E15-03-0136

Balak, C., Benard, M., Schaefer, E., Iqbal, S., Ramsey, K., Ernoult-Lange, M., et al. (2019). Rare *de novo* missense variants in RNA helicase DDX6 cause intellectual disability and dysmorphic features and Lead to P-body defects and RNA dysregulation. *Am. J. Hum. Genet.* 105, 509–525. doi: 10.1016/j.ajhg.2019.07.010

Bohnsack, K. E., Yi, S., Venus, S., Jankowsky, E., and Bohnsack, M. T. (2023). Cellular functions of eukaryotic RNA helicases and their links to human diseases. *Nat. Rev. Mol. Cell Biol.* 24, 749–769. doi: 10.1038/s41580-023-00628-5

Bosco, B., Rossi, A., Rizzotto, D., Hamadou, M. H., Bisio, A., Giorgetta, S., et al. (2021). DHX30 coordinates cytoplasmic translation and mitochondrial function contributing to cancer cell survival. *Cancers* 13:4412. doi: 10.3390/cancers13174412

Bourgeron, T. (2009). A synaptic trek to autism. *Curr. Opin. Neurobiol.* 19, 231–234. doi: 10.1016/j.conb.2009.06.003

Burns, W., Bird, L. M., Heron, D., Keren, B., Ramachandra, D., Thiffault, I., et al. (2021). Syndromic neurodevelopmental disorder associated with de novo variants in DDX23. *Am. J. Med. Genet. A* 185, 2863–2872. doi: 10.1002/ajmg.a.62359

Cajigas, I. J., Tushev, G., Will, T. J., tom Dieck, S., Fuerst, N., and Schuman, E. M. (2012). The local transcriptome in the synaptic neuropil revealed by deep sequencing and high-resolution imaging. *Neuron* 74, 453–466. doi: 10.1016/j.neuron.2012.02.036

Calame, D. G., Guo, T., Wang, C., Garrett, L., Jolly, A., Dawood, M., et al. (2023). Monoallelic variation in DHX9, the gene encoding the DExH-box helicase DHX9, underlies neurodevelopment disorders and Charcot-Marie-tooth disease. *Am. J. Hum. Genet.* 110, 1394–1413. doi: 10.1016/j.ajhg.2023.06.013

Calo, E., Flynn, R. A., Martin, L., Spitale, R. C., Chang, H. Y., and Wysocka, J. (2015). RNA helicase DDX21 coordinates transcription and ribosomal RNA processing. *Nature* 518, 249–253. doi: 10.1038/nature13923

Calviello, L., Venkataramanan, S., Rogowski, K. J., Wyler, E., Wilkins, K., Tejura, M., et al. (2021). DDX3 depletion represses translation of mRNAs with complex 5' UTRs. *Nucleic Acids Res.* 49, 5336–5350. doi: 10.1093/nar/gkab287

Chakraborty, P., Huang, J. T. J., and Hiom, K. (2018). DHX9 helicase promotes R-loop formation in cells with impaired RNA splicing. *Nat. Commun.* 9:4346. doi: 10.1038/s41467-018-06677-1

Chalupnikova, K., Lattmann, S., Selak, N., Iwamoto, F., Fujiki, Y., and Nagamine, Y. (2008). Recruitment of the RNA helicase RHAU to stress granules via a unique RNA-binding domain. *J. Biol. Chem.* 283, 35186–35198. doi: 10.1074/jbc.M804857200

Chen, Y., Dawes, R., Kim, H. C., Stenton, S. L., Walker, S., Ljungdahl, A., et al. (2024). De novo variants in the non-coding spliceosomal snRNA gene RNU4-2 are a frequent cause of syndromic neurodevelopmental disorders. *medRxiv*. doi: 10.1101/2024.04.07.24305438

Chen, S., Francioli, L. C., Goodrich, J. K., Collins, R. L., Kanai, M., Wang, Q., et al. (2024). A genomic mutational constraint map using variation in 76,156 human genomes. *Nature* 625, 92–100. doi: 10.1038/s41586-023-06045-0

Chuang, R. Y., Weaver, P. L., Liu, Z., and Chang, T. H. (1997). Requirement of the DEAD-box protein ded1p for messenger RNA translation. *Science* 275, 1468–1471. doi: 10.1126/science.275.5305.1468

Cordin, O., Banroques, J., Tanner, N. K., and Linder, P. (2006). The DEAD-box protein family of RNA helicases. *Gene* 367, 17–37. doi: 10.1016/j.gene.2005.10.019

Cougot, N., Bhattacharyya, S. N., Tapia-Arancibia, L., Bordonne, R., Filipowicz, W., Bertrand, E., et al. (2008). Dendrites of mammalian neurons contain specialized P-body-like structures that respond to neuronal activation. *J. Neurosci.* 28, 13793–13804. doi: 10.1523/JNEUROSCI.4155-08.2008

Cristini, A., Groh, M., Kristiansen, M. S., and Gromak, N. (2018). RNA/DNA hybrid Interactome identifies DXH9 as a molecular player in transcriptional termination and R-loop-associated DNA damage. *Cell Rep.* 23, 1891–1905. doi: 10.1016/j.celrep.2018.04.025

Cruciat, C. M., Dolde, C., de Groot, R. E., Ohkawara, B., Reinhard, C., Korswagen, H. C., et al. (2013). RNA helicase DDX3 is a regulatory subunit of casein kinase 1 in Wnt-beta-catenin signaling. *Science* 339, 1436–1441. doi: 10.1126/science.1231499

Dorner, K., and Hondele, M. (2024). The story of RNA unfolded: the molecular function of DEAD-and DExH-box ATPases and their complex relationship with membraneless organelles. *Annu. Rev. Biochem.* 93:11.1-11.30. doi: 10.1146/annurev-biochem-052521-121259

English, M. A., Lei, L., Blake, T., Wincovitch, S. M. Sr., Sood, R., Azuma, M., et al. (2012). Incomplete splicing, cell division defects, and hematopoietic blockage in dhx8 mutant zebrafish. *Dev. Dyn.* 241, 879–889. doi: 10.1002/dvdy.23774

Fairman-Williams, M. E., Guenther, U. P., and Jankowsky, E. (2010). SF1 and SF2 helicases: family matters. *Curr. Opin. Struct. Biol.* 20, 313–324. doi: 10.1016/j.sbi.2010.03.011

Favaro, F. P., Alvizi, L., Zechi-Ceide, R. M., Bertola, D., Felix, T. M., de Souza, J., et al. (2014). A noncoding expansion in EIF4A3 causes Richieri-Costa-Pereira syndrome, a craniofacial disorder associated with limb defects. *Am. J. Hum. Genet.* 94, 120–128. doi: 10.1016/j.ajhg.2013.11.020

Fleckner, J., Zhang, M., Valcarcel, J., and Green, M. R. (1997). U2AF65 recruits a novel human DEAD box protein required for the U2 snRNP-branchpoint interaction. *Genes Dev.* 11, 1864–1872. doi: 10.1101/gad.11.14.1864

Gencheva, M., Kato, M., Newo, A. N., and Lin, R.-J. (2010). Contribution of DEAH-box protein DHX16 in human pre-mRNA splicing. *Biochem. J.* 429, 25–32. doi: 10.1042/BJ20100266

Gilissen, C., Hehir-Kwa, J. Y., Thung, D. T., van de Vorst, M., van Bon, B. W., Willemsen, M. H., et al. (2014). Genome sequencing identifies major causes of severe intellectual disability. *Nature* 511, 344–347. doi: 10.1038/nature13394

Glock, C., Biever, A., Tushev, G., Nassim-Assir, B., Kao, A., Bartnik, I., et al. (2021). The translatome of neuronal cell bodies, dendrites, and axons. *Proc. Natl. Acad. Sci. U. S. A.* 118:e2113929118. doi: 10.1073/pnas.2113929118

Gorbalenya, A. E., and Koonin, E. V. (1993). Helicases: amino acid sequence comparisons and structure-function relationships. *Curr. Opin. Struct. Biol.* 3, 419–429. doi: 10.1016/S0959-440X(05)80116-2

Greene, D., Genomics England Research, C., Pirri, D., Frudd, K., Sackey, E., Al-Owain, M., et al. (2023). Genetic association analysis of 77,539 genomes reveals rare disease etiologies. *Nat. Med.* 29, 679–688. doi: 10.1038/s41591-023-02211-z

Greene, D., Thys, C., Berry, I. R., Jarvis, J., Ortibus, E., Mumford, A. D., et al. (2024). Mutations in the U4 snRNA gene RNU4-2 cause one of the most prevalent monogenic neurodevelopmental disorders. *Nat. Med.* doi: 10.1038/s41591-024-03085-5

Hafner, A. S., Donlin-Asp, P. G., Leitch, B., Herzog, E., and Schuman, E. M. (2019). Local protein synthesis is a ubiquitous feature of neuronal pre-and postsynaptic compartments. *Science* 364:eaau3644. doi: 10.1126/science.aau3644

Hautbergue, G. M., Hung, M.-L., Walsh, M. J., Snijders, A. P., Chang, C.-T., Jones, R., et al. (2009). UIF, a new mRNA export adaptor that works together with REF/ALY, requires FACT for recruitment to mRNA. *Curr. Biol.* 19, 1918–1924. doi: 10.1016/j.cub.2009.09.041

Hilliker, A., Gao, Z., Jankowsky, E., and Parker, R. (2011). The DEAD-box protein Ded1 modulates translation by the formation and resolution of an eIF4F-mRNA complex. *Mol. Cell* 43, 962–972. doi: 10.1016/j.molcel.2011.08.008

Hondele, M., Sachdev, R., Heinrich, S., Wang, J., Vallotton, P., Fontoura, B. M. A., et al. (2019). DEAD-box ATPases are global regulators of phase-separated organelles. *Nature* 573, 144–148. doi: 10.1038/s41586-019-1502-y

Hoye, M. L., Calviello, L., Poff, A. J., Ejimogu, N. E., Newman, C. R., Montgomery, M. D., et al. (2022). Aberrant cortical development is driven by impaired cell cycle and translational control in a DDX3X syndrome model. *eLife* 11:e78203. doi: 10.7554/eLife.78203

Hug, N., Aitken, S., Longman, D., Raab, M., Armes, H., Mann, A. R., et al. (2022). A dual role for the RNA helicase DHX34 in NMD and pre-mRNA splicing and its function in hematopoietic differentiation. *RNA* 28, 1224–1238. doi: 10.1261/rna.079277.122

Jagtap, P. K. A., Muller, M., Kiss, A. E., Thomae, A. W., Lapouge, K., Beck, M., et al. (2023). Structural basis of RNA-induced autoregulation of the DExH-type RNA helicase maleless. *Mol. Cell* 83, 4318–4333.e10. doi: 10.1016/j.molcel.2023.10.026

Jalal, C., Uhlmann-Schiffler, H., and Stahl, H. (2007). Redundant role of DEAD box proteins p68 (Ddx5) and p72/p82 (Ddx17) in ribosome biogenesis and cell proliferation. *Nucleic Acids Res.* 35, 3590–3601. doi: 10.1093/nar/gkm058

Kanai, Y., Dohmae, N., and Hirokawa, N. (2004). Kinesin transports RNA: isolation and characterization of an RNA-transporting granule. *Neuron* 43, 513–525. doi: 10.1016/j.neuron.2004.07.022

Kang, H. J., Kawasawa, Y. I., Cheng, F., Zhu, Y., Xu, X., Li, M., et al. (2011). Spatio-temporal transcriptome of the human brain. *Nature* 478, 483–489. doi: 10.1038/nature10523

Karczewski, K. J., Francioli, L. C., Tiao, G., Cummings, B. B., Alfoldi, J., Wang, Q., et al. (2020). The mutational constraint spectrum quantified from variation in 141,456 humans. *Nature* 581, 434–443. doi: 10.1038/s41586-020-2308-7

Kellner, M., Rohrmoser, M., Forné, I., Voss, K., Burger, K., Mühl, B., et al. (2015). DEAD-box helicase DDX27 regulates 3′ end formation of ribosomal 47S RNA and stably associates with the PeBoW-complex. *Exp. Cell Res.* 334, 146–159. doi: 10.1016/j.yexcr.2015.03.017

Khodosevich, K., and Sellgren, C. M. (2023). Neurodevelopmental disorders-high-resolution rethinking of disease modeling. *Mol. Psychiatry* 28, 34–43. doi: 10.1038/s41380-022-01876-1

Kim, J., Muraoka, M., Okada, H., Toyoda, A., Ajima, R., and Saga, Y. (2022). The RNA helicase DDX6 controls early mouse embryogenesis by repressing aberrant inhibition of BMP signaling through miRNA-mediated gene silencing. *PLoS Genet.* 18:e1009967. doi: 10.1371/journal.pgen.1009967

Kindler, S., and Kreienkamp, H. J. (2012). Dendritic mRNA targeting and translation. *Adv. Exp. Med. Biol.* 970, 285–305. doi: 10.1007/978-3-7091-0932-8_13

Kota, K. P., Wagner, S. R., Huerta, E., Underwood, J. M., and Nickerson, J. A. (2008). Binding of ATP to UAP56 is necessary for mRNA export. *J. Cell Sci.* 121, 1526–1537. doi: 10.1242/jcs.021055

Lahn, B. T., and Page, D. C. (1997). Functional coherence of the human Y chromosome. *Science* 278, 675–680. doi: 10.1126/science.278.5338.675

Lee, C. S., Dias, A. P., Jedrychowski, M., Patel, A. H., Hsu, J. L., and Reed, R. (2008). Human DDX3 functions in translation and interacts with the translation initiation factor eIF3. *Nucleic Acids Res.* 36, 4708–4718. doi: 10.1093/nar/gkn454

Lek, M., Karczewski, K. J., Minikel, E. V., Samocha, K. E., Banks, E., Fennell, T., et al. (2016). Analysis of protein-coding genetic variation in 60,706 humans. *Nature* 536, 285–291. doi: 10.1038/nature19057

Lennox, A. L., Hoye, M. L., Jiang, R., Johnson-Kerner, B. L., Suit, L. A., Venkataramanan, S., et al. (2020). Pathogenic DDX3X mutations impair RNA metabolism and neurogenesis during fetal cortical development. *Neuron* 106, 404–420.e8. doi: 10.1016/j.neuron.2020.01.042

Lessel, D., Schob, C., Kury, S., Reijnders, M. R. F., Harel, T., Eldomery, M. K., et al. (2017). De novo missense mutations in DHX30 impair global translation and cause a neurodevelopmental disorder. *Am. J. Hum. Genet.* 101, 716–724. doi: 10.1016/j.ajhg.2017.09.014

Lessel, D., Zeitler, D. M., Reijnders, M. R. F., Kazantsev, A., Hassani Nia, F., Bartholomaus, A., et al. (2020). Germline AGO2 mutations impair RNA interference and human neurological development. *Nat. Commun.* 11:5797. doi: 10.1038/s41467-020-19572-5

Li, Y., Wang, X., Zhang, X., and Goodrich, D. W. (2005). Human hHpr1/p84/Thoc1 regulates transcriptional elongation and physically links RNA polymerase II and RNA processing factors. *Mol. Cell. Biol.* 25, 4023–4033. doi: 10.1128/MCB.25.10.4023-4033.2005

Li, Q., Zhang, P., Zhang, C., Wang, Y., Wan, R., Yang, Y., et al. (2014). DDX3X regulates cell survival and cell cycle during mouse early embryonic development. *J. Biomed. Res.* 28, 282–291. doi: 10.7555/JBR.27.20130047

Linder, P., and Jankowsky, E. (2011). From unwinding to clamping - the DEAD box RNA helicase family. *Nat. Rev. Mol. Cell Biol.* 12, 505–516. doi: 10.1038/nrm3154

Lupan, B. M., Solecki, R. A., Musso, C. M., Alsina, F. C., and Silver, D. L. (2023). The exon junction complex component EIF4A3 is essential for mouse and human cortical progenitor mitosis and neurogenesis. *Development* 150:dev201619. doi: 10.1242/dev.201619

Mannucci, I., Dang, N. D. P., Huber, H., Murry, J. B., Abramson, J., Althoff, T., et al. (2021). Genotype-phenotype correlations and novel molecular insights into the DHX30-associated neurodevelopmental disorders. *Genome Med.* 13:90. doi: 10.1186/s13073-021-00900-3

Martin, R., Hackert, P., Ruprecht, M., Simm, S., Brüning, L., Mirus, O., et al. (2014). A pre-ribosomal RNA interaction network involving snoRNAs and the Rok1 helicase. *RNA* 20, 1173–1182. doi: 10.1261/rna.044669.114

Martin, R., Straub, A. U., Doebele, C., and Bohnsack, M. T. (2013). DExD/H-box RNA helicases in ribosome biogenesis. *RNA Biol.* 10, 4–18. doi: 10.4161/rna.21879

Mikhailova, T., Shuvalova, E., Ivanov, A., Susorov, D., Shuvalov, A., Kolosov, P. M., et al. (2017). RNA helicase DDX19 stabilizes ribosomal elongation and termination complexes. *Nucleic Acids Res.* 45, 1307–1318. doi: 10.1093/nar/gkw1239

Morris-Rosendahl, D. J., and Crocq, M. A. (2020). Neurodevelopmental disorders-the history and future of a diagnostic concept. *Dialogues Clin. Neurosci.* 22, 65–72. doi: 10.31887/DCNS.2020.22.1/macrocq

Murakami, K., Nakano, K., Shimizu, T., and Ohto, U. (2017). The crystal structure of human DEAH-box RNA helicase 15 reveals a domain organization of the mammalian DEAH/RHA family. *Acta Crystallogr. F Struct. Biol. Commun.* 73, 347–355. doi: 10.1107/S2053230X17007336

Naineni, S. K., Robert, F., Nagar, B., and Pelletier, J. (2023). Targeting DEAD-box RNA helicases: the emergence of molecular staples. *Wiley Interdiscip. Rev. RNA* 14:e1738. doi: 10.1002/wrna.1738

Nicklas, S., Okawa, S., Hillje, A. L., Gonzalez-Cano, L., Del Sol, A., and Schwamborn, J. C. (2015). The RNA helicase DDX6 regulates cell-fate specification in neural stem cells via miRNAs. *Nucleic Acids Res.* 43, 2638–2654. doi: 10.1093/nar/gkv138

Niemi, M. E. K., Martin, H. C., Rice, D. L., Gallone, G., Gordon, S., Kelemen, M., et al. (2018). Common genetic variants contribute to risk of rare severe neurodevelopmental disorders. *Nature* 562, 268–271. doi: 10.1038/s41586-018-0566-4

Nissan, T., Rajyaguru, P., She, M., Song, H., and Parker, R. (2010). Decapping activators in *Saccharomyces cerevisiae* act by multiple mechanisms. *Mol. Cell* 39, 773–783. doi: 10.1016/j.molcel.2010.08.025

Obuća, M., Cvačková, Z., Kubovčiak, J., Kolář, M., and Staněk, D. (2022). Retinitis pigmentosa-linked mutation in DHX38 modulates its splicing activity. *PLoS One* 17:e0265742. doi: 10.1371/journal.pone.0265742

Ostareck, D. H., Naarmann-de Vries, I. S., and Ostareck-Lederer, A. (2014). DDX6 and its orthologs as modulators of cellular and viral RNA expression. *Wiley Interdiscip. Rev. RNA* 5, 659–678. doi: 10.1002/wrna.1237

Paine, I., Posey, J. E., Grochowski, C. M., Jhangiani, S. N., Rosenheck, S., Kleyner, R., et al. (2019). Paralog studies augment gene discovery: DDX and DHX genes. *Am. J. Hum. Genet.* 105, 302–316. doi: 10.1016/j.ajhg.2019.06.001

Parenti, I., Rabaneda, L. G., Schoen, H., and Novarino, G. (2020). Neurodevelopmental disorders: from genetics to functional pathways. *Trends Neurosci.* 43, 608–621. doi: 10.1016/j.tins.2020.05.004

Paul, M. S., Duncan, A. R., Genetti, C. A., Pan, H., Jackson, A., Grant, P. E., et al. (2023). Rare EIF4A2 variants are associated with a neurodevelopmental disorder characterized by intellectual disability, hypotonia, and epilepsy. *Am. J. Hum. Genet.* 110, 120–145. doi: 10.1016/j.ajhg.2022.11.011

Pause, A., and Sonenberg, N. (1992). Mutational analysis of a DEAD box RNA helicase: the mammalian translation initiation factor eIF-4A. *EMBO J.* 11, 2643–2654. doi: 10.1002/j.1460-2075.1992.tb05330.x

Perooli, F. M., Wilkinson, K. A., Pring, K., and Hanley, J. G. (2024). An essential role for the RNA helicase DDX6 in NMDA receptor-dependent gene silencing and dendritic spine shrinkage. *Sci. Rep.* 14:3066. doi: 10.1038/s41598-024-53484-4

Prabu, J. R., Muller, M., Thomae, A. W., Schussler, S., Bonneau, F., Becker, P. B., et al. (2015). Structure of the RNA helicase MLE reveals the molecular mechanisms for uridine specificity and RNA-ATP coupling. *Mol. Cell* 60, 487–499. doi: 10.1016/j.molcel.2015.10.011

Pyle, A. M. (2008). Translocation and unwinding mechanisms of RNA and DNA helicases. *Annu. Rev. Biophys.* 37, 317–336. doi: 10.1146/annurev.biophys.37.032807.125908

Rajendran, R. R., Nye, A. C., Frasor, J., Balsara, R. D., Martini, P. G., and Katzenellenbogen, B. S. (2003). Regulation of nuclear receptor transcriptional activity by a novel DEAD box RNA helicase (DP97). *J. Biol. Chem.* 278, 4628–4638. doi: 10.1074/jbc.M210066200

Rosa, E. S. I., Smetana, J. H. C., and de Oliveira, J. F. (2024). A comprehensive review on DDX3X liquid phase condensation in health and neurodevelopmental disorders. *Int. J. Biol. Macromol.* 259:129330. doi: 10.1016/j.ijbiomac.2024.129330

Rossow, K. L., and Janknecht, R. (2003). Synergism between p68 RNA helicase and the transcriptional coactivators CBP and p300. *Oncogene* 22, 151–156. doi: 10.1038/sj.onc.1206067

Rouya, C., Siddiqui, N., Morita, M., Duchaine, T. F., Fabian, M. R., and Sonenberg, N. (2014). Human DDX6 effects miRNA-mediated gene silencing via direct binding to CNOT1. *RNA* 20, 1398–1409. doi: 10.1261/rna.045302.114

Sahoo, P. K., Lee, S. J., Jaiswal, P. B., Alber, S., Kar, A. N., Miller-Randolph, S., et al. (2018). Axonal G3BP1 stress granule protein limits axonal mRNA translation and nerve regeneration. *Nat. Commun.* 9:3358. doi: 10.1038/s41467-018-05647-x

Samocha, K. E., Robinson, E. B., Sanders, S. J., Stevens, C., Sabo, A., McGrath, L. M., et al. (2014). A framework for the interpretation of de novo mutation in human disease. *Nat. Genet.* 46, 944–950. doi: 10.1038/ng.3050

Sauer, M., Juranek, S. A., Marks, J., De Magis, A., Kazemier, H. G., Hilbig, D., et al. (2019). DHX36 prevents the accumulation of translationally inactive mRNAs with G4-structures in untranslated regions. *Nat. Commun.* 10:2421. doi: 10.1038/s41467-019-10432-5

Shamseldin, H. E., Rajab, A., Alhashem, A., Shaheen, R., Al-Shidi, T., Alamro, R., et al. (2013). Mutations in DDX59 implicate RNA helicase in the pathogenesis of orofaciodigital syndrome. *Am. J. Hum. Genet.* 93, 555–560. doi: 10.1016/j.ajhg.2013.07.012

Shen, H.-H. (2009). UAP56-a key player with surprisingly diverse roles in pre-mRNA splicing and nuclear export. *BMB Rep.* 42, 185–188. doi: 10.5483/BMBRep.2009.42.4.185

Shen, H., Yanas, A., Owens, M. C., Zhang, C., Fritsch, C., Fare, C. M., et al. (2022). Sexually dimorphic RNA helicases DDX3X and DDX3Y differentially regulate RNA metabolism through phase separation. *Mol. Cell* 82, 2588–2603.e9. doi: 10.1016/j.molcel.2022.04.022

Sheng, Y., Tsai-Morris, C. H., Gutti, R., Maeda, Y., and Dufau, M. L. (2006). Gonadotropin-regulated testicular RNA helicase (GRTH/Ddx25) is a transport protein involved in gene-specific mRNA export and protein translation during spermatogenesis. *J. Biol. Chem.* 281, 35048–35056. doi: 10.1074/jbc.M605086200

Shih, C. Y., Chen, Y. C., Lin, H. Y., and Chu, C. Y. (2023). RNA helicase DDX6 regulates A-to-I editing and neuronal differentiation in human cells. *Int. J. Mol. Sci.* 24:3197. doi: 10.3390/ijms24043197

Shiina, N., Shinkura, K., and Tokunaga, M. (2005). A novel RNA-binding protein in neuronal RNA granules: regulatory machinery for local translation. *J. Neurosci.* 25, 4420–4434. doi: 10.1523/JNEUROSCI.0382-05.2005

Siletti, K., Hodge, R., Mossi Albiach, A., Lee, K. W., Ding, S. L., Hu, L., et al. (2023). Transcriptomic diversity of cell types across the adult human brain. *Science* 382:eadd7046. doi: 10.1126/science.add7046

Snijders Blok, L., Madsen, E., Juusola, J., Gilissen, C., Baralle, D., Reijnders, M. R., et al. (2015). Mutations in DDX3X are a common cause of unexplained intellectual disability with gender-specific effects on Wnt signaling. *Am. J. Hum. Genet.* 97, 343–352. doi: 10.1016/j.ajhg.2015.07.004

Soto-Rifo, R., Rubilar, P. S., Limousin, T., de Breyne, S., Decimo, D., and Ohlmann, T. (2012). DEAD-box protein DDX3 associates with eIF4F to promote translation of selected mRNAs. *EMBO J.* 31, 3745–3756. doi: 10.1038/emboj.2012.220

Steward, O., and Schuman, E. M. (2001). Protein synthesis at synaptic sites on dendrites. *Annu. Rev. Neurosci.* 24, 299–325. doi: 10.1146/annurev.neuro.24.1.299

Sun, C., Nold, A., Fusco, C. M., Rangaraju, V., Tchumatchenko, T., Heilemann, M., et al. (2021). The prevalence and specificity of local protein synthesis during neuronal synaptic plasticity. *Sci. Adv.* 7:eabj0790. doi: 10.1126/sciadv.abj0790

Tanner, N. K., Cordin, O., Banroques, J., Doere, M., and Linder, P. (2003). The Q motif: a newly identified motif in DEAD box helicases may regulate ATP binding and hydrolysis. *Mol. Cell* 11, 127–138. doi: 10.1016/s1097-2765(03)00006-6

Tanner, N. K., and Linder, P. (2001). DExD/H box RNA helicases: from generic motors to specific dissociation functions. *Mol. Cell* 8, 251–262. doi: 10.1016/s1097-2765(01)00329-x

Valentin-Vega, Y. A., Wang, Y. D., Parker, M., Patmore, D. M., Kanagaraj, A., Moore, J., et al. (2016). Cancer-associated DDX3X mutations drive stress granule assembly and impair global translation. *Sci. Rep.* 6:25996. doi: 10.1038/srep25996

van der Lelij, P., Chrzanowska, K. H., Godthelp, B. C., Rooimans, M. A., Oostra, A. B., Stumm, M., et al. (2010). Warsaw breakage syndrome, a cohesinopathy associated with mutations in the XPD helicase family member DDX11/ChlR1. *Am. J. Hum. Genet.* 86, 262–266. doi: 10.1016/j.ajhg.2010.01.008

Weber, R., Wohlbold, L., and Chang, C.-T. (2024). Human DDX6 regulates translation and decay of inefficiently translated mRNAs. *Elife* 13:RP92426. doi: 10.7554/eLife.92426

Weston, A., and Sommerville, J. (2006). Xp54 and related (DDX6-like) RNA helicases: roles in messenger RNP assembly, translation regulation and RNA degradation. *Nucleic Acids Res.* 34, 3082–3094. doi: 10.1093/nar/gkl409

Xing, Z., Ma, W. K., and Tran, E. J. (2019). The DDX5/Dbp2 subfamily of DEAD-box RNA helicases. *Wiley Interdiscip. Rev. RNA* 10:e1519. doi: 10.1002/wrna.1519

Yamada, M., Nitta, Y., Uehara, T., Suzuki, H., Miya, F., Takenouchi, T., et al. (2023). Heterozygous loss-of-function DHX9 variants are associated with neurodevelopmental disorders: human genetic and experimental evidences. *Eur. J. Med. Genet.* 66:104804. doi: 10.1016/j.ejmg.2023.104804

Yan, X., Mouillet, J.-F., Ou, Q., and Sadovsky, Y. (2003). A novel domain within the DEAD-box protein DP103 is essential for transcriptional repression and helicase activity. *Mol. Cell. Biol.* 23, 414–423. doi: 10.1128/MCB.23.1.414-423.2003

Yang, L., Liu, Q., Zhao, Y., Lin, N., Huang, Y., Wang, Q., et al. (2024). DExH-box helicase 9 modulates hippocampal synapses and regulates neuropathic pain. *iScience* 27:109016. doi: 10.1016/j.isci.2024.109016

Yang, S., Winstone, L., Mondal, S., and Wu, Y. (2023). Helicases in R-loop formation and resolution. *J. Biol. Chem.* 299:105307. doi: 10.1016/j.jbc.2023.105307

Zanini, I. M., Soneson, C., Lorenzi, L. E., and Azzalin, C. M. (2017). Human cactin interacts with DHX8 and SRRM2 to assure efficient pre-mRNA splicing and sister chromatid cohesion. *J. Cell Sci.* 130, 767–778. doi: 10.1242/jcs.194068

Zeitelhofer, M., Karra, D., Macchi, P., Tolino, M., Thomas, S., Schwarz, M., et al. (2008). Dynamic interaction between P-bodies and transport ribonucleoprotein particles in dendrites of mature hippocampal neurons. *J. Neurosci.* 28, 7555–7562. doi: 10.1523/JNEUROSCI.0104-08.2008

Zheng, H. J., Tsukahara, M., Liu, E., Ye, L., Xiong, H., Noguchi, S., et al. (2015). The novel helicase helG (DHX30) is expressed during gastrulation in mice and has a structure similar to a human DExH box helicase. *Stem Cells Dev.* 24, 372–383. doi: 10.1089/scd.2014.0077

TYPE Brief Research Report
PUBLISHED 23 August 2024
DOI 10.3389/fnmol.2024.1393779

Check for updates

OPEN ACCESS

EDITED BY
Oriane Mauger,
Max Planck Institute of Psychiatry, Germany

REVIEWED BY
Florence Rage,
Délégation Languedoc Roussillon (CNRS), France
Eugene V. Makeyev,
King's College London, United Kingdom

*CORRESPONDENCE
Michael Briese
✉ Briese_M@ukw.de

RECEIVED 29 February 2024
ACCEPTED 08 August 2024
PUBLISHED 23 August 2024

CITATION
Salehi S, Zare A, Gandhi G, Sendtner M and Briese M (2024) Ptbp2 re-expression rescues axon growth defects in Smn-deficient motoneurons.
Front. Mol. Neurosci. 17:1393779.
doi: 10.3389/fnmol.2024.1393779

Ptbp2 re-expression rescues axon growth defects in Smn-deficient motoneurons

Saeede Salehi, Abdolhossein Zare, Gayatri Gandhi, Michael Sendtner and Michael Briese*

Institute of Clinical Neurobiology, University Hospital Würzburg, Würzburg, Germany

Spinal muscular atrophy (SMA) is a neuromuscular disorder caused by mutations or deletions in the survival motoneuron 1 (*SMN1*) gene, resulting in deficiency of the SMN protein that is essential for motoneuron function. Smn depletion in mice disturbs axonal RNA transport and translation, thereby contributing to axon growth impairment, muscle denervation, and motoneuron degeneration. However, the mechanisms whereby Smn loss causes axonal defects remain unclear. RNA localization and translation in axons are controlled by RNA-binding proteins (RBP) and we recently observed that the neuronal RBP Ptbp2 modulates axon growth in motoneurons. Here, we identify Smn as an interactor of Ptbp2 in the cytosolic compartments of motoneurons. We show that the expression level of Ptbp2 is reduced in axons but not in the somata of Smn-depleted motoneurons. This is accompanied by reduced synthesis of the RBP hnRNP R in axons. Re-expression of Ptbp2 in axons compensates for the deficiency of Smn and rescues the defects in axon elongation and growth cone maturation observed in Smn-deficient motoneurons. Our data suggest that Ptbp2 and Smn are components of cytosolic mRNP particles, contributing to the precise spatial and temporal control of protein synthesis within axons and axon terminals.

KEYWORDS
spinal muscular atrophy, SMN, axonal RNA transport, axonal translation, axon growth, Ptbp2

Introduction

Spinal muscular atrophy (SMA) is a severe neuromuscular disorder characterized by lower motoneuron degeneration and caused by reduced expression of the survival motor neuron (SMN) protein due to mutations or deletions in the *SMN1* gene (Lefebvre et al., 1995). In the cytosol, SMN assembles spliceosomal small nuclear ribonucleoproteins (snRNPs) (Fischer et al., 1997; Liu et al., 1997; Pellizzoni et al., 1998). Additionally, granules containing Smn have been observed in axons and axon terminals of motoneurons (Jablonka et al., 2001; Giavazzi et al., 2006; Dombert et al., 2014). Several RNA binding proteins (RBPs) interact with SMN including hnRNP R, and this interaction is necessary for the transport of mRNAs such as *Actb* mRNA encoding β-actin into axons (Rossoll et al., 2003; Glinka et al., 2010). Impaired axonal RNA localization and translation have been linked to SMA, and motoneurons cultured from an SMA mouse model show defects in axon growth

(Rossoll et al., 2003; Jablonka et al., 2007). However, the molecular mechanism underlying the axon growth defects caused by Smn deficiency remains unclear.

Recently, we demonstrated that Ptbp2, a neuronal RBP, facilitates the axonal localization and translation of the *Hnrnpr* transcript encoding hnRNP R in motoneurons, thereby supporting axon growth (Salehi et al., 2023). Here, we show that Smn is associated with Ptbp2 not only in the cell body but also in axons and growth cones of motoneurons, and this interaction is RNA-independent. We found that the level of Ptbp2 protein is significantly reduced in axons but not cell bodies of Smn knockout motoneurons cultured from an SMA mouse model. The reduction in Ptbp2 was accompanied by decreased levels of hnRNP R in axonal compartments of Smn-deficient motoneurons. Re-introducing Ptbp2 could rescue axon elongation and growth cone maturation defects in Smn-depleted motoneurons. Altogether, our data suggest that Smn and Ptbp2 are components of cytosolic granules in motoneurons that control axonal localization and translation of proteins such as hnRNP R.

Materials and methods

Animals and ethical approval

All of the experimental procedures in this study were performed according to the regulations on animal protection of the German federal law and the Association for Assessment and Accreditation of Laboratory Animal Care, in agreement with and under the control of the local veterinary authority. Mice were housed in the animal facility of the Institute of Clinical Neurobiology at the University Hospital of Wuerzburg. The CD1 and *Smn* knockout mice were maintained on a 12 h/12 h day/night cycle under controlled conditions at 20–22°C and 55–65% humidity with food and water in abundant supply.

Isolation and enrichment of primary embryonic mouse motoneurons

Isolation and enrichment of primary mouse motoneurons were performed as previously described (Wiese et al., 2010). Lumbar spinal cords were dissected from E13 mouse embryos, and motoneurons were isolated by panning using a $p75^{NTR}$ antibody. Cells were plated on coverslips or culture dishes coated with poly-DL-ornithine hydrobromide (PORN) (P8638, Sigma) and laminin-111 (23017-015, Thermo Fisher Scientific). Motoneurons were maintained at 37°C, 5% CO_2 in neurobasal medium (Gibco) supplemented with 2% B27 (Gibco), 2% heat-inactivated horse serum (Linaris), 500 µM GlutaMAX (Gibco) and 5 ng/ml of brain-derived neurotrophic factor (BDNF). Medium was replaced one day after plating and then every other day.

Plasmid construction

To generate the construct for expressing EGFP-tagged Ptbp2 (EGFP-Ptbp2), the mouse Ptbp2 coding sequence and the coding sequence of EGFP were PCR-amplified from mouse cDNA and the pSIH-HI plasmid, respectively. Subsequently, the PCR products were inserted into pSIH-H1 digested with SalI (FD0644, Thermo Fisher Scientific) and NheI (FD0973, Thermo Fisher Scientific) using the NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs).

Lentiviral transduction

Lentiviral particles were packaged in HEK293TN cells (System Biosciences, cat. no. LV900A-1) cells with pCMV-pRRE, pCMV-pRSV, and pCMV-pMD2G as described before (Subramanian et al., 2012). Transduction was performed by incubation of motoneurons with lentiviruses in a total volume of 50 µl for 10 min at room temperature before plating at on day *in vitro* (DIV) 0.

Co-immunoprecipitation

Primary mouse motoneurons were grown on laminin-111-coated 6 cm dishes for 7 DIV. Cells were washed once with Dulbecco's Phosphate Buffered Saline (DPBS, without $MgCl_2$, $CaCl_2$; D8537, Sigma-Aldrich) and lysed in lysis buffer (10 mM HEPES pH 7.0, 100 mM KCl, 5 mM $MgCl_2$, 0.5% NP-40) on ice for 15 min and cleared via centrifugation at 20,000 × *g* for 15 min at 4°C. The supernatant was then divided into two microtubes and 0.1 µg RNase A (EN0531, Thermo Fisher Scientific) was added to the microtube labeled +RNase and incubated for 15 min at room temperature. Protein G Dynabeads were bound to either 1 µg of normal rabbit IgG (500-P00, PeproTech) or 1 µg of anti-Ptbp2 antibody (55186-1-AP, Proteintech) by rotating for 60 min at room temperature. 300 µl lysate was added to the antibody-bound beads and rotated for overnight at 4°C. Beads were washed twice with 500 µl lysis buffer and proteins were eluted in 1 × Laemmli buffer. Proteins were size-separated by SDS-PAGE and analyzed by immunoblotting.

Proximity ligation assay (PLA)

PLA was carried out using the Duolink In Situ Orange Starter Kit Mouse/Rabbit (DUO92102, Sigma-Aldrich) according to the manufacturer's recommendations. Briefly, motoneurons were grown for 6 DIV on laminin-111-coated glass coverslips and washed twice with DPBS. Cells were fixed in paraformaldehyde lysine phosphate (PLP) buffer (pH 7.4) containing 4% paraformaldehyde (PFA) (28908, Thermo Fisher Scientific), 5.4% glucose and 0.01 M sodium metaperiodate for 10 min, then permeabilized. After permeabilization and washing, cells were blocked in blocking buffer for 1 h at 37°C and incubated with antibodies against Ptbp2 (1:100; 55186-1-AP, Proteintech) and Smn (1:100; 610647, BD Biosciences) diluted in blocking buffer overnight at 4°C. PLA probes were applied at 1:5 dilution for 1 h at 37°C, followed by ligation and amplification for 30 and 100 min, respectively. Cells were fixed again for 10 min at room temperature in PLP, washed with DPBS, and stained with FITC-conjugated anti-Tubb3 antibody (130-131-158, Miltenyi Biotec).

Puromycylation-PLA

Motoneurons isolated from $Smn^{-/-}$,*SMN2*$^{tg/tg}$ and +/+ mice were grown for 6 DIV on laminin-111-coated glass coverslips. Cells were treated with 10 µg/ml puromycin (Sigma-Aldrich, P8833) supplemented in the medium for 8 min at 37°C in a cell culture incubator. In negative control experiments, puromycin was omitted. Cells were washed twice with prewarmed Hanks' Balanced Salt Solution (HBSS; Gibco) and fixed for 10 min in PLP. After fixation, cells were washed and permeabilized for a proximity ligation assay (PLA) using antibodies against puromycin (Sigma-Aldrich, MABE343, 1:200 dilution) and the N-terminus of hnRNP R (Sigma-Aldrich, HPA026092, 1:200 dilution).

Immunofluorescence staining

Motoneurons were cultured on laminin-111- and PORN-coated glass coverslips for 7 DIV. Cells were washed twice with DPBS and fixed with 4% paraformaldehyde (PFA) at room temperature for 15 min followed by permeabilization with 0.3% Triton X-100 at room temperature for 20 min. Cells were washed three times with DPBS, blocked in a blocking buffer containing 4% BSA at room temperature for 1 h and then incubated in primary antibodies [anti-Ptbp2, 1:250 (55186-1-AP, Proteintech); anti-tubulin, 1:500 (T5168, Sigma-Aldrich); anti-tau, 1:500 (T6402, Sigma-Aldrich)] at 4°C overnight. This was followed by incubation with secondary antibodies [all at 1:500; for anti-tubulin: donkey polyclonal anti-mouse (DyLight 488-conjugated; SA5-10166, Thermo Fisher Scientific); for anti-Ptbp2 and anti-tau: donkey polyclonal anti-rabbit (Alexa Fluor® 647-conjugated; A31573, Thermo Fisher Scientific)] at room temperature for 1 h and counterstaining with 4′,6-diamidino-2-phenylindole (DAPI). Alexa Fluor 546 phalloidin (A22283, Invitrogen) was added at 1:50 in DPBS during incubation with secondary antibodies. Coverslips were washed and mounted using FluorSave Reagent (Merck, 345789) and subsequently imaged.

Image acquisition and data analysis

Images were acquired on an Olympus Fluoview 1000 confocal system equipped with the following objectives: 10 × (NA: 0.25), 20 × (NA: 0.75), 40 × (oil differential interference contrast, NA: 1.30), or 60 × (oil differential interference contrast, NA: 1.35). Fluorescence excitation was achieved with using 405, 473, 559, and 633 nm lasers. Images were obtained with the corresponding Olympus FV10-ASW (RRID:SCR_014215) imaging software for visualization. The resulting images (Olympus.oib format) were processed by maximum intensity projection and were adjusted in brightness and contrast using ImageJ as part of the Fiji package (Schindelin et al., 2012).

For quantification of immunofluorescence signals of Ptbp2, raw images were projected using ImageJ and mean gray values were measured after background subtraction. For axon length measurements, transduced motoneurons were plated on laminin-111 and immunostained at DIV 7 with an anti-tau antibody. The images were acquired with a Keyence BZ-8000K fluorescence microscope equipped with a standard color camera using a 20 × 0.7-NA objective. The length of the longest axon branch was quantified using ImageJ software. Axon collaterals were not considered for the analysis. Motoneurons were only scored when designated axons were at least three times longer than the corresponding dendrites ensuring an unambiguous distinction between axons and dendrites. For growth cone size analysis, cells were plated on laminin-221 (CC085; Merck) for 7 DIV and stained with anti-tau and phalloidin. The area of the growth cone was measured using ImageJ software. Images from control and *Smn* knockout motoneurons were acquired with identical settings (laser intensity and photomultiplier voltage).

Protein extraction and western blotting

Total protein was extracted from primary motoneurons with RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.05% sodium deoxycholate, 0.1% SDS). Protein concentration was quantified using a BCA protein assay kit (23227, Thermo Fisher Scientific). Equal amounts of proteins were size-separated by SDS-PAGE gel electrophoresis followed by transfer onto nitrocellulose membrane and immunoblotting with primary antibodies [anti-Ptbp2, 1:2,000; anti-Smn, 1:2,000; anti-Histone H3, 1:10,000 (ab1791, Abcam); anti-β-actin, 1:10,000 (GTX26276, GeneTex)] diluted in Tris-buffered saline with Tween 20 (TBST) (50 mM Tris–HCl pH 7.6, 150 mM NaCl, 1% Tween 20) overnight at 4°C. Following three washes with TBST, peroxidase-conjugated secondary antibodies [all at 1:10,000; for anti-Ptbp2 and anti-Histone H3: mouse monoclonal anti-rabbit (211-032-171, Jackson ImmunoResearch); for anti-β-actin and anti-Smn: goat polyclonal anti-mouse IgG (115-035-174, Jackson ImmunoResearch)] were added for 1 h at room temperature. Blots were washed three times with TBST and incubated with ECL Western blotting substrate (32106, Thermo Fisher Scientific) followed by exposure on X-ray film (Fuji super RX). Blots were scanned and quantified by densitometry analysis using ImageJ.

Statistics and reproducibility

All statistical analyses were performed using GraphPad Prism version 9 for Windows (GraphPad Software, San Diego, CA, USA). No statistical method was used to predetermine the sample size. No data were excluded from the analyses. Two groups were compared using unpaired two-tailed Student's t-test, two-tailed one-sample t-test or Mann–Whitney test. For multiple

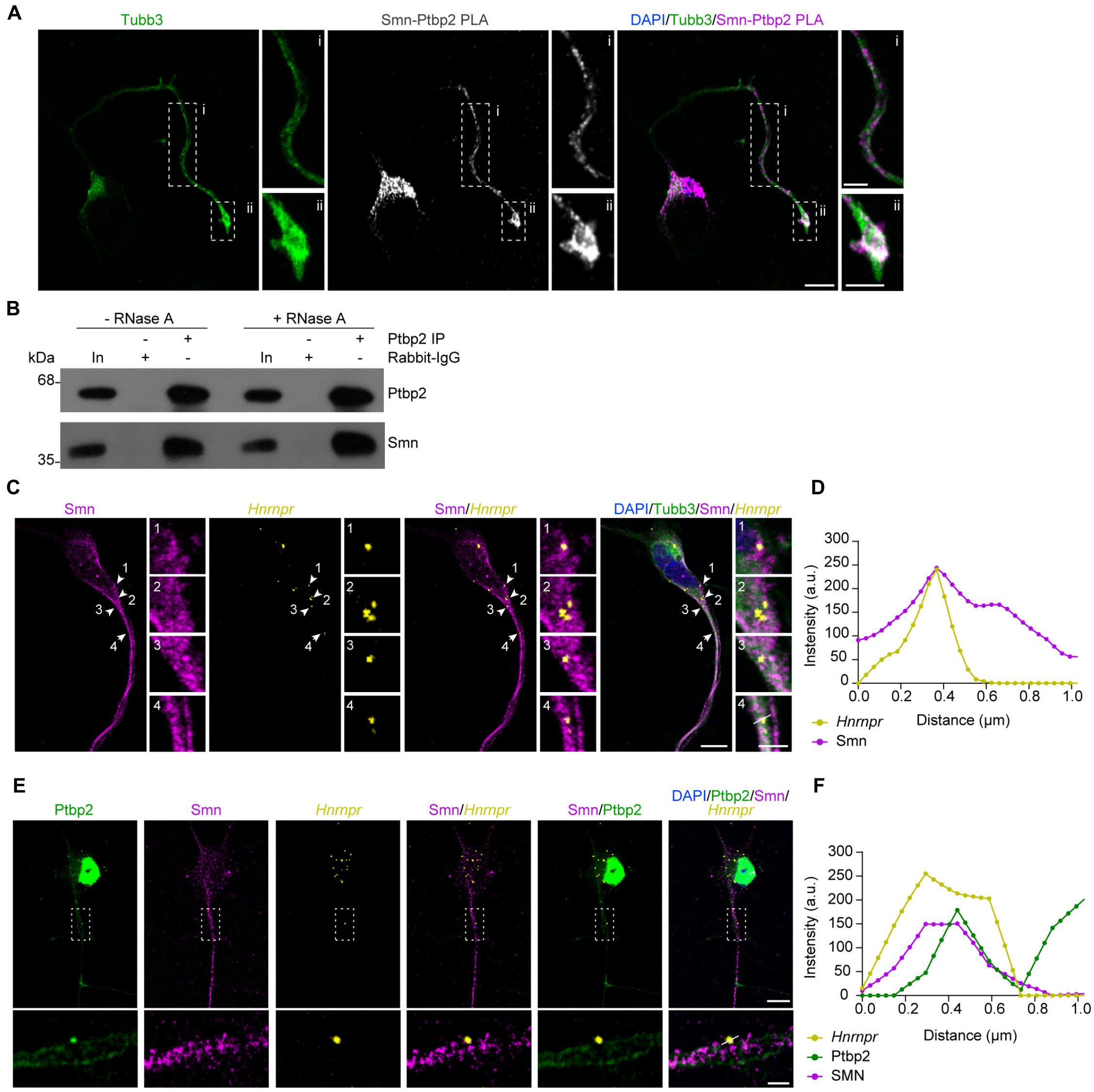


FIGURE 1
Ptbp2 interacts with Smn in motoneurons. **(A)** Representative images of Smn-Ptbp2 PLA signal in cultured motoneurons at DIV 6 using anti-Smn and anti-Ptbp2 antibodies. Motoneuron morphology was visualized with anti-Tubb3 antibody. Scale bars, 10 and 5 μm (magnified areas). **(B)** Co-immunoprecipitation of Smn by anti-Ptbp2 from motoneuron lysate pre-treated with RNase A as indicated. **(C)** Representative images showing Smn immunofluorescence and *Hnrnpr* FISH in cultured motoneurons at DIV 6. Arrowheads indicate colocalization of Smn and *Hnrnpr* in granules. Scale bars, 10 and 2 μm (magnified areas). **(D)** Fluorescence intensity profiles of Smn and *Hnrnpr* at the location indicated by arrow 4 in **(C)**. **(E)** Representative images showing Ptbp2 and Smn immunofluorescence and *Hnrnpr* FISH in cultured motoneurons at DIV 6. Scale bars, 10 and 2 μm (magnified areas). **(F)** Fluorescence intensity profiles of Ptbp2, Smn and *Hnrnpr* at the location indicated by a line in **(E)**.

independent groups, Kruskal-Wallis test with Dunn's multiple comparisons test. Details of replicate numbers, quantification, and statistics for each experiment are specified in the figure legends.

Results

Smn is associated with Ptbp2 in axons of motoneurons

Guided by our recent study demonstrating that depletion of Ptbp2 leads to axon growth defects similar to Smn-deficient motoneurons (Salehi et al., 2023), we investigated whether Ptbp2 is associated with Smn in cultured primary mouse motoneurons. For this purpose, we evaluated the interaction between Ptbp2 and Smn *in situ* by performing a proximity ligation assay (PLA) using antibodies against Ptbp2 and Smn. We observed that the Ptbp2-Smn PLA signal was detectable in the cytosol of the somata as well as in axons and growth cones of cultured motoneurons (Figure 1A). As negative controls, no signal was detected when either anti-Ptbp2 or anti-Smn antibody was omitted (Supplementary Figure 1). To further validate the association between Ptbp2 and Smn, we performed immunoprecipitation from motoneuron lysates using anti-Ptbp2 antibody and evaluated Smn co-immunoprecipitation by immunoblot analysis. We assessed the RNA-dependence of the

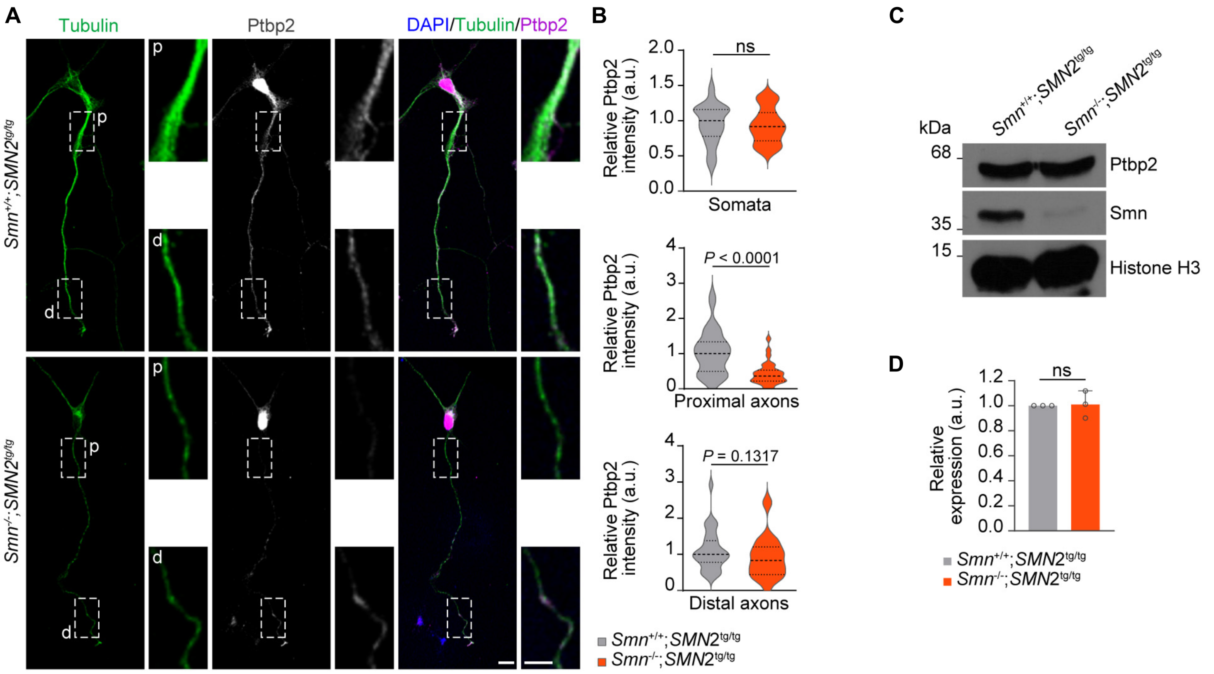


FIGURE 2
Reduction of Ptbp2 in axons of Smn-deficient motoneurons. **(A)** Immunofluorescence imaging of Ptbp2 in motoneurons cultured from *Smn*$^{+/+}$; *SMN2*$^{tg/tg}$ and *Smn*$^{-/-}$; *SMN2*$^{tg/tg}$ mice at DIV 7. Scale bars, 10 and 5 μm (magnified areas). **(B)** Ptbp2 immunosignals in somata, proximal (p) and distal (d) axons. *n* (*Smn*$^{+/+}$; *SMN2*$^{tg/tg}$) = 31 and *n* (*Smn*$^{-/-}$; *SMN2*$^{tg/tg}$) = 28 motoneurons from three biological replicates. Mann–Whitney and unpaired two-tailed Student's *t*-test. **(C)** Immunoblot of Ptbp2 in *Smn*$^{+/+}$; *SMN2*$^{tg/tg}$ and *Smn*$^{-/-}$; *SMN2*$^{tg/tg}$ motoneurons at DIV 7. Histone H3 was used as loading control. **(D)** Quantitative analysis of Western blots as shown in **(C)** for Ptbp2. Two-tailed one-sample *t*-test. Data are mean ± s.d. of n = 3 biological replicates.

interaction by treating the motoneuron lysate with RNase A. We found that Smn co-precipitated with Ptbp2 without and with RNase A treatment, indicating that the interaction between Ptbp2 and Smn is RNA-independent (Figure 1B). Together, these data show that Smn interacts with Ptbp2 in axons of motoneurons.

We previously showed that Ptbp2 and *Hnrnpr* mRNA are components of cytosolic mRNP particles in axons of motoneurons. To investigate whether Smn is associated with this complex, we visualized *Hnrnpr* mRNA by fluorescent *in situ* hybridization (FISH) and Smn by immunostaining in motoneurons. We observed that Smn-positive punctae were in close proximity to the FISH signal for *Hnrnpr* mRNA in axons (Figures 1C, D). Next, we assessed whether Smn is associated with Ptbp2 complexes containing *Hnrnpr* mRNA in the axons of motoneurons. To do so, we performed FISH for *Hnrnpr* visualization together with Smn and Ptbp2 immunostaining. Smn-positive punctae were observed close to the Ptbp2-positive punctae that co-localized with *Hnrnpr* mRNA in axons (Figures 1E, F). These data suggest that Smn is associated with cytosolic Ptbp2 complexes containing *Hnrnpr* mRNA.

Ptbp2 is reduced in axons of Smn-deficient motoneurons

Having shown that Ptbp2 is associated with Smn in axons of motoneurons, we next addressed the question whether the axonal localization of Ptbp2 is regulated by Smn. To do so, we performed Ptbp2 immunostaining on primary motoneurons cultured from a severe SMA mouse model. In these mice, deletion of murine *Smn* is partially compensated for by expression of human SMN from an *SMN2* transgene (Monani et al., 2000). We observed that the level of Ptbp2 was significantly reduced in proximal axons of motoneurons cultured from *Smn*$^{-/-}$; *SMN2*$^{tg/tg}$ mice while Ptbp2 levels in the somata were unchanged. Distally, we observed a tendency toward Ptbp2 reduction in axons of Smn-deficient motoneurons (Figures 2A, B). In line with this result, the total level of Ptbp2 was not affected in *Smn*-deficient motoneurons (Figures 2C, D). Thus, Smn regulates the axonal localization of Ptbp2.

Loss of Smn affects the local synthesis of hnRNP R in axons

We previously showed that Ptbp2 promotes axonal hnRNP R translation in motoneurons (Salehi et al., 2023). Therefore, we investigated whether the reduction of Ptbp2 in axons of Smn-depleted motoneurons affects the axonal levels of hnRNP R. For this purpose, we first performed puromycin treatment coupled with PLA (Puro-PLA) to measure newly synthesized hnRNP R in both control and Smn-depleted motoneurons. In this assay, puromycin is incorporated into nascent polypeptides such that PLA with antibodies against the N-terminus of hnRNP R and puromycin can visualize newly synthesized hnRNP R (Tom Dieck et al., 2015). We observed that the number of hnRNP R Puro-PLA

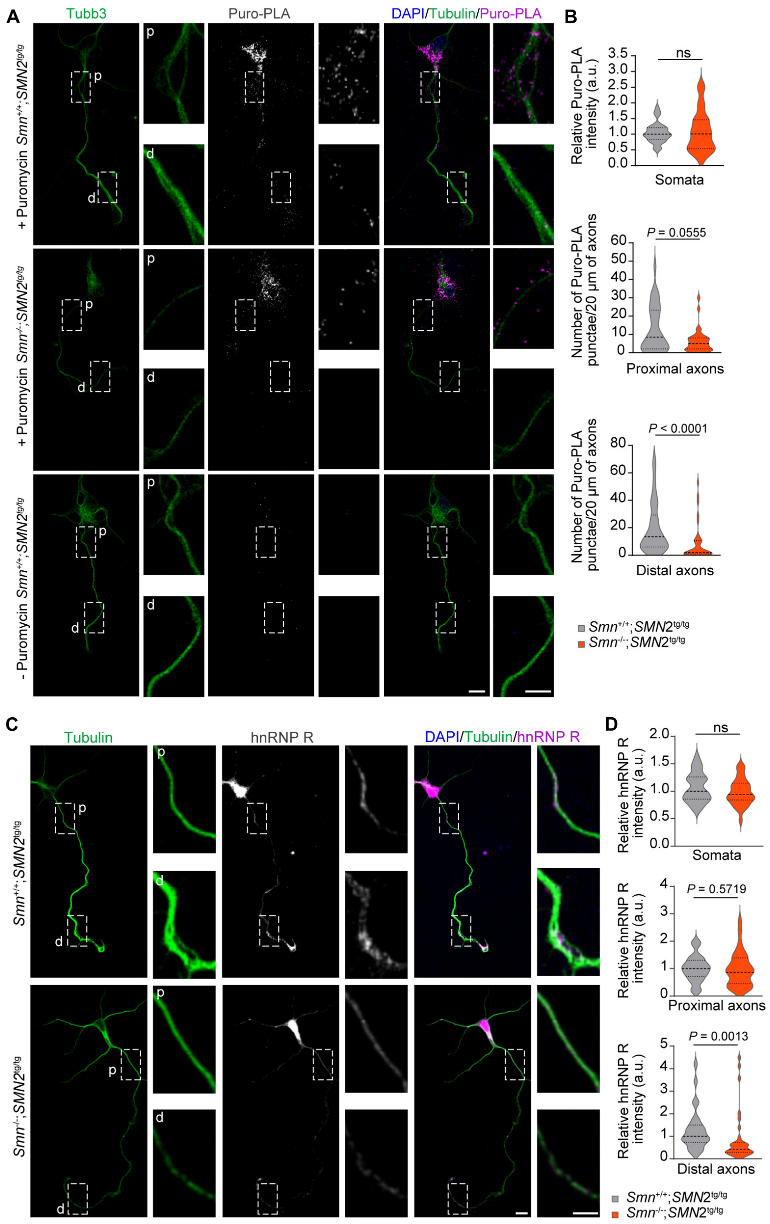


FIGURE 3
Reduction of hnRNP R in axons of Smn-deficient motoneurons. **(A)** Puro-PLA of hnRNP R in control and Smn-deficient motoneurons. Scale bars, 10 and 5 µm (magnified areas). **(B)** Quantification of relative Puro-PLA intensity in somata and the number of Puro-PLA punctae in 20 µm of proximal and distal axons of motoneurons cultured from *Smn*$^{+/+}$; *SMN2*$^{tg/tg}$ and *Smn*$^{-/-}$; *SMN2*$^{tg/tg}$ mice at DIV 6. *n* (*Smn*$^{+/+}$; *SMN2*$^{tg/tg}$) = 34 and *n* (*Smn*$^{-/-}$; *SMN2*$^{tg/tg}$) = 36 motoneurons from three biological replicates. Mann–Whitney and unpaired *t*-test. **(C)** Immunofluorescence imaging of hnRNP R in motoneurons cultured from *Smn*$^{+/+}$; *SMN2*$^{tg/tg}$ and *Smn*$^{-/-}$; *SMN2*$^{tg/tg}$ mice at DIV 7. Scale bars, 10 and 5 µm (magnified areas). **(D)** hnRNP R immunosignals in somata, proximal and distal axons. *n* (*Smn*$^{+/+}$; *SMN2*$^{tg/tg}$) = 28 and *n* (*Smn*$^{-/-}$; *SMN2*$^{tg/tg}$) = 31 motoneurons from three biological replicates. Mann–Whitney and unpaired *t*-test.

punctae was significantly reduced in the distal axons of Smn-depleted motoneurons (Figures 3A, B). We also found a tendency for a reduced number of Puro-PLA punctae in proximal axons of Smn-depleted motoneurons but there was no change in somata (Figures 3A, B). These data suggest that Smn is involved in axonal hnRNP R translation. Consistent with these results, hnRNP R immunostaining revealed that the level of hnRNP R was reduced in the distal axons of Smn-deficient motoneurons while it remained unchanged in the somata (Figures 3C, D). Together, our findings suggest that Smn deficiency affects local hnRNP R synthesis in axons.

Re-expression of Ptbp2 rescues axon growth in Smn-deficient motoneurons

Previous studies have shown that loss of Smn in SMA mouse models and patients with SMA results in axonal and synaptic defects (Jablonka et al., 2022). Furthermore primary motoneurons

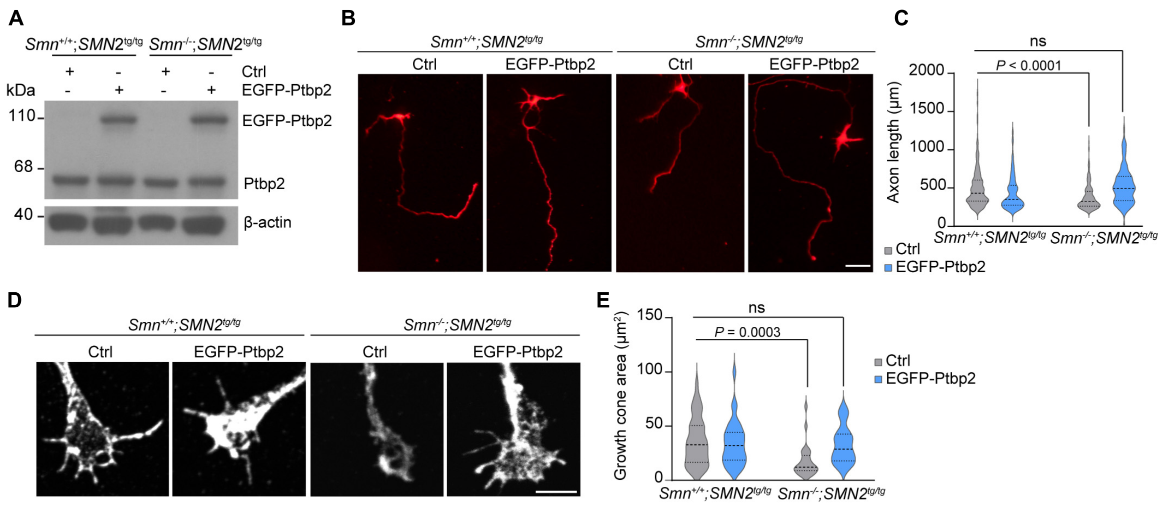


FIGURE 4

Re-introducing Ptbp2 restores impaired axon growth in *Smn*-deficient motoneurons. **(A)** Immunoblot analysis of Ptbp2 in *Smn*$^{+/+}$; *SMN2*$^{tg/tg}$ and *Smn*$^{-/-}$; *SMN2*$^{tg/tg}$ motoneurons transduced with lentivirus expressing either EGFP (Ctrl) or EGFP-Ptbp2 at DIV 7. β-actin was used as a loading control. **(B)** Cultured DIV 7 motoneurons from *Smn*$^{+/+}$; *SMN2*$^{tg/tg}$ and *Smn*$^{-/-}$; *SMN2*$^{tg/tg}$ mice immunostained for tau. Scale bar, 50 μm. **(C)** Quantification of axon lengths. Kruskal-Wallis test with Dunn's multiple comparisons test. For Ctrl, *n* (*Smn*$^{+/+}$; *SMN2*$^{tg/tg}$) = 462 and *n* (*Smn*$^{-/-}$; *SMN2*$^{tg/tg}$) = 229 motoneurons; for EGFP-Ptbp2, *n* (*Smn*$^{+/+}$; *SMN2*$^{tg/tg}$) = 398 and *n* (*Smn*$^{-/-}$; *SMN2*$^{tg/tg}$) = 99 from three biological replicates. **(D)** Representative images of growth cones of *Smn*$^{+/+}$; *SMN2*$^{tg/tg}$ and *Smn*$^{-/-}$; *SMN2*$^{tg/tg}$ motoneurons expressing either EGFP (Ctrl) or EGFP-Ptbp2 at DIV 7. **(E)** Quantification of growth cone size. Kruskal-Wallis test with Dunn's multiple comparisons test. For Ctrl, *n* (*Smn*$^{+/+}$; *SMN2*$^{tg/tg}$) = 44 and *n* (*Smn*$^{-/-}$; *SMN2*$^{tg/tg}$) = 25 motoneurons; for EGFP-Ptbp2, *n* (*Smn*$^{+/+}$; *SMN2*$^{tg/tg}$) = 33 and *n* (*Smn*$^{-/-}$; *SMN2*$^{tg/tg}$) = 25 from three biological replicates.

cultured from *Smn*$^{-/-}$; *SMN2*$^{tg/tg}$ exhibit decreased growth cone size and impaired axon elongation (Rossoll et al., 2003; Jablonka et al., 2007). To examine whether re-introducing Ptbp2 can rescue impaired axon growth in Smn-deficient motoneurons, primary motoneurons cultured from *Smn*$^{-/-}$; *SMN2*$^{tg/tg}$ and *Smn*$^{+/+}$; *SMN2*$^{tg/tg}$ mice were transduced with lentiviruses expressing an EGFP-Ptbp2 fusion protein (Figure 4A). We found that re-expression of Ptbp2 could restore axon length and growth cone size in Smn-depleted motoneurons (Figures 4B–E). These results indicate that the reduction of axonal Ptbp2 contributes to the impairment of axon growth in Smn-deficient motoneurons.

Discussion

The SMN protein has a canonical function in spliceosomal snRNP biogenesis in the cytoplasm (Fischer et al., 1997). This, however, raises the question why loss of SMN in SMA particularly affects lower motoneurons in the spinal cord (Briese et al., 2005). In motoneurons, SMN is also present in axons and growth cones (Jablonka et al., 2001; Zhang et al., 2003, 2006; Giavazzi et al., 2006). SMN is associated with a variety of RBPs known to be involved in mRNA transport, stability, and local translation in neurons including hnRNP R, hnRNP Q, FMRP and HuD (Rossoll et al., 2002; Piazzon et al., 2008; Fallini et al., 2011). The expanding list of RBPs identified as SMN interactors together with the observation that SMN localizes in axons has put forward the hypothesis that SMN complexes distinct from those involved in snRNP biogenesis localize to axons to facilitate mRNA delivery followed by local protein synthesis to support axon growth and maintenance. Our study reveals that Smn interacts with the RBP Ptbp2 in axons. Ptbp2 primarily localizes to the cell body of neuronal cells but also is present in axons and growth cones, where it is involved in localization and translation of the *Hnrnpr* mRNA (Salehi et al., 2023). We observed that Smn regulates the axonal levels of Ptbp2 and the local synthesis of hnRNP R. This finding reveals an additional layer of complexity as the local production of RBPs such as hnRNP R might fine-tune local mRNA processing and translation. Loss of these regulatory pathways might destabilize axons, thereby contributing to the axonal pathology observed as an early pathological event in SMA. Conspicuously, we observed that Ptbp2 was reduced in proximal axons of Smn-deficient motoneurons while hnRNP R levels were more reduced in distal regions. While the exact mechanisms underlying this discrepancy are not known, it is possible that axonally localized Ptbp2 is not only derived from axonal transport, which is affected by Smn loss, but also from local synthesis of Ptbp2 in distal regions, which might not be affected by Smn deficiency.

How Smn modulates the axonal transport of Ptbp2 is currently not clear. It is possible that Smn associates with Ptbp2 already in the somata of motoneurons and that Smn-Ptbp2 complexes, together with the *Hnrnpr* mRNA, are transported in axons toward distal regions. In agreement with this notion, it has been shown previously that SMN interacts with the RBP HuD and that these proteins are actively co-transported in axons (Fallini et al., 2011). Alternatively, it is also conceivable that Ptbp2 bound to *Hnrnpr* mRNA associates with Smn locally in axons to facilitate hnRNP R synthesis. However, our PLA results show that Ptbp2 binds to Smn already in the somata of motoneurons, and it is thus more likely that Smn-Ptbp2 complexes are pre-assembled prior to transport. Either way, Smn-Ptbp2 complexes might be remodeled locally to induce translation of *Hnrnpr* mRNA, which might be kept in a translationally silent state during transport. In addition to *Hnrnpr* mRNA, the axonal localization of many other mRNAs might be regulated by Smn and Ptbp2. This possibility is supported by previous studies showing that there is a broad reduction of mRNAs in axons of Smn-deficient motoneurons (Fallini et al., 2011; Saal et al., 2014; Hennlein et al., 2023). Future experiments identifying the protein and RNA composition of axonal Smn and Ptbp2 complexes might reveal novel insights into the mechanisms whereby mRNAs are transported in axons to contribute to the proteomics diversity present at axon terminals.

In axons, Smn granules co-localize with ribosomal RNAs and control translation through interaction with other RBPs (Zhang et al., 2003; Lauria et al., 2020). An important finding of our study is that re-introducing Ptbp2 can rescue the axon growth defect of Smn-deficient motoneurons. We provide evidence that loss of Smn affects the local synthesis of hnRNP R and it is thus possible that deficiency of hnRNP R itself contributes to the axonal defects seen in motoneurons lacking Smn. Multiple lines of evidence indicate that hnRNP R is important for axon development. First, hnRNP R interacts with many mRNAs encoding proteins involved in axon growth and maturation including cytoskeletal components such as β-actin and synaptic proteins (Briese et al., 2018). Second, hnRNP R regulates the axonal localization of such transcripts to axons and it is possible that hnRNP R also controls their local translation (Briese et al., 2018). Third, depletion of hnRNP R reduces axon growth without affecting motoneuron survival (Glinka et al., 2010; Briese et al., 2018). Thus, by facilitating the local synthesis of hnRNP R, itself a multi-functional RBP, Smn together with Ptbp2 might stimulate axon growth. In addition to *Hnrnpr* mRNA, Ptbp2 has been shown to interact with many mRNAs (Licatalosi et al., 2012) suggesting that Smn-Ptbp2 complexes potentially regulate the axonal transport and local translation of several mRNAs.

We have previously shown that Ptbp2 promotes axonal translation of hnRNP R in cultured motoneurons by regulating the association of *Hnrnpr* mRNA with translating ribosomes (Salehi et al., 2023). Together with our finding that hnRNP R levels were reduced in axons but not cell bodies of Smn-deficient motoneurons, this suggests that Smn, through interaction with Ptbp2, promotes the axonal translation of hnRNP R in motoneurons for axon growth. Considering that neuromuscular connectivity is affected early in the course of SMA, our results thus highlight the importance of mechanisms for local protein synthesis for motoneuron development and functionality.

Data availability statement

The original contributions presented in this study are included in this article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by the Animal Care and Ethic Committee of the University of Wuerzburg, the local veterinary authority and the Regierung von Unterfranken. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SS: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. AZ: Formal analysis, Investigation, Methodology, Writing – review & editing. GG: Investigation, Writing – review & editing. MS: Conceptualization, Funding acquisition, Supervision, Writing – review & editing. MB: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Funding

The authors declare that financial support was received for the research, authorship, and/or publication of this article. This work was supported by the Deutsche Forschungsgemeinschaft [BR4910/2–2 (SPP1935) to MB and SE697/5–2 (SPP1935) to MS].

Acknowledgments

We thank Flinders University for providing the p75NTR antibody.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2024.1393779/full#supplementary-material

SUPPLEMENTARY FIGURE 1

Smn is associated with Ptbp2 in motoneurons. **(A,B)** Representative images of PLA signal in motoneurons at DIV 6 with either Ptbp2 antibody **(A)** or Smn antibody **(B)** alone as a negative control. Scale bars, 10 and 5 μm (magnified areas).

References

Briese, M., Esmaeili, B., and Sattelle, D. B. (2005). Is spinal muscular atrophy the result of defects in motor neuron processes? *Bioessays* 27, 946–957. doi: 10.1002/bies.20283

Briese, M., Saal-Bauernschubert, L., Ji, C., Moradi, M., Ghanawi, H., Uhl, M., et al. (2018). hnRNP R and its main interactor, the noncoding RNA 7SK, coregulate the axonal transcriptome of motoneurons. *Proc. Natl. Acad. Sci. U.S.A.* 115, E2859–E2868. doi: 10.1073/pnas.1721670115

Dombert, B., Sivadasan, R., Simon, C. M., Jablonka, S., and Sendtner, M. (2014). Presynaptic localization of Smn and hnRNP R in axon terminals of embryonic and postnatal mouse motoneurons. *PLoS One* 9:e110846. doi: 10.1371/journal.pone.0110846

Fallini, C., Zhang, H., Su, Y., Silani, V., Singer, R. H., Rossoll, W., et al. (2011). The survival of motor neuron (SMN) protein interacts with the mRNA-binding protein HuD and regulates localization of poly (A) mRNA in primary motor neuron axons. *J. Neurosci.* 31, 3914–3925. doi: 10.1523/JNEUROSCI.3631-10.2011

Fischer, U., Liu, Q., and Dreyfuss, G. (1997). The SMN–SIP1 complex has an essential role in spliceosomal snRNP biogenesis. *Cell* 90, 1023–1029. doi: 10.1016/S0092-8674(00)80368-2

Giavazzi, A., Setola, V., Simonati, A., and Battaglia, G. (2006). Neuronal-specific roles of the survival motor neuron protein: Evidence from survival motor neuron expression patterns in the developing human central nervous system. *J. Neuropathol. Exp. Neurol.* 65, 267–277. doi: 10.1097/01.jnen.0000205144.54457.a3

Glinka, M., Herrmann, T., Funk, N., Havlicek, S., Rossoll, W., Winkler, C., et al. (2010). The heterogeneous nuclear ribonucleoprotein-R is necessary for axonal β-actin mRNA translocation in spinal motor neurons. *Hum. Mol. Genet.* 19, 1951–1966. doi: 10.1093/hmg/ddq073

Hennlein, L., Ghanawi, H., Gerstner, F., Palominos García, E., Yildirim, E., Saal-Bauernschubert, L., et al. (2023). Plastin 3 rescues cell surface translocation and activation of TrkB in spinal muscular atrophy. *J. Cell Biol.* 222:e202204113. doi: 10.1083/jcb.202204113

Jablonka, S., Bandilla, M., Wiese, S., Bühler, D., Wirth, B., Sendtner, M., et al. (2001). Co-regulation of survival of motor neuron (SMN) protein and its interactor SIP1 during development and in spinal muscular atrophy. *Hum. Mol. Genet.* 10, 497–505. doi: 10.1093/hmg/10.5.497

Jablonka, S., Beck, M., Lechner, B. D., Mayer, C., and Sendtner, M. (2007). Defective Ca2+ channel clustering in axon terminals disturbs excitability in motoneurons in spinal muscular atrophy. *J. Cell Biol.* 179, 139–149. doi: 10.1083/jcb.200703187

Jablonka, S., Hennlein, L., and Sendtner, M. (2022). Therapy development for spinal muscular atrophy: Perspectives for muscular dystrophies and neurodegenerative disorders. *Neurol. Res. Pract.* 4:2. doi: 10.1186/s42466-021-00162-9

Lauria, F., Bernabò, P., Tebaldi, T., Groen, E. J. N., Perenthaler, E., Maniscalco, F., et al. (2020). SMN-primed ribosomes modulate the translation of transcripts related to spinal muscular atrophy. *Nat. Cell Biol.* 22, 1239–1251. doi: 10.1038/s41556-020-00577-7

Lefebvre, S., Bürglen, L., Reboullet, S., Clermont, O., Burlet, P., Viollet, L., et al. (1995). Identification and characterization of a spinal muscular atrophy-determining gene. *Cell* 80, 155–165. doi: 10.1016/0092-8674(95)90460-3

Licatalosi, D. D., Yano, M., Fak, J. J., Mele, A., Grabinski, S. E., Zhang, C., et al. (2012). Ptbp2 represses adult-specific splicing to regulate the generation of neuronal precursors in the embryonic brain. *Genes Dev.* 26, 1626–1642. doi: 10.1101/gad.191338.112

Liu, Q., Fischer, U., Wang, F., and Dreyfuss, G. (1997). The spinal muscular atrophy disease gene product, SMN, and its associated protein Sip1 are in a complex with spliceosomal snRNP proteins. *Cell* 90, 1013–1021. doi: 10.1016/S0092-8674(00)80367-0

Monani, U. R., Sendtner, M., Coovert, D. D., Parsons, D. W., Andreassi, C., Le, T. T., et al. (2000). The human centromeric survival motor neuron gene (SMN2) rescues embryonic lethality in Smn−/−mice and results in a mouse with spinal muscular atrophy. *Hum. Mol. Genet.* 9, 333–339. doi: 10.1093/hmg/9.3.333

Pellizzoni, L., Kataoka, N., Charroux, B., and Dreyfuss, G. (1998). A novel function for SMN, the spinal muscular atrophy disease gene product, in pre-mRNA splicing. *Cell* 95, 615–624. doi: 10.1016/S0092-8674(00)81632-3

Piazzon, N., Rage, F., Schlotter, F., Moine, H., Branlant, C., and Massenet, S. (2008). In vitro and in cellulo evidences for association of the survival of motor neuron complex with the fragile X mental retardation protein. *J. Biol. Chem.* 283, 5598–5610. doi: 10.1074/jbc.M707304200

Rossoll, W., Jablonka, S., Andreassi, C., Kröning, A.-K., Karle, K., Monani, U. R., et al. (2003). Smn, the spinal muscular atrophy–determining gene product, modulates axon growth and localization of β-actin mRNA in growth cones of motoneurons. *J. Cell Biol.* 163, 801–812. doi: 10.1083/jcb.200304128

Rossoll, W., Kröning, A.-K., Ohndorf, U.-M., Steegborn, C., Jablonka, S., and Sendtner, M. (2002). Specific interaction of Smn, the spinal muscular atrophy determining gene product, with hnRNP-R and gry-rbp/hnRNP-Q: A role for Smn in RNA processing in motor axons? *Hum. Mol. Genet.* 11, 93–105. doi: 10.1093/hmg/11.1.93

Saal, L., Briese, M., Kneitz, S., Glinka, M., and Sendtner, M. (2014). Subcellular transcriptome alterations in a cell culture model of spinal muscular atrophy point to widespread defects in axonal growth and presynaptic differentiation. *RNA* 20, 1789–1802. doi: 10.1261/rna.047373.114

Salehi, S., Zare, A., Prezza, G., Bader, J., Schneider, C., Fischer, U., et al. (2023). Cytosolic Ptbp2 modulates axon growth in motoneurons through axonal localization and translation of Hnrnpr. *Nat. Commun.* 14:4158. doi: 10.1038/s41467-023-39787-6

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., et al. (2012). Fiji: An open-source platform for biological-image analysis. *Nat. Methods* 9, 676–682. doi: 10.1038/nmeth.2019

Subramanian, N., Wetzel, A., Dombert, B., Yadav, P., Havlicek, S., Jablonka, S., et al. (2012). Role of Nav1. 9 in activity-dependent axon growth in motoneurons. *Hum. Mol. Genet.* 21, 3655–3667. doi: 10.1093/hmg/dds195

Tom Dieck, S., Kochen, L., Hanus, C., Heumüller, M., Bartnik, I., Nassim-Assir, B., et al. (2015). Direct visualization of newly synthesized target proteins in situ. *Nat. Methods* 12, 411–414. doi: 10.1038/nmeth.3319

Wiese, S., Herrmann, T., Drepper, C., Jablonka, S., Funk, N., Klausmeyer, A., et al. (2010). Isolation and enrichment of embryonic mouse motoneurons from the lumbar spinal cord of individual mouse embryos. *Nat. Protoc.* 5, 31–38. doi: 10.1038/nprot.2009.193

Zhang, H. L., Pan, F., Hong, D., Shenoy, S. M., Singer, R. H., and Bassell, G. J. (2003). Active transport of the survival motor neuron protein and the role of exon-7 in cytoplasmic localization. *J. Neurosci.* 23, 6627–6637. doi: 10.1523/JNEUROSCI.23-16-06627.2003

Zhang, H., Xing, L., Rossoll, W., Wichterle, H., Singer, R. H., and Bassell, G. J. (2006). Multiprotein complexes of the survival of motor neuron protein SMN with Gemins traffic to neuronal processes and growth cones of motor neurons. *J. Neurosci.* 26, 8622–8632. doi: 10.1523/JNEUROSCI.3967-05.2006

frontiers | Frontiers in Molecular Neuroscience

TYPE Original Research
PUBLISHED 29 August 2024
DOI 10.3389/fnmol.2024.1392408

Check for updates

OPEN ACCESS

EDITED BY
Clémence Bernard,
University of Exeter, United Kingdom

REVIEWED BY
Takatoshi Iijima,
Tokai University, Japan
Leena Ali Ibrahim,
King Abdullah University of Science and Technology, Saudi Arabia

*CORRESPONDENCE
Jing Qiu
✉ jing.qiu@ed.ac.uk
Owen Dando
✉ owen.dando@ed.ac.uk
Giles E. Hardingham
✉ giles.hardingham@ed.ac.uk

RECEIVED 27 February 2024
ACCEPTED 05 August 2024
PUBLISHED 29 August 2024

CITATION
Dando O, McQueen J, Burr K, Kind PC, Chandran S, Hardingham GE and Qiu J (2024) A comparison of basal and activity-dependent exon splicing in cortical-patterned neurons of human and mouse origin.
Front. Mol. Neurosci. 17:1392408.
doi: 10.3389/fnmol.2024.1392408

A comparison of basal and activity-dependent exon splicing in cortical-patterned neurons of human and mouse origin

Owen Dando[1,2,3]*, Jamie McQueen[1,3], Karen Burr[1,2], Peter C. Kind[1,3], Siddharthan Chandran[1,4], Giles E. Hardingham[1,2,3]* and Jing Qiu[1,3]*

[1]Edinburgh Medical School, UK Dementia Research Institute at the University of Edinburgh, Edinburgh, United Kingdom, [2]Simons Initiative for the Developing Brain, University of Edinburgh, Edinburgh, United Kingdom, [3]Centre for Discovery Brain Sciences, Hugh Robson Building, University of Edinburgh,, Edinburgh, United Kingdom, [4]Centre for Clinical Brain Sciences, Edinburgh Medical School, Edinburgh, United Kingdom

Rodent studies have shown that alternative splicing in neurons plays important roles in development and maturity, and is regulatable by signals such as electrical activity. However, rodent-human similarities are less well explored. We compared basal and activity-dependent exon splicing in cortical-patterned human ESC-derived neurons with that in cortical mouse ESC-derived neurons, primary mouse cortical neurons at two developmental stages, and mouse hippocampal neurons, focussing on conserved orthologous exons. Both basal exon inclusion levels and activity-dependent changes in splicing showed human-mouse correlation. Conserved activity regulated exons are enriched in RBFOX, SAM68, NOVA and PTBP targets, and centered on cytoskeletal organization, mRNA processing, and synaptic signaling genes. However, human-mouse correlations were weaker than inter-mouse comparisons of neurons from different brain regions, developmental stages and origin (ESC vs. primary), suggestive of some inter-species divergence. The set of genes where activity-dependent splicing was observed only in human neurons were dominated by those involved in lipid biosynthesis, signaling and trafficking. Study of human exon splicing in mouse Tc1 neurons carrying human chromosome-21 showed that neuronal basal exon inclusion was influenced by cis-acting sequences, although may not be sufficient to confer activity-responsiveness in an allospecific environment. Overall, these comparisons suggest that neuronal alternative splicing should be confirmed in a human-relevant system even when exon structure is evolutionarily conserved.

KEYWORDS

RNA-seq—RNA sequencing, gene expression, neuronal activity, calcium signaling, evolutionary conservation and divergence, alternative splicing

Introduction

Humans and mice diverged from their common ancestor approximately 80 million years ago. Nevertheless, over 90% of human genes have 1:1 orthologs in mice (Monaco et al., 2015). However, whether the expression of these orthologs in response to signaling pathways is conserved or otherwise was not well understood. A classic example of this type of response is found in CNS neurons, where electrical activity dynamically controls gene expression to influence neuronal development, neuroprotection, neurophysiological properties, and ultimately cognitive function (Soriano and Hardingham, 2007; Bell and Hardingham, 2011; Hardingham and Lipton, 2011; West and Greenberg, 2011; Hardingham et al., 2018; Lee and Fields, 2021). Neuronal activity regulates the transcription of hundreds of genes whose promoters recruit transcription factors and coactivators that are controlled by Ca^{2++}-activated signaling pathways, including CREB, SRF, AP-1, FOXO, ATF4, Notch, Jacob, PGC1-α and CBP (Sheng et al., 1990; Hardingham et al., 1997; Hardingham and Bading, 1998; Cruzalegui et al., 1999; McKenzie et al., 2005; Al-Mubarak et al., 2009; Puddifoot et al., 2012; Karpova et al., 2013; Lewerenz et al., 2014).

We previously reported that activity-responsiveness of expression levels of 1:1 orthologs in mouse and human cortical neurons showed evidence of divergence (Qiu et al., 2016). The human system employed was glutamatergic cortical-patterned neurons from human embryonic stem cells (hESC^{CORT}-neurons), and comparisons were made to mouse primary cortical neurons (Mus-PRIM^{CORT} neurons) at day in vitro (DIV) 4 and DIV10, as well as cortical-patterned neurons from mouse embryonic stem cells (Mus-ESC^{CORT}-neurons). The rationale for employing multiple mouse neuronal preparations was to get a clearer idea of the extent of non-species-dependent differences such as developmental stage or cellular origin (primary tissue vs. stem cell). Mechanistically, we concluded that human-mouse differences in activity-responsiveness involved changes in cis-acting gene promoter regions that contain binding sites for activity-responsive transcription factors (Qiu et al., 2016). Other studies published shortly after also provided evidence of divergence of gene activity-responsiveness and showed that it could influence lineage-specific aspects of neuronal development (Ataman et al., 2016; Pruunsild et al., 2017), reviewed in (Hardingham et al., 2018).

While the basic property of whether a gene's transcription is up- or down-regulated in neurons in response to electrical activity is of importance in determining the physiological outcome, so is post-transcriptional regulation. Eukaryotic genes possess coding exons interspersed with non-coding introns, the former of which are spliced to create protein-coding open reading frames. Many genes exhibit variable usage of exons (a form of alternative splicing) which enable a variety of related proteins to be encoded at a single genomic locus. In neurons, alternative splicing plays a key role in development (Saito et al., 2016, 2019; Wamsley et al., 2018). Moreover, many exons are subject to signal-dependent inclusion (or exclusion). Neuronal electrical activity is known to control exon usage, mediated by several RNA binding factors including the RBFOX proteins (1–3), SAM68 (Furlanis and Scheiffele, 2018; Jacko et al., 2018; Farini et al., 2020) and NOVA (Eom et al., 2013; Ibrahim et al., 2023). Moreover, activity-dependent alternative splicing regulates neurophysiological and other properties by determining the function of specific proteins (Furlanis and Scheiffele, 2018). Aberrant alternative splicing in neurons is thought to contribute to the pathogenesis of human brain disorders including Parkinson's and Alzheimer's diseases, and ALS-FTD (Lopez Soto et al., 2019; Li et al., 2021), and misregulation of activity-dependent exon usage is implicated in autism spectrum disorder phenotypes (Parikshak et al., 2016; Quesnel-Vallières et al., 2016; Quesnel-Vallieres et al., 2019).

However, despite mice being widely used to model human brain disease, comparisons of alternative splicing in mouse vs. human neurons are lacking. Alternative splicing has previously been compared across several species (including human and mouse) in a variety of organs, including the brain (Barbosa-Morais et al., 2012; Merkin et al., 2012; Verta and Jacobs, 2022). However, this does not give complete insight into neurons specifically since the brain is a mixture of cell types (neurons, macroglia, immune cells and vascular cells) which will have distinct alternative splicing profiles and which may contribute different proportions to the brain in different species. Furthermore, in these studies dynamic changes in alternative splicing as a result of neuronal activity was not addressed.

Here we have compared alternative splicing in cortical-patterned neurons of human and mouse origin, considering both basal levels of exon inclusion as well as changes that occur in response to neuronal activity, and focusing on exons which are directly orthologous in the human and mouse genomes. We studied splicing in human hESC^{CORT}-neurons, with comparisons made to mouse primary cortical neurons (Mus-PRIM^{CORT} neurons) at day in vitro (DIV) 4 and DIV10, cortical-patterned neurons from mouse embryonic stem cells (Mus-ESC^{CORT}-neurons), and also mouse primary neurons from a different brain region (hippocampus, Mus-PRIM^{HIPP} neurons). Additionally we took advantage of a mouse model Tc1, which carries a copy of human chromosome 21 (Hsa21), albeit with certain regions disrupted (O'Doherty et al., 2005; Gribble et al., 2013), enabling the study of splicing of certain human genes in a mouse neuronal environment, which can point to the importance of cis-acting DNA sequences in dictating exon splicing behavior.

Results

Human-mouse comparison of basal exon inclusion in cortical neurons

We compared basal exon inclusion in human vs. mouse cortical neurons, before analyzing any activity-dependent changes. We first made a genome-wide comparison between basal exon inclusion levels between Hum-ESC^{CORT} and DIV4 Mus-PRIM^{CORT} neurons that we had previously performed RNA-seq on Qiu et al. (2016). Comparisons were restricted to "orthologous" exon inclusion/exclusion events, namely those whose upstream exon end, downstream exon start, and start and end of the alternatively spliced exon could be matched to within ten base-pairs, after translating co-ordinates between the mm10 and hg38 genome assemblies (28.9% and 27.4%, respectively, of mouse and human events detected in 1:1 orthologous genes). We used the "percent spliced in" (PSI) term to describe exon inclusion levels: 100% is a constitutively spliced exon; 0% is a constitutively skipped exon. We observed a significant correlation between Hum-ESC^{CORT} and DIV4 Mus-PRIM^{CORT} neurons when comparing the PSI of orthologous exons (Figures 1A, E). For each sample set we also classified every exon as primarily included (PI, PSI > 80), primarily skipped (PS, PS < 20) or alternatively-spliced (AS, 80 > PSI > 20). In Hum-ESC^{CORT} neurons, alternatively-spliced exons were enriched 13-fold in those exons also alternatively-spliced in DIV4 Mus-PRIM^{CORT} neurons (Figure 1G). Collectively this suggests that the basal level of orthologous exon inclusion in human and mouse cortical neuronal mRNA transcripts exhibits significant conservation, although the correlation was far from perfect. Figure 1F illustrates two orthologous exons (from *ZMYND11* and *HNRNPAB*), one of which (from *ZYMYND11*) has a similar PSI in Hum-ESC^{CORT} and DIV4 Mus-PRIM^{CORT} neurons, and one (from *HNRNPAB*) is quite different.

We wanted to gain a better indication as to whether the imperfect correlation in exon inclusion levels observed (Figure 1A) may be in part due to evolutionary divergence. Non species-specific differences could in theory be responsible, such as the two populations of neurons being at a different developmental stage, derived from different sources (embryonic stem cell line vs. primary tissue), or even simple experimental variation. We therefore performed identical analyses between DIV4 Mus-PRIM^{CORT} neurons and more mature DIV10 Mus-PRIM^{CORT} neurons as well as with mouse ES cell-derived cortical neurons (Mus-ESC^{CORT}-neurons) to determine the approximate influence of developmental stage (DIV4 vs DIV10) and tissue origin (primary vs. stem cell) on PSI. We had previously subjected these samples to RNA-seq analysis (Qiu et al., 2016). These inter-mouse comparisons showed a higher correlation between each other (Figures 1B, C, E) and higher enrichment of alternatively spliced genes than that observed in the Hum-ESC^{CORT} vs. DIV4 Mus-PRIM^{CORT} comparison (around 50-fold, Figure 1G). We also compared the PSI in our DIV4 Mus-PRIM^{CORT} neurons to that in DIV10 neurons generated and analysed by a different laboratory and from a different brain region [hippocampus, DIV10 Mus-PRIMHIPP, (Quesnel-Vallières et al., 2016)]. We observed a good correlation and strong enrichment: similar to that observed between mouse neuronal preparations made in our laboratory (Figures 1D, E, G).

Aware that our comparisons thus far involve only one human-derived dataset, we compared basal exon PSI levels in our samples to those calculated from a published transcriptome (RNA-seq) of human iPSC-derived neurons (Pruunsild et al., 2017). We found that exon PSI levels in these human iPSC-derived neurons correlated well with our Hum-ESC^{CORT} neurons that was substantially stronger than comparing to our mouse neuronal samples (Supplementary Figure 2 and Figures 1H). Thus, two independently derived human neuron samples show stronger correlation with each other than with any of the mouse samples.

A heat map summary showing the correlation coefficients of all possible pairwise comparisons of the data relating to the six aforementioned neuronal samples (four mouse, two human) illustrates that all intra-mouse and intra-human correlations are higher than all human-mouse correlations (Figure 1H). This suggests that maturation state and origin (tissue vs. stem cell) or the particular cell line chosen are unlikely to account for all of the changes in splicing observed between Hum-ESC^{CORT} and mouse neurons.

We next wanted to determine whether the inter-species differences in exon PSI were diminished when only considering genes whose expression is similar. Taking only data from genes expressed at similar levels in Hum-ESC^{CORT} and DIV 4 mouse neurons (within 20% in either direction) we observed similar correlation coefficients (Supplementary Figure 2A) as the full data set (Figure 1E), with mouse-mouse comparisons stronger than human-mouse. This suggests that divergence in exon PSI is not associated with divergence in expression level.

Although our study is focussed on exon inclusion events, which represent the majority of AS events in neurons (> 70%), we wanted proof-of-principle that other types of event follow a similar pattern of divergence/conservation, choosing "retained intron" events, which represent around 5% of AS events. Despite there being far fewer events, there were sufficient to show a correlation in "percent retained intron" between DIV4 mouse neurons and other mouse neuronal populations, and a weaker correlation between DIV4 mouse neurons and Hum-ESC^{CORT} neurons (Supplementary Figures 1A–E).

Collectively these data support a model whereby basal PSI of orthologous exons in mRNAs from human vs. mouse cortical neurons exhibit some evolutionary divergence. Interestingly, the mouse cortical neuronal exon usage pattern was found to be more similar to that in human cortical neurons than that in mouse cortical astrocytes (Figures 1E, F and Supplementary Figure 2C). That the splicing landscape in mouse neurons is more similar to human neurons than mouse astrocytes (a closely related neural cell) illustrates that there is significant conservation in cortical neuronal alternative splicing as well as the aforementioned divergence. It also underlines the usefulness in studying alternative splicing in individual cell types rather than whole tissues such as brain (Barbosa-Morais et al., 2012; Merkin et al., 2012; Verta and Jacobs, 2022).

Human-mouse comparison of activity-dependent alternative splicing

We next studied changes in exon inclusion within neurons in response to to L-type Ca^{2+} channel activation, an important mediator of activity-dependent gene regulation (Sheng et al., 1990; Bito et al., 1997; Deisseroth et al., 2003; West and Greenberg, 2011; Wheeler et al., 2012; Ma et al., 2014). To do this, hESC^{CORT}-neurons and DIV4 Mus-PRIM^{CORT} neurons were treated ± KCl-induced membrane depolarization in the presence of the L-type Ca^{2+} channel agonist FPL64176, plus NMDA receptor antagonist MK-801 (hereafter KCl) for 4 h (MK-801 is used as standard to prevent any excitotoxicity and associated gene expression (Schramm et al., 1990; Ramnath et al., 1992; Xia et al., 1996; Wahl et al., 2009; Zhou et al., 2009; Gupta et al., 2013). After 4 h RNA was harvested and RNA-seq performed, followed by analysis of KCl-induced changes in exon usage. In both DIV4 Mus-PRIM^{CORT} neurons (Figure 2A) and hESC^{CORT}-neurons (Figure 2B) splicing levels changed significantly in 800–900 orthologous exons, with KCl treatment causing an increase in exclusion of certain exons, and inclusion of others. Others have

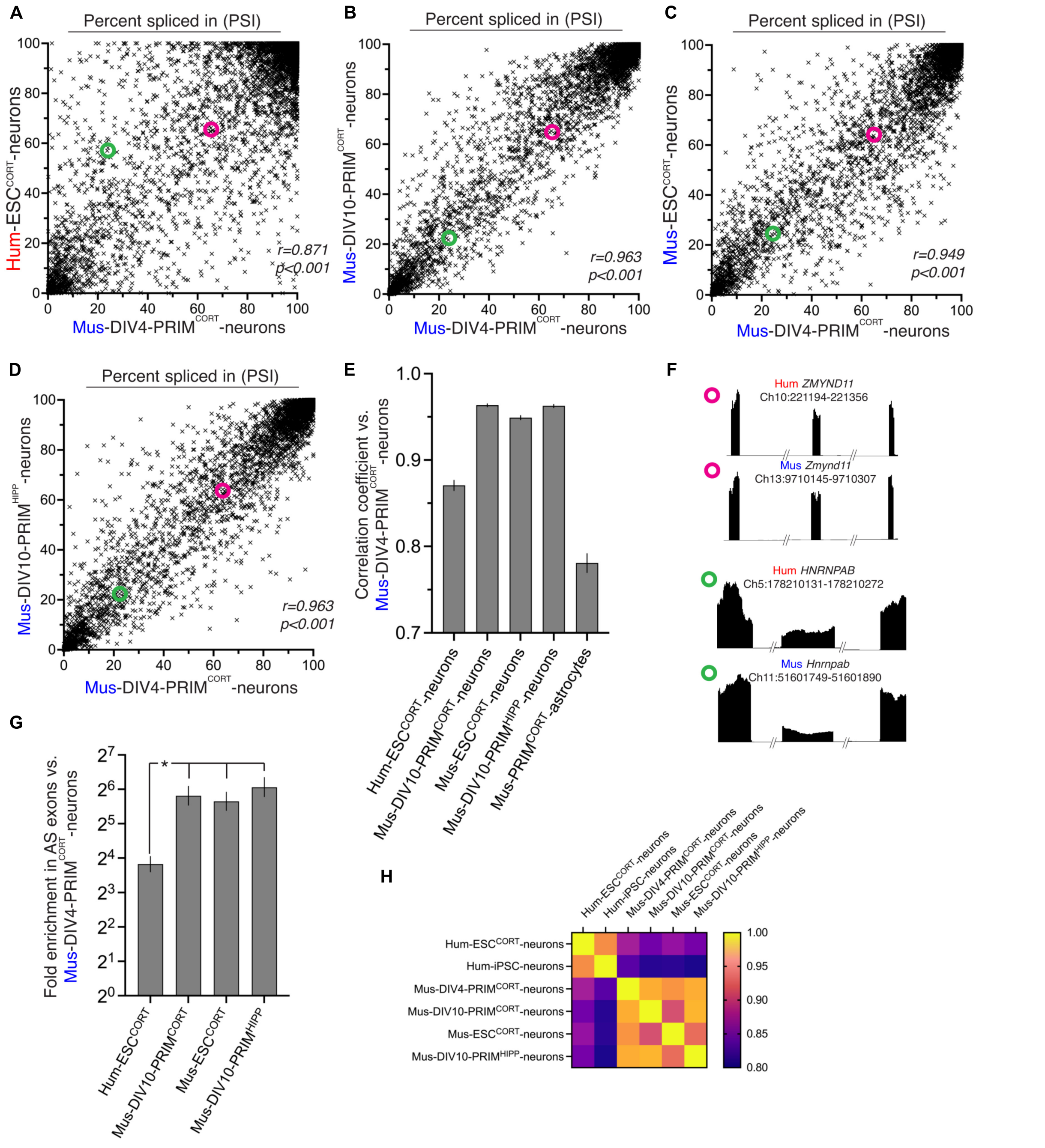


FIGURE 1

Comparison of basal exon inclusion in cortical-patterned neurons of human and mouse origin. **(A–D)** The exon inclusion ratio, otherwise known as "percent spliced in" (PSI) in DIV4 Mus-PRIM^{CORT} neurons plotted against the corresponding PSI in the indicated cell types (mean PSI, n = 3 independent biological replicates here and throughout the figure). All exons plotted have a 1:1 human-mouse ortholog, the mean of 3 independent biological replicates is shown. **(E)** Pearson r correlation coefficients for the comparisons made in **(A–D)**, and Supplementary Figure 2C. Error bars indicate the 95% confidence limits and in all cases p < 0.0001. For data relating to this figure see Source_Data.xlsx. **(F)** Examples of two 1:1 orthologous exons (coordinates relate to this exon), plus flanking exons, showing relative RNA-seq read density. One exon (from *ZMYND11*) has a similar PSI in human and mouse neurons, while one (from *HNRNPAB*) has a different PSI in human and mouse neurons. The PSI of the *ZMYND11* exon and *HNRNPAB* exon is highlighted in the scatter graphs **A–D** with magenta and green circles, respectively. **(G)** Fold enrichment of exons classed as alternatively spliced in DIV4 Mus-PRIM^{CORT} neurons in exons which are also classed as alternatively spliced in the indicated neuronal cell types (defined as 80 > mean PSI > 20, n = 3). Error bars indicate the 95% confidence limits of the enrichment factor. In all cases p < 0.0001 (Fisher's exact test). *P < 0.05 (normal approximation to difference in log odds ratios). **(H)** A heat map summary showing the correlation coefficients of all possible pairwise comparisons as indicated.

reported previously that in mouse hippocampal neurons, activity-induced exclusion/skipping of exons in response to neuronal activity is more prevalent than activity-induced increases in exon inclusion (Quesnel-Vallières et al., 2016). This is also something we observe, not only with mouse neurons but human neurons too (Figures 2A, B). Since neuronal activity also regulates genes

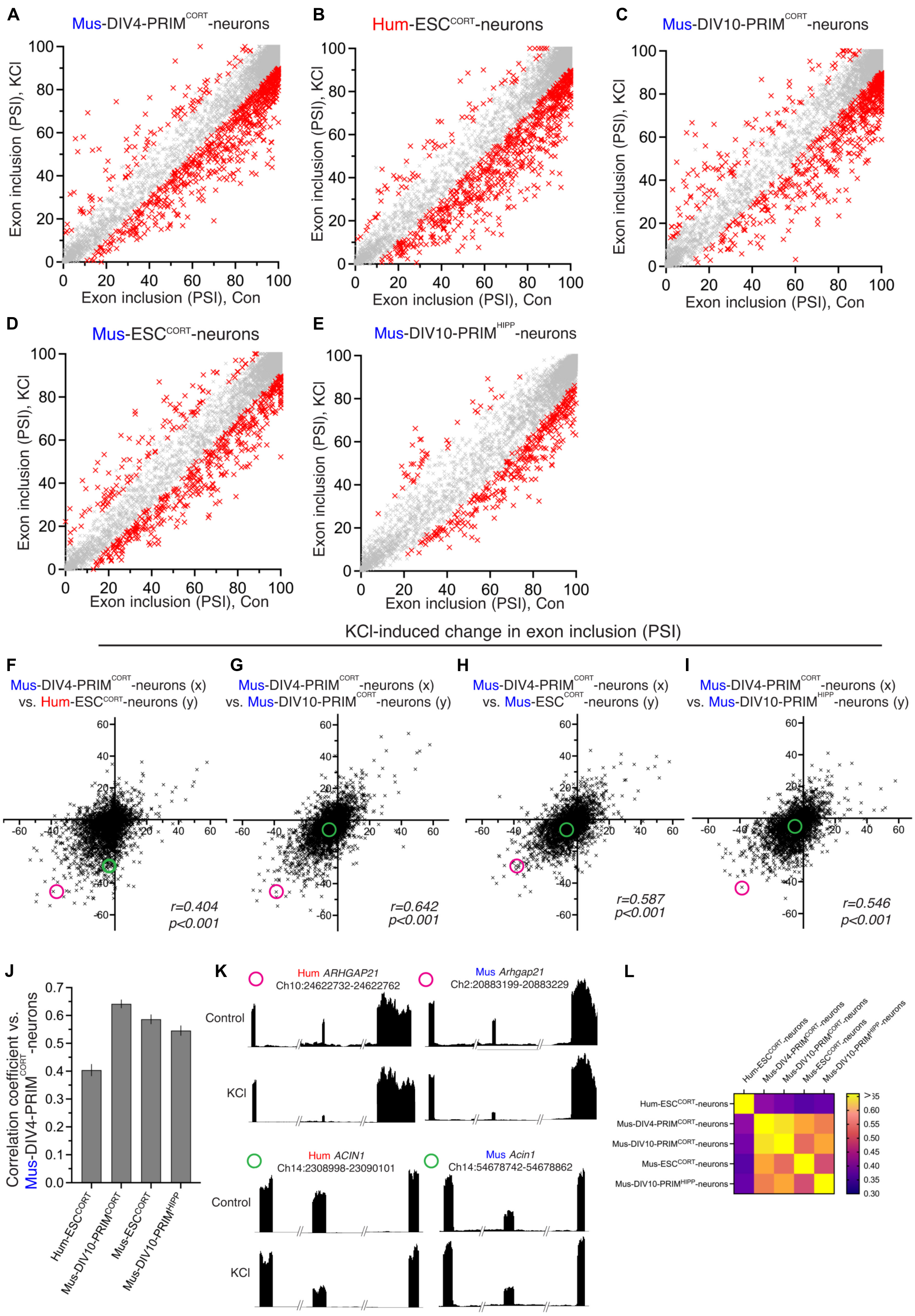


FIGURE 2
Comparison of activity-dependent alternative splicing in human and mouse cortical neurons. **(A–E)** For the indicated neuronal preparations, PSI of exons in control neurons is plotted against that in KCl-stimulated neurons [**(A–D)**: 4h; E: 3h-data were generated by another lab (Quesnel-Vallières et al., 2016)]. All exons plotted have a 1:1 human-mouse ortholog. Red crosses indicate a significant difference in PSI ($p < 0.05$, read count for exon inclusion or exclusion in all samples > 5, PSI difference > 10, $n = 3$). **(F)** The KCl-induced change in PSI in DIV4 Mus-PRIM^{CORT} neurons is plotted against the corresponding change in Hum-ESC^{CORT} neurons. **(G–I)** The KCl-induced change in PSI in exons in DIV4 Mus-PRIM^{CORT} neurons is plotted against the corresponding change in the indicated cell types. **(J)** Correlation coefficients for the comparisons made in **(F–I)**. Error bars indicate the 95% confidence limits and in all cases. $p < 0.0001$ For data points relating to this figure see Source_Data.xlsx. **(K)** Examples of two 1:1 orthologous exons (coordinates relate to this exon), plus flanking exons, showing relative RNA-seq read density. One exon (from *ARHGAP21*) has a similar KCl-induced PSI change in human and mouse neurons, while one exon (from *ACIN1*) is only subject to activity-dependent alternative splicing in human neurons. The KCl-induced PSI change of the *ARHGAP21* exon and *ACIN1* exon is highlighted in (F,G,H and I) with a magenta and a green circle, respectively. **(L)** A heat map summary showing the correlation coefficients of all possible pairwise comparisons as indicated.

at the transcriptional level, we plotted changes in exon PSI against the Log2 fold change at the gene mRNA level. We found no correlation (Supplementary Figure 3) which indicates that changes in splicing are not coupled to changes in gene expression, (which is expected as they are mediated by distinct cis-acting sequences).

Similar alternative splicing analyses were then performed for DIV10 Mus-PRIM^{CORT} neurons ± KCl (Figure 2C), Mus-ESC^{CORT}-neurons ± KCl (Figure 2D) and on RNA-seq data obtained by another laboratory: a 3h KCl stimulation of DIV10 Mus-PRIM^{HIPP} neurons (Quesnel-Vallières et al., 2016; Figure 2E). Plotting KCl-induced changes in PSI in hESC^{CORT} vs. DIV4 Mus-PRIM^{CORT} neurons revealed a correlation, albeit quite weak (Figure 2F). Figure 2K shows examples of RNA-seq read density in two 1:1 orthologous exons for both species ± KCl treatment. One exon (from *ARHGAP21*) has a similar KCl-induced PSI change in human and mouse neurons, while one exon (from *ACIN1*) is only subject to activity-dependent alternative splicing in human neurons. Globally, the correlation between KCl-induced changes in PSI of exons in DIV4 Mus-PRIM^{CORT} neurons vs. DIV10 Mus-PRIM^{CORT} neurons and vs. mESC^{CORT}-neurons are significantly higher (Figures 2G, H, J). We also compared KCl-induced changes in PSI in DIV10 Mus-PRIM^{CORT} neurons with activity-dependent changes in PSI calculated from similar RNA-seq data obtained by another laboratory: a 3h KCl stimulation of DIV10 mouse hippocampal neurons (Quesnel-Vallières et al., 2016), also revealing a correlation similar to our comparisons of DIV10 Mus-PRIM^{CORT} neurons vs. DIV4 Mus-PRIM^{CORT} neurons and mESC^{CORT}-neurons (Figures 2I, J). A heat map showing the correlation coefficients of all possible pairwise comparisons of the data relating to the five neuronal samples (four mouse, one human, Figure 2L) illustrates that all inter-mouse correlations are higher than all human-mouse correlations (Figure 1H), indicative of species-specific differences in activity-dependent splicing in human vs. mouse cortical neurons.

One factor to consider when assessing differences in KCl-induced changes in PSI in different neuronal preparations is that the magnitude of change may be influenced by differences in basal PSI, which we know shows human-mouse differences (Figure 1). For example, a mouse exon with a basal PSI of 40 that increases upon KCl stimulation has a theoretical maximal PSI change of 60, whereas if the orthologous human exon has a basal PSI of 70 then the maximal PSI change possible is only 30. We therefore performed additional comparisons of KCl-induced inclusion level differences calculated as a % of the maximum possible inclusion level difference. We restricted our analysis to orthologous exons where basal PSI was > 20 and < 80 to eliminate excessive skewing of the data caused by modest absolute changes in PSI giving very high percentage figures (e.g., a PSI changing from 95 to 98 would give a figure of 60%). Our comparisons (Figures 3A–F) mirrored those made in Figures 2F–J and yielded similar results: KCl-induced changes in exon inclusion in hESC^{CORT} vs. mouse neurons showed significant correlation, but it was weaker than when comparing the different mouse neuronal preparations with each other. These observations are consistent with their being evolutionary divergence in activity-dependent alternative splicing of orthologous exons between mice and humans.

Ontology of human-specific vs. conserved activity-dependent alternatively spliced genes

There are several examples of activity-dependent changes in exon inclusion influencing the function of the protein encoded by the alternatively spliced transcript (Furlanis and Scheiffele, 2018). This can require that an exon encodes a functionally autonomous part of a protein so it can be included or excluded to alter a protein's function without causing non-specific loss of function (e.g. through protein misfolding or removing part of a key structural domain). We reasoned that the organization of exons in a gene is more likely to be conserved in those exons subject to signal-dependent regulation. Indeed, taking exons that are subject to activity-dependent regulation in mouse neurons (DIV4 and DIV10 Mus-PRIM^{CORT}, mESC^{CORT}-neurons) we observed that they are enriched 2.7-fold in 1:1 human-mouse orthologs ($p < 0.0001$ (Fisher's exact test)). The fact that exons subject to activity-dependent regulation in mouse neurons are more likely to have a direct human ortholog points to evolutionary pressure to maintain genetic structure where the exon is subject to alternative splicing.

Taking genes containing orthologous exons subject to activity-dependent changes in PSI in both human and mouse neurons, ontological analysis revealed three main functional areas (Figures 4A–C). Prominent processes and functions are associated with cytoskeletal organization and transport along cytoskeletal tracks (Figure 4A). The second major area is in the control of gene expression including transcriptional control, epigenetic regulation, and post-transcriptional mRNA processing, including polyadenylation and RNA splicing itself (Figure 4B). A third prominent area of conserved activity-responsive AS functions involve synaptic signaling and action potential firing (Figure 4C), and associated subcellular components such as synaptic vesicles, the pre- and post-synaptic membrane, and specialist structures including the post-synaptic density, AMPA receptor complex and the axon initial segment. These processes have been highlighted recently as subject to regulation by activity-dependent AS, particularly in the context of homeostatic plasticity in mice (Iijima and Yoshimura, 2019; Thalhammer et al., 2020) and our data suggests that activity-dependent AS may play a similar role in human neurons.

We were also interested in identifying putative regulators of this conserved group of activity-dependent exons. Dynamic changes in exon usage in developing neurons are controlled by several RNA binding factors, prominent among them being the RBFOX proteins (1–3), SAM68, NOVA, and PTBP (Eom et al., 2013; Saito et al., 2016, 2019; Furlanis and Scheiffele, 2018; Jacko et al., 2018; Wamsley et al., 2018; Farini et al., 2020; Ibrahim et al., 2023), with SAM68 and NOVA particularly implicated in activity-dependent exon usage (Iijima et al., 2011; Eom et al., 2013; Farini et al., 2020; Ibrahim et al., 2023).

Consistent with conserved importance of exons regulated by these factors, their targets are enriched in orthologous exons compared to non-orthologous ones (Figure 4D). Moreover, even after allowing for this enrichment, orthologous exons exhibiting activity-dependent changes in PSI in both human and mouse neurons are further enriched in targets of these splicing factors

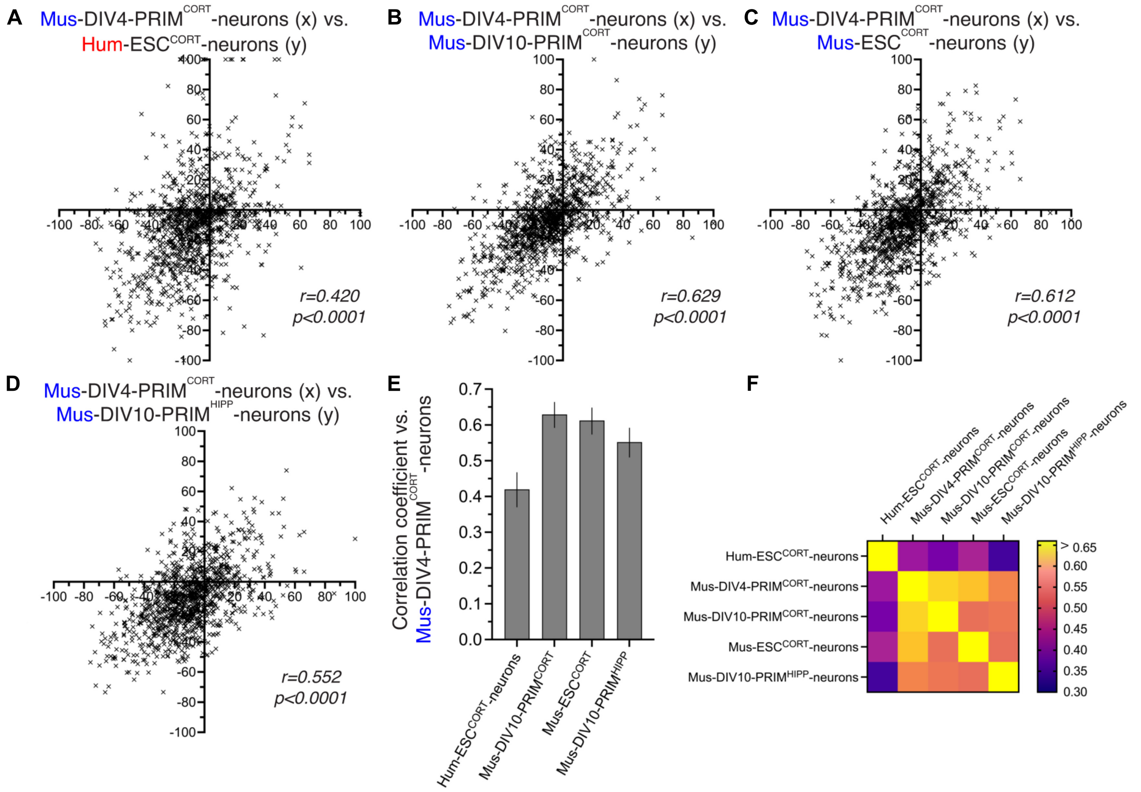


FIGURE 3
Comparison of activity-dependent alternative splicing as a percentage of possible change. **(A–D)** For exons classed as alternatively spliced (80 > PSI > 20) in DIV4 Mus-PRIM^{CORT} neurons, the effect of KCl stimulation on PSI was calculated as a percentage of the maximum possible PSI change and plotted (x-axis) against the corresponding value for the other cell types (y-axis) as indicated. **(E)** Correlation coefficients for the comparisons made in **(A–D)**. Error bars indicate the 95% confidence limits and in all cases $p < 0.0001$. For data points relating to this figure see Source_Data.xlsx. **(F)** A heat map summary showing the correlation coefficients of all possible pairwise comparisons as indicated.

(Figure 4E). Thus, exons regulated by RBFOX, SAM68, NOVA, and PTBP are both conserved in terms of gene structure and additionally form a significant element of conserved exons subject to activity-dependent exon inclusion in human and mouse neurons.

We also wanted to determine whether genes subject to "human-specific" activity-dependent exon inclusion were enriched in any specific biological processes. We studied genes that have a 1:1 human-mouse orthologue which are subject to human neuron-specific activity-dependent alternative splicing. "Human-specific" regulation was defined as genes that in Hum-ESC^{CORT} neurons had 1 or more exons whose PSI in the mature transcript changed > 10% (up or down, $p < 0.05$) upon KCl treatment, but whose mouse ortholog did not meet these criteria in any of the mouse neuronal preparations used in this study. 1104 genes were found to be subject to "human-specific" activity-dependent alternative splicing (by the criteria above) and were subject to ontological analysis. Biological processes and molecular function terms enriched in genes subject to human-specific activity-dependent alternative splicing are dominated by lipid biology (Figures 4F, G). This includes gene sets involved in lipid/phospholipid biosynthesis, regulation of lipid biosynthesis (e.g. signaling to the sterol regulatory element) and lipid interaction, as well as lipid transport and processes associated with lipid bilayer dynamics such as synaptic vesicle endocytosis and membrane ruffle assembly. The prominence of a wide range of gene sets relating to lipid biology (Figures 4F, G) is also clear when reviewing all pathways that are significantly enriched (see Source_Data.xlsx). The functional consequences of activity-dependent AS of so many lipid pathway genes will require further investigation.

Cis vs. trans determinants of basal and activity-dependent alternative splicing

Our human-mouse and mouse-mouse comparisons suggest that both basal exon inclusion levels and activity-dependent changes in exon inclusion show a degree of evolutionary divergence. We wanted to get an indication as to whether putative species-specific differences are due to changes in cis-acting factors (i.e. DNA sequence) or trans-acting factors, such as splicing factors or signal transduction machinery. RNA-seq data from cortical neurons cultured from the Tc1 transchromosomic mouse strain (O'Doherty et al., 2005) was analysed, since these neurons contain an extrachromosomal copy of human chromosome 21 (O'Doherty et al., 2005), albeit an incomplete copy (Gribble et al., 2013), enabling us to assess the PSI of human chromosome 21 exons in parallel with their mouse ortholog in the same cellular environment. We used our *in silico* species-specific RNA-seq read analysis workflow (Qiu et al., 2018) to distinguish between human

A

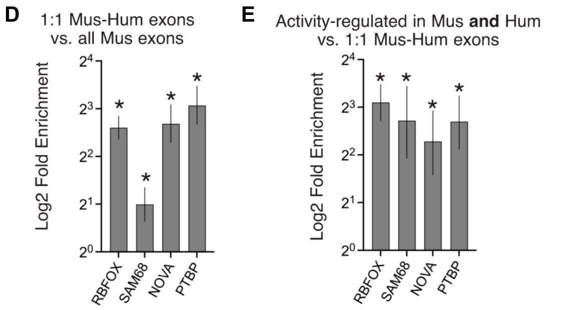
CYTOSKELETAL ORGANISATION & CYTOSKELETON-MEDIATED TRANSPORT				Odds	
GO reference	GO Term	Annotated	Significant	Ratio	P_adj
GO:0030050	vesicle transport along actin filament (BP)	10	5	5.15	1.44E-03
GO:0090042	tubulin deacetylation (BP)	10	5	5.15	2.65E-02
GO:0099519	dense core granule cytoskeletal transport (BP)	11	5	4.67	2.65E-02
GO:0034453	microtubule anchoring (BP)	20	8	4.10	2.40E-03
GO:1903861	positive regulation of dendrite extension (BP)	14	5	3.68	8.22E-03
GO:0031116	positive regulation of microtubule polymerization (MF)	20	7	3.59	2.02E-03
GO:0051010	microtubule plus-end binding (MF)	18	7	4.00	9.60E-04
GO:0030507	spectrin binding (MF)	19	6	3.26	7.35E-03
GO:0005200	structural constituent of cytoskeleton (MF)	48	9	1.93	3.88E-02
GO:0001725	stress fiber (CC)	34	12	3.68	4.20E-05
GO:0035371	microtubule plus-end (CC)	20	5	2.60	3.69E-02
GO:0005881	cytoplasmic microtubule (CC)	45	10	2.31	3.80E-02

B

TRANSCRIPTIONAL CONTROL, mRNA PROCESSING & SPLICING				Odds	
GO reference	Term	Annotated	Significant	Ratio	P_adj
GO:0043981	histone H4-K5 acetylation (BP)	15	5	3.42	1.14E-02
GO:0006342	chromatin silencing (BP)	30	10	3.42	3.97E-02
GO:0006378	mRNA polyadenylation (BP)	37	9	2.49	6.36E-03
GO:0031124	mRNA 3'-end processing (BP)	71	16	2.31	2.18E-02
GO:0000381	regulation of alternative mRNA splicing, via spliceosome (BP)	58	11	1.95	2.26E-02
GO:0035925	mRNA 3'-UTR AU-rich region binding (MF)	14	5	3.68	8.07E-03
GO:0033613	activating transcription factor binding (MF)	37	9	2.51	2.77E-03
GO:0001103	RNA polymerase II repressing transcription factor binding (MF)	27	6	2.29	4.12E-02
GO:0003729	mRNA binding (MF)	205	34	1.71	1.12E-02
GO:0016607	nuclear speckles	269	42	1.63	9.90E-04

C

ELECTROPHYSIOLOGY & SYNAPTIC FUNCTION				Odds	
GO reference	Term	Annotated	Significant	Ratio	P_adj
GO:0086010	membrane depolarization during action potential (BP)	19	8	4.32	2.43E-03
GO:0048791	calcium ion-regulated exocytosis of neurotransmitter (BF	15	5	3.42	2.38E-02
GO:0019228	neuronal action potential (BP)	16	5	3.21	1.52E-02
GO:0035235	ionotropic glutamate receptor signaling pathway (BP)	19	5	2.70	3.17E-02
GO:0005248	voltage-gated sodium channel activity (MF)	14	5	3.68	8.07E-03
GO:0005244	voltage-gated ion channel activity (MF)	70	16	2.36	7.22E-03
GO:0043194	axon initial segment (CC)	14	6	4.48	1.17E-03
GO:0001518	voltage-gated sodium channel complex (CC)	8	5	6.49	3.50E-04
GO:0032281	AMPA glutamate receptor complex (CC)	19	5	2.75	3.00E-02
GO:0042734	presynaptic membrane (CC)	107	22	2.14	2.32E-03
GO:0008021	synaptic vesicle (CC)	137	19	1.44	3.29E-02
GO:0045211	postsynaptic membrane (CC)	168	22	1.36	3.29E-02
GO:0014069	postsynaptic density (CC)	254	33	1.35	2.08E-02



F

LIPID BIOSYNTHESIS, METABOLISM, TRANSPORT, AND INTERACTION				Odds	
GO reference	Term	Annotated	Significant	Ratio	P_adj
GO:1900029	positive regulation of ruffle assembly (BP)	10	6	4.41	8.00E-04
GO:0006646	phosphatidylethanolamine biosynthetic process (BP	10	5	3.68	6.40E-03
GO:0032933	SREBP signaling pathway (BP)	13	6	3.39	8.40E-03
GO:0002092	positive regulation of receptor internalization (BP)	14	5	2.63	3.20E-02
GO:0048488	synaptic vesicle endocytosis (BP)	40	11	2.02	3.48E-02
GO:0015918	sterol transport (BP)	50	10	1.47	3.61E-02
GO:0006687	glycosphingolipid metabolic process (BP)	40	8	1.47	1.05E-02
GO:0031957	very long-chain fatty acid-CoA ligase activity (MF)	6	5	6.10	2.60E-04
GO:0015485	cholesterol binding (MF)	28	8	2.08	3.02E-02
GO:0035091	phosphatidylinositol binding (MF)	163	34	1.52	8.25E-03
GO:0005543	phospholipid binding (MF)	254	51	1.46	1.63E-02
GO:0044232	organelle membrane contact site (CC)	21	9	3.14	1.52E-02
GO:0044233	mitochondria-associated ER membrane (CC)	13	5	2.81	2.35E-02
GO:0009898	cytoplasmic side of plasma membrane (CC)	83	17	1.50	2.94E-02

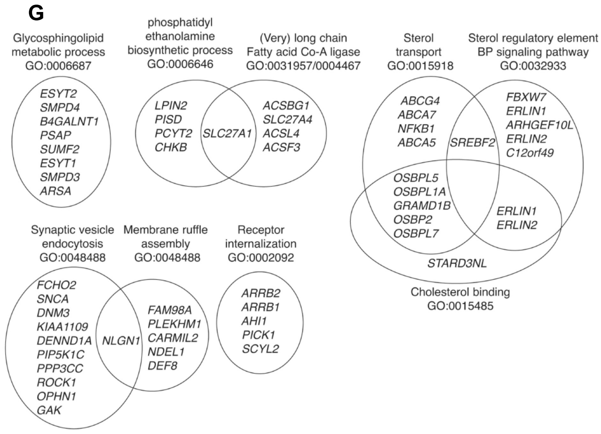


FIGURE 4
Ontology of genes subject to human-mouse conserved and "human-specific" activity-dependent exon usage. **(A–C)** Selected GO terms are shown which are enriched (Fisher's weighted $p < 0.05$) in genes that have one or more 1:1 human-mouse orthologous exon which are subject to activity-dependent inclusion/exclusion in both Hum-ESC^{CORT} neurons and in one or more of our mouse cortical neuronal preparations (DIV4 and DIV10 Mus-PRIM^{CORT}, mESC^{CORT}-neurons). 782 genes contain exons that qualify as being regulated in a conserved manner by the above criteria, out of a background of 8039 genes (defined as 1:1 orthologous genes possessing $\geq$ 1 orthologous exons expressed in human and mouse neurons). The nature of the GO term is shown (BP, Biological Process; MF, Molecular Function; CC, Cellular Component). **(D,E)** Enrichment tests were performed for RBFOX and SAM68 target cassette exon splicing events (Jacko et al., 2018; Farini et al., 2020) (see Methods, *$p < 0.0001$ (Fisher's exact test)). For **(D)** the presence of these target exons was compared between the set of all exons expressed in mouse neurons, and the set of exons expressed in mouse neurons for which there is a 1:1 human ortholog. For **(E)** the presence of these target exons was compared between the set of 1:1 orthologous exons subject to activity-dependent splicing in both human and mouse neurons [as per **(A–C)**], and the whole set of expressed 1:1 orthologous exons. **(F,G)** Selected GO terms are shown which are enriched (Fisher's weighted $p < 0.05$) in genes that have a 1:1 human-mouse orthologue which are subject to activity-dependent alternative splicing in human neurons but not mouse neurons. In **(G)** the genes within selected GO terms that account for the enrichment are shown, and any genes in more than one GO term indicated by the overlapping nature of the Venn diagram. Note that while selected pathways are shown in this figure, all significantly enriched pathways are shown in Source_Data.xlsx.

and mouse genome-derived RNA-seq reads within the same RNA sample. We first considered the basal PSI of the human exons in Tc1 mouse neurons and compared them to the PSI of those same exons in their normal human cellular environment (hESC^{CORT}-neurons). Of the 45 hCh21 exons which both had a 1:1 mouse orthologue and passed an expression level threshold, there was a significant correlation comparing PSI of human exons in Tc1 cortical neurons vs. the human cellular environment of Hum-ESC^{CORT} neurons (Figure 5A). As expected, the corresponding mouse exons showed near-identical PSI in Tc1 cortical neurons vs. DIV10 Mus-PRIM^{CORT} neurons (Figure 5B). We also wanted to determine whether human-mouse differences in PSI of orthologous exons observed when comparing the transcriptomes of human and mouse neurons were also observed when those exons were studied in the same cellular environment (mouse Tc1 neurons). We observed a correlation between human-mouse differences in PSI when studying them in their own cellular environment vs. a common Tc1 cellular environment (Figure 5C). This supports a model whereby basal PSI is dictated by cis-acting DNA elements and that human-mouse differences may be driven at least in part by divergence in cis-acting DNA elements.

We next investigated activity-dependent changes in splicing in human Ch21 exons within the mouse Tc1 neurons, and this was compared to changes of the same exons in Hum-ESC^{CORT}

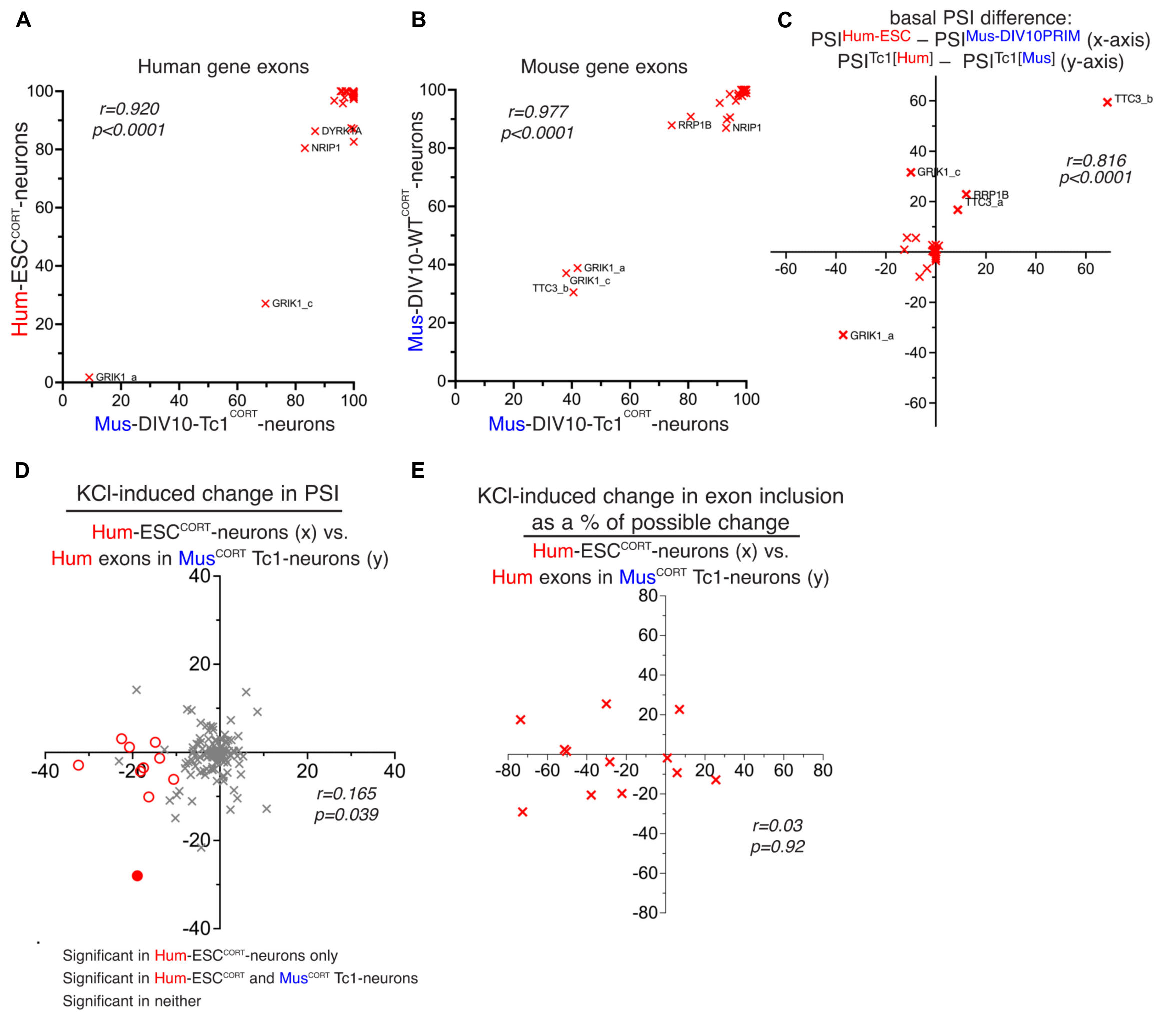


FIGURE 5

Study of human and mouse gene basal and activity-dependent alternative splicing in mouse Tc1 neurons. **(A)** PSI of hCh21 exons (with a 1:1 mouse/human ortholog) in Hum-ESC^{CORT} neurons vs. the PSI of the same human exon in mouse Tc1 neurons. **(B)** PSI of the mouse orthologs of the exons from Figure 5A in mouse Tc1 neurons (x-axis) vs. PSI of the same exons in DIV10 Mus-PRIM^{CORT} neurons. **(C)** A comparison of the difference in basal PSI in orthologous exons within mouse Tc1 neurons compared to the difference in the same exons between mouse (DIV10 Mus-PRIM^{CORT}) and human (hESC^{CORT}) neurons. Correlation coefficient r is shown. **(D)** A comparison of KCl-induced PSI changes in human Ch21 exons in Hum-ESC^{CORT} neurons vs. mouse Tc1 neurons. **(E)** For alternatively spliced exons (80 > PSI > 20) the effect of KCl stimulation on PSI as a percentage of the maximum possible PSI change was compared between Hum-ESC^{CORT} neurons vs. mouse Tc1 neurons. For data points relating to this figure see Source_Data.xlsx.

neurons. This revealed a very poor correlation (Figure 5D). There were 10 hCh21 exons (spanning 9 different genes) that undergo activity-dependent changes in inclusion in Hum-ESC^{CORT} neurons (PSI change $\geq$ 10 in any direction, $p < 0.05$) and whose regulation could be studied in mouse Tc1 neurons. Only 1 out of those 10 human exons were controlled by KCl treatment of Tc1 neurons (Figure 5D). We also performed additional comparisons of activity-induced splicing changes calculated as a percentage of the maximum possible change, restricting our analysis to orthologous exons where basal PSI was > 20 and < 80 as before (a similar approach to that taken in Figure 3). This also revealed no correlation between activity-dependent changes in human exon inclusion in a human (Hum-ESC^{CORT}) vs. mouse (Tc1) cellular environment (Figure 5E).

Thus, unlike basal inclusion levels of human exons which in mouse Tc1 neurons appeared to correlate quite well with that observed in Hum-ESC^{CORT} neurons, activity-dependent changes in human gene exon inclusion were not recapitulated in mouse Tc1 neurons. This suggests that cis-acting DNA elements may not be sufficient to direct activity-dependent alternative splicing and "trans-acting factors" such as a human neuron's activity-responsive signaling or splicing machinery may be required. However, the relatively small number of activity-responsive exons studied means that we cannot rule out that certain activity-responsive human exons can also be similarly regulated in a mouse cellular environment. Of the activity-responsive human exons that could be analyzed in Tc1 neurons none were putative targets of RBFOX, SAM68, NOVA or PTBP. It is possible that conserved exon targets of these factors can be controlled by mouse splicing factors directed by cis-acting binding sites for these factors, although whether these splicing factors have cross-species activity is not clear.

Concluding remarks

To conclude, our study indicates that there is significant conservation of both basal and activity-dependent exon usage between cortical-patterned human and mouse neurons. The classes of genes previously identified as being subject to activity-dependent alternative splicing in mouse neurons: synaptic, electrophysiology, cytoskeletal (Furlanis and Scheiffele, 2018; Jacko et al., 2018; Iijima and Yoshimura, 2019; Farini et al., 2020), also feature strongly in genes whose splicing is similarly regulated in human neurons. These genes whose activity-dependent splicing is conserved are also enriched in regulatory targets of splicing factors such as RBFOX and SAM68. However our study also supports the notion that there are both quantitative and qualitative differences in orthologous exon usage in human neurons, compared to their mouse ortholog. Moreover, differences in both basal exon usage and the activity-dependency of exon usage are apparent, although cis-acting sequences may be sufficient to drive the former, but not the latter. It is conceivable that the functional impact of neuronal activity on human forebrain neurons is different to those from mice, and that these differences may arise from alternative exon usage and not just differential regulation at the transcriptional level (Hardingham et al., 2018; Qiu et al., 2018). The prominence of lipid biology in genes with exons subject to human-specific control by neuronal activity is intriguing and provides a basis for further functional investigation.

Materials and methods

Splicing analysis

We used RNA-seq data from the following accessions: E-MTAB-5489 (Qiu et al., 2016), E-MTAB-5514 (Hasel et al., 2017), GSE89984 (Quesnel-Vallières et al., 2016) and GSE88773 (Pruunsild et al., 2017). Samples containing RNA-seq reads from only a single species were mapped to their respective genome using the STAR version 2.7.0f (Dobin et al., 2013); reads were mapped to the primary assemblies of the human (hg38) or mouse (mm10) reference genomes contained in Ensembl release 99 (Cunningham et al., 2022). Samples containing RNA-seq reads derived from both the human and mouse genomes (or single-species samples which were to be compared with these) were processed with Sargasso version 2.1 (Qiu et al., 2018) to disambiguate reads between the two species, using a conservative filtering strategy, to prioritize minimizing the number of read mis-assigned to the wrong species. In order to measure levels of exon inclusion, and differences in exon inclusion between experimental conditions, data were then processed with the differential splicing tool rMATS, version 4.1.0 (Shen et al., 2014), focusing on the "skipped exon" category of splicing events. Significance for differential inclusion events was generally defined as $p < 0.05$, read count for exon inclusion or exclusion in all samples > 5, inclusion level difference > 10 PSI (percent spliced in).

To match orthologous skipped exon events, the co-ordinates of mouse events were transformed from mm10 to hg38 co-ordinates using the command-line version of the UCSC liftOver tool.[1] Mouse and human exon inclusion/exclusion events were then considered to be orthologous if the human co-ordinates and lifted mouse co-ordinates of the upstream exon end, downstream exon start, and the start and end of the alternatively spliced exon could be matched to within ten base-pairs.

Enrichment analysis

Enrichment analyses were performed as follows. For gene ontology enrichments, a background gene set was constructed consisting of all human genes for which an event with average read count for exon inclusion or exclusion over all samples greater than 5 was tested for differential splicing, which had a 1:1 orthologous mouse gene, and for which the mouse gene had an event with average read count > 5 tested in at least one of the DIV4, DIV10 or mESC KCl vs basal comparisons. Then gene ontology enrichment was tested in (i) those human genes which had a significant differential splicing event (according to the definition above), for which the 1:1 orthologous mouse gene had a significant differential splicing event in at least one of the DIV4, DIV10 or mESC KCl vs basal comparisons; (ii) those human genes which had a significant differential splicing event for which the 1:1 orthologous mouse gene did not have a significant differential splicing event in any of the DIV4, DIV10 or mESC KCl vs basal comparisons. Gene ontology enrichment analyses were performed using topGO, R package version 2.42.0 (Alexa et al., 2006).

At the level of splicing events themselves, enrichments for targets of particular splicing factors were tested using Fisher's exact test. For each splicing factor three enrichment tests were performed: (i) in the background of all mouse events with average read count for exon inclusion or exclusion over all samples greater than 5 which were tested for differential splicing in at least one of the DIV4, DIV10 or mESC KCl vs. basal comparisons, enrichment for those events with an orthologous human event with average read count greater than 5 which was tested for differential splicing; (ii) in the background corresponding to the enrichment set in (i), enrichment for those events which were significant in the human KCl vs basal comparison, and also in at least one of the mouse DIV4, DIV10 or mESC KCl vs basal comparisons. Enrichment tests were performed for (i) Rbfox target cassette exon splicing events determined by RNA-seq profiling after 10 days of maturation in Rbfox triple KO vs. WT neurons as reported (Jacko et al., 2018); (ii) the union of Sam68 cassette exon splicing events determined by RNA-seq from Sam68 KO vs. WT neurons at P1 and P10 (Farini et al., 2020), and (iii) direct target exons of NOVA and PTBP determined using an integrative modeling approach as reported (Weyn-Vanhentenryck et al., 2018).

Statistical analysis

For comparisons between basal exon inclusion, or activity-dependent exon inclusion level difference, Pearson correlation

1 https://genome.ucsc.edu/cgi-bin/hgLiftOver

coefficients were calculated. Enrichment for targets of particular splicing factors were tested using Fisher's exact test. Gene ontology enrichment analyses were performed using topGO's default *weight01* algorithm, which integrates GO graph topology to supplement the Fisher's exact test used for individual GO terms. Significance of changes in exon inclusion in response to activity were calculated by rMATS, which uses a generalized linear modeling approach to incorporate estimates of per-sample uncertainty, and inter-replicate variability, in PSI into estimates of differential alternative splicing.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/ena, E-MTAB-5489, https://www.ebi.ac.uk/ena, E-MTAB-5514.

Ethics statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used. The animal study was approved by University of Edinburgh Ethical Review Board. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

OD: Formal analysis, Writing–original draft, Writing–review and editing. JM: Investigation, Writing–review and editing. KB: Investigation, Writing–review and editing. PK: Supervision, Writing–review and editing. SC: Supervision, Writing–review and editing. GH: Conceptualization, Supervision, Writing–original draft, Writing–review and editing. JQ: Conceptualization, Formal analysis, Investigation, Writing–original draft, Writing–review and editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of the article. This work was funded by the UK Dementia Research Institute (GEH, SC) which receives its funding from DRI Ltd., funded by the UK Medical Research Council, Alzheimer's Society, and Alzheimer's Research UK.

Acknowledgments

We acknowledge the work of all the authors of the studies who generated the primary data analyzed in this study (Qiu et al., 2016; Quesnel-Vallières et al., 2016; Hasel et al., 2017; Pruunsild et al., 2017; Jacko et al., 2018; Weyn-Vanhentenryck et al., 2018; Farini et al., 2020).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2024.1392408/full#supplementary-material

References

Alexa, A., Rahnenführer, J., and Lengauer, T. (2006). Improved scoring of functional groups from gene expression data by decorrelating GO graph structure. *Bioinformatics* 22, 1700–1707. doi: 10.1093/bioinformatics/btl140

Al-Mubarak, B., Soriano, F. X., and Hardingham, G. E. (2009). Synaptic NMDAR activity suppresses FOXO1 expression via a cis-acting FOXO binding site: FOXO1 is a FOXO target gene. *Channels* 3, 233–238.

Ataman, B., Boulting, G., Harmin, D., Yang, M., Baker-Salisbury, M., Yap, E., et al. (2016). Evolution of Osteocrin as an activity-regulated factor in the primate brain. *Nature* 539, 242–247. doi: 10.1038/nature20111

Barbosa-Morais, N., Irimia, M., Pan, Q., Xiong, H., Gueroussov, S., Lee, L., et al. (2012). The evolutionary landscape of alternative splicing in vertebrate species. *Science* 338, 1587–1593. doi: 10.1126/science.1230612

Bell, K. F., and Hardingham, G. E. (2011). The influence of synaptic activity on neuronal health. *Curr. Opin. Neurobiol.* 21, 299–305. doi: 10.1016/j.conb.2011.01.002

Bito, H., Deisseroth, K., and Tsien, R. W. (1997). Ca2+-dependent regulation in neuronal gene expression. *Curr. Opin. Neurobiol.* 7, 419–429.

Cruzalegui, F. H., Hardingham, G. E., and Bading, H. (1999). c-Jun functions as a calcium-regulated transcriptional activator in the absence of JNK/SAPK1 activation. *EMBO J.* 18, 1335–1344.

Cunningham, F., Allen, J., Allen, J., Alvarez-Jarreta, J., Amode, M., Armean, I., et al. (2022). Ensembl 2022. *Nucleic Acids Res.* 50, D988–D995. doi: 10.1093/nar/gkab1049

Deisseroth, K., Mermelstein, P. G., Xia, H., and Tsien, R. W. (2003). Signaling from synapse to nucleus: The logic behind the mechanisms. *Curr. Opin. Neurobiol.* 13, 354–365.

Dobin, A., Davis, C., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., et al. (2013). STAR: Ultrafast universal RNA-seq aligner. *Bioinformatics* 29, 15–21. doi: 10.1093/bioinformatics/bts635

Eom, T., Zhang, C., Wang, H., Lay, K., Fak, J., Noebels, J., et al. (2013). NOVA-dependent regulation of cryptic NMD exons controls synaptic protein levels after seizure. *Elife* 2:e00178. doi: 10.7554/eLife.00178

Farini, D., Cesari, E., Weatheritt, R., La Sala, G., Naro, C., Pagliarini, V., et al. (2020). A dynamic splicing program ensures proper synaptic connections in the developing cerebellum. *Cell Rep.* 31:107703. doi: 10.1016/j.celrep.2020.107703

Furlanis, E., and Scheiffele, P. (2018). Regulation of neuronal differentiation, function, and plasticity by alternative splicing. *Annu. Rev. Cell Dev. Biol.* 34, 451–469. doi: 10.1146/annurev-cellbio-100617-062826

Gribble, S., Wiseman, F., Clayton, S., Prigmore, E., Langley, E., Yang, F., et al. (2013). Massively parallel sequencing reveals the complex structure of an irradiated human chromosome on a mouse background in the Tc1 model of Down syndrome. *PLoS One* 8:e60482. doi: 10.1371/journal.pone.0060482

Gupta, K., Hardingham, G. E., and Chandran, S. (2013). NMDA receptor-dependent glutamate excitotoxicity in human embryonic stem cell-derived neurons. *Neurosci. Lett.* 543, 95–100. doi: 10.1016/j.neulet.2013.03.010

Hardingham, G. E., and Bading, H. (1998). Nuclear calcium: A key regulator of gene expression. *Biometals* 11, 345–358.

Hardingham, G. E., and Lipton, S. A. (2011). Regulation of neuronal oxidative and nitrosative stress by endogenous protective pathways and disease processes. *Antioxid. Redox Signal.* 14, 1421–1424. doi: 10.1089/ars.2010.3573

Hardingham, G. E., Chawla, S., Johnson, C. M., and Bading, H. (1997). Distinct functions of nuclear and cytoplasmic calcium in the control of gene expression. *Nature* 385, 260–265.

Hardingham, G., Pruunsild, P., Greenberg, M., and Bading, H. (2018). Lineage divergence of activity-driven transcription and evolution of cognitive ability. *Nat. Rev. Neurosci.* 19, 9–15. doi: 10.1038/nrn.2017.138

Hasel, P., Dando, O., Jiwaji, Z., Baxter, P., Todd, A., Heron, S., et al. (2017). Neurons and neuronal activity control gene expression in astrocytes to regulate their development and metabolism. *Nat. Commun.* 8:15132. doi: 10.1038/ncomms15132

Ibrahim, L., Wamsley, B., Alghamdi, N., Yusuf, N., Sevier, E., Hairston, A., et al. (2023). Nova proteins direct synaptic integration of somatostatin interneurons through activity-dependent alternative splicing. *Elife* 12:e86842. doi: 10.7554/eLife.86842

Iijima, T., and Yoshimura, T. (2019). A perspective on the role of dynamic alternative rna splicing in the development, specification, and function of axon initial segment. *Front. Mol. Neurosci.* 12:295. doi: 10.3389/fnmol.2019.00295

Iijima, T., Wu, K., Witte, H., Hanno-Iijima, Y., Glatter, T., Richard, S., et al. (2011). SAM68 regulates neuronal activity-dependent alternative splicing of neurexin-1. *Cell* 147, 1601–1614. doi: 10.1016/j.cell.2011.11.028

Jacko, M., Weyn-Vanhentenryck, S., Smerdon, J., Yan, R., Feng, H., Williams, D., et al. (2018). Rbfox splicing factors promote neuronal maturation and axon initial segment assembly. *Neuron* 97:853–868.e6. doi: 10.1016/j.neuron.2018.01.020

Karpova, A., Mikhaylova, M., Bera, S., Bär, J., Reddy, P., Behnisch, T., et al. (2013). Encoding and transducing the synaptic or extrasynaptic origin of NMDA receptor signals to the nucleus. *Cell* 152, 1119–1133. doi: 10.1016/j.cell.2013.02.002

Lee, P. R., and Fields, R. D. (2021). Activity-dependent gene expression in neurons. *Neuroscientist* 27, 355–366. doi: 10.1177/1073858420943515

Lewerenz, J., Baxter, P., Kassubek, R., Albrecht, P., Van Liefferinge, J., Westhoff, M., et al. (2014). Phosphoinositide 3-kinases upregulate system xc(-) via eukaryotic initiation factor 2α and activating transcription factor 4 - A pathway active in glioblastomas and epilepsy. *Antioxid. Redox Signal.* 20, 2907–2922. doi: 10.1089/ars.2013.5455

Li, D., McIntosh, C. S., Mastaglia, F. L., Wilton, S. D., and Aung-Htut, M. T. (2021). Neurodegenerative diseases: A hotbed for splicing defects and the potential therapies. *Transl. Neurodegener.* 10:16. doi: 10.1186/s40035-021-00240-7

Lopez Soto, E., Gandal, M., Gonatopoulos-Pournatzis, T., Heller, E., Luo, D., and Zheng, S. (2019). Mechanisms of neuronal alternative splicing and strategies for therapeutic interventions. *J. Neurosci.* 39, 8193–8199. doi: 10.1523/JNEUROSCI.1149-19.2019

Ma, H., Groth, R., Cohen, S., Emery, J., Li, B., Hoedt, E., et al. (2014). γCaMKII shuttles Ca^{2+}/CaM to the nucleus to trigger CREB phosphorylation and gene expression. *Cell* 159, 281–294. doi: 10.1016/j.cell.2014.09.019

McKenzie, G., Stevenson, P., Ward, G., Papadia, S., Bading, H., Chawla, S., et al. (2005). Nuclear Ca2+ and CaM kinase IV specify hormonal- and Notch-responsiveness. *J. Neurochem.* 93, 171–185. doi: 10.1111/j.1471-4159.2005.03010.x

Merkin, J., Russell, C., Chen, P., and Burge, C. B. (2012). Evolutionary dynamics of gene and isoform regulation in Mammalian tissues. *Science* 338, 1593–1599. doi: 10.1126/science.1228186

Monaco, G., van Dam, S., Casal Novo Ribeiro, J. L., Larbi, A., and de Magalhães, J. P. (2015). A comparison of human and mouse gene co-expression networks reveals conservation and divergence at the tissue, pathway and disease levels. *BMC Evol. Biol.* 15:259. doi: 10.1186/s12862-015-0534-7

O'Doherty, A., Ruf, S., Mulligan, C., Hildreth, V., Errington, M., Cooke, S., et al. (2005). An aneuploid mouse strain carrying human chromosome 21 with Down syndrome phenotypes. *Science* 309, 2033–2037. doi: 10.1126/science.1114535

Parikshak, N., Swarup, V., Belgard, T., Irimia, M., Ramaswami, G., Gandal, M., et al. (2016). Genome-wide changes in lncRNA, splicing, and regional gene expression patterns in autism. *Nature* 540, 423–427. doi: 10.1038/nature20612

Pruunsild, P., Bengtson, C. P., and Bading, H. (2017). Networks of cultured iPSC-derived neurons reveal the human synaptic activity-regulated adaptive gene program. *Cell Rep.* 18, 122–135. doi: 10.1016/j.celrep.2016.12.018

Puddifoot, C., Martel, M., Soriano, F., Camacho, A., Vidal-Puig, A., Wyllie, D., et al. (2012). PGC-1α negatively regulates extrasynaptic NMDAR activity and excitotoxicity. *J. Neurosci.* 32, 6995–7000. doi: 10.1523/JNEUROSCI.6407-11.2012

Qiu, J., Dando, O., Baxter, P., Hasel, P., Heron, S., Simpson, T., et al. (2018). Mixed-species RNA-seq for elucidation of non-cell-autonomous control of gene transcription. *Nat. Protoc.* 13, 2176–2199. doi: 10.1038/s41596-018-0029-2

Qiu, J., McQueen, J., Bilican, B., Dando, O., Magnani, D., Punovuori, K., et al. (2016). Evidence for evolutionary divergence of activity-dependent gene expression in developing neurons. *Elife* 5:e20337. doi: 10.7554/eLife.20337

Quesnel-Vallières, M., Dargaei, Z., Irimia, M., Gonatopoulos-Pournatzis, T., Ip, J., Wu, M., et al. (2016). Misregulation of an activity-dependent splicing network as a common mechanism underlying autism spectrum disorders. *Mol. Cell* 64, 1023–1034. doi: 10.1016/j.molcel.2016.11.033

Quesnel-Vallieres, M., Weatheritt, R. J., Cordes, S. P., and Blencowe, B. J. (2019). Autism spectrum disorder: Insights into convergent mechanisms from transcriptomics. *Nat. Rev.* 20, 51–63. doi: 10.1038/s41576-018-0066-2

Ramnath, R. R., Strange, K., and Rosenberg, P. A. (1992). Neuronal injury evoked by depolarizing agents in rat cortical cultures. *Neuroscience* 51, 931–939.

Saito, Y., Miranda-Rottmann, S., Ruggiu, M., Park, C., Fak, J., Zhong, R., et al. (2016). NOVA2-mediated RNA regulation is required for axonal pathfinding during development. *Elife* 5:e14371. doi: 10.7554/eLife.14371

Saito, Y., Yuan, Y., Zucker-Scharff, I., Fak, J., Jereb, S., Tajima, Y., et al. (2019). Differential NOVA2-mediated splicing in excitatory and inhibitory neurons regulates cortical development and cerebellar function. *Neuron* 101:707-720.e5. doi: 10.1016/j.neuron.2018.12.019

Schramm, M., Eimerl, S., and Costa, E. (1990). Serum and depolarizing agents cause acute neurotoxicity in cultured cerebellar granule cells: Role of the glutamate receptor responsive to N-methyl-D-aspartate. *Proc. Natl. Acad. Sci. U.S.A.* 87, 1193–1197.

Shen, S., Park, J., Lu, Z., Lin, L., Henry, M., Wu, Y., et al. (2014). rMATS: Robust and flexible detection of differential alternative splicing from replicate RNA-Seq data. *Proc. Natl. Acad. Sci. U.S.A.* 111, E5593–E5601. doi: 10.1073/pnas.1419161111

Sheng, M., McFadden, G., and Greenberg, M. E. (1990). Membrane depolarization and calcium induce c-fos transcription via phosphorylation of transcription factor CREB. *Neuron* 4, 571–582.

Soriano, F. X., and Hardingham, G. E. (2007). Compartmentalized NMDA receptor signalling to survival and death. *J. Physiol.* 584, 381–387.

Thalhammer, A., Jaudon, F., and Cingolani, L. A. (2020). Emerging roles of activity-dependent alternative splicing in homeostatic plasticity. *Front. Cell Neurosci* 14:104. doi: 10.3389/fncel.2020.00104

Verta, J. P., and Jacobs, A. (2022). The role of alternative splicing in adaptation and evolution. *Trends Ecol. Evol.* 37, 299–308. doi: 10.1016/j.tree.2021.11.010

Wahl, A., Buchthal, B., Rode, F., Bomholt, S., Freitag, H., Hardingham, G., et al. (2009). Hypoxic/ischemic conditions induce expression of the putative pro-death gene Clca1 via activation of extrasynaptic N-methyl-D-aspartate receptors. *Neuroscience* 158, 344–352. doi: 10.1016/j.neuroscience.2008.06.018

Wamsley, B., Jaglin, X., Favuzzi, E., Quattrocolo, G., Nigro, M., Yusuf, N., et al. (2018). Rbfox1 mediates cell-type-specific splicing in cortical interneurons. *Neuron* 100:846–859.e7. doi: 10.1016/j.neuron.2018.09.026

West, A. E., and Greenberg, M. E. (2011). Neuronal activity-regulated gene transcription in synapse development and cognitive function. *Cold Spring Harb. Perspect. Biol.* 3:5744. doi: 10.1101/cshperspect.a005744

Weyn-Vanhentenryck, S. M., Feng, H., Ustianenko, D., Duffié, R., Yan, Q., Jacko, M., et al. (2018). Precise temporal regulation of alternative splicing during neural development. *Nat. Commun.* 9:2189. doi: 10.1038/s41467-018-04559-0

Wheeler, D., Groth, R., Ma, H., Barrett, C., Owen, S., Safa, P., et al. (2012). Ca(V)1 and Ca(V)2 channels engage distinct modes of Ca(2+) signaling to control CREB-dependent gene expression. *Cell* 149, 1112–1124. doi: 10.1016/j.cell.2012.03.041

Xia, Z., Dudek, H., Miranti, C., and Greenberg, M. (1996). Calcium influx via the NMDA receptor induces immediate early gene transcription by a MAP kinase/ERK-dependent mechanism. *J. Neurosci.* 16, 5425–5436. doi: 10.1523/JNEUROSCI.16-17-05425.1996

Zhou, X., Moon, C., Zheng, F., Luo, Y., Soellner, D., Nuñez, J., et al. (2009). N-methyl-D-aspartate-stimulated ERK1/2 signaling and the transcriptional up-regulation of plasticity-related genes are developmentally regulated following in vitro neuronal maturation. *J. Neurosci. Res.* 87, 2632–2644. doi: 10.1002/jnr.22103

frontiers | Frontiers in Molecular Neuroscience

TYPE Mini Review
PUBLISHED 02 September 2024
DOI 10.3389/fnmol.2024.1398048

Check for updates

OPEN ACCESS

EDITED BY
Clémence Bernard,
University of Exeter, United Kingdom

REVIEWED BY
Beatriz Alvarez,
Complutense University of Madrid, Spain
Hanna Hörnberg,
Helmholtz Association of German Research Centers (HZ), Germany

*CORRESPONDENCE
Susana R. Louros
✉ slouros@ed.ac.uk

RECEIVED 08 March 2024
ACCEPTED 12 August 2024
PUBLISHED 02 September 2024

CITATION
Elu N, Subash S and R. Louros S (2024) Crosstalk between ubiquitination and translation in neurodevelopmental disorders. *Front. Mol. Neurosci.* 17:1398048.
doi: 10.3389/fnmol.2024.1398048

Crosstalk between ubiquitination and translation in neurodevelopmental disorders

Nagore Elu[1,2], Srividya Subash[1,2] and Susana R. Louros[1,2]*

[1]Centre for Discovery Brain Sciences, University of Edinburgh, Edinburgh, United Kingdom, [2]Simons Initiative for the Developing Brain, Patrick Wild Centre, University of Edinburgh, Edinburgh, United Kingdom

Ubiquitination is one of the most conserved post-translational modifications and together with mRNA translation contributes to cellular protein homeostasis (proteostasis). Temporal and spatial regulation of proteostasis is particularly important during synaptic plasticity, when translation of specific mRNAs requires tight regulation. Mutations in genes encoding regulators of mRNA translation and in ubiquitin ligases have been associated with several neurodevelopmental disorders. RNA metabolism and translation are regulated by RNA-binding proteins, critical for the spatial and temporal control of translation in neurons. Several ubiquitin ligases also regulate RNA-dependent mechanisms in neurons, with numerous ubiquitination events described in splicing factors and ribosomal proteins. Here we will explore how ubiquitination regulates translation in neurons, from RNA biogenesis to alternative splicing and how dysregulation of ubiquitin signaling can be the underlying cause of pathology in neurodevelopmental disorders, such as Fragile X syndrome. Finally we propose that targeting ubiquitin signaling is an attractive novel therapeutic strategy for neurodevelopmental disorders where mRNA translation and ubiquitin signaling are disrupted.

KEYWORDS

ubiquitin, translation, splicing, ribosome, neurodevelopmental disorders, FMRP, UBE3A

Introduction

Protein abundance is regulated by the coordination of synthesis and degradation, and these two processes sculpt the molecular architecture of a neuron during development and plasticity. The fate of proteins within cells is directly regulated by ubiquitination. This post-translational modification involves the covalent attachment of a small protein, ubiquitin, to target proteins. Ubiquitination is a cascade of events that start with the activation of ubiquitin by the E1 activating enzyme. Active ubiquitin is then transferred to the E2 conjugating enzyme, which is in charge of interacting with the E3 ligase to ultimately transfer ubiquitin into E3 ligase substrates. Mono-ubiquitination of proteins can regulate cellular processes such as gene transcription, signal transduction and DNA damage response by altering protein localization, protein-protein interactions or endocytosis (Greer et al., 2003; Pelzer et al., 2013; Fukushima et al., 2015; Zhou et al., 2017; Wang et al., 2019). In addition, ubiquitin can generate different types of chains via its seven lysine residues, and each type of poly-ubiquitination (N1, K6, K11, K27, K29, K33, K48, and K63) will have a distinct intracellular role. For instance, the K48 chain-type has been widely described to target proteins into degradation by the Ubiquitin Proteasome System (UPS; Thrower et al., 2000), while K63 ubiquitin chains can drive changes in protein localization or regulate endocytosis and innate immune response (Richard et al., 2020; Madiraju et al., 2022; Saeed et al., 2023).

Ubiquitin signaling has been widely described to be involved in receptor trafficking, synapse formation (Haas and Broadie, 2008; Iwai, 2021; Lei et al., 2021; Pérez-Villegas et al., 2022; Dikic and Schulman, 2023) and remodeling (Mei et al., 2020) by regulating the turnover of synaptic proteins at synapses (Tai and Schuman, 2008; Bingol and Sheng, 2011). Disruption of any ubiquitin-mediated pathways leads to aberrant neuronal morphology, connectivity or synapse formation, which are hallmark features of neurodevelopmental disorders (NDDs; Louros and Osterweil, 2016; Batool et al., 2019).

Neurodevelopmental disorders affect more than 15% of children worldwide (Romero-Ayuso, 2021), and include intellectual disability (ID) and autism spectrum disorder (ASD; Ismail and Shapiro, 2019). Large-scale sequencing studies contributed to the understanding of NDDs pathophysiology by unveiling their genetic etiology. More precisely, these studies report mutations in genes involved in synaptic function and structure, transcriptional and translational regulators (De Rubeis et al., 2014; Krumm et al., 2014; de la Torre-Ubieta et al., 2016), as well as mutations in genes involved in ubiquitin-dependent protein degradation (Louros and Osterweil, 2016; Trost et al., 2022). Several components of the UPS implicated in NDDs, also play crucial roles in RNA synthesis and splicing (Cho et al., 2014; Jewell et al., 2015; Saez et al., 2020; Pitts et al., 2022). Therefore, there is a growing interest in exploring the crosstalk between protein synthesis and protein degradation in the context of NDDs. This mini-review aims to compile all pertinent information on the regulatory role of ubiquitination in RNA biogenesis in the context of NDDs.

Ubiquitin ligases as new players in translation control

Neurons maintain efficient crosstalk between protein translation and degradation to adjust to their physiological needs. Among the intermediaries between protein synthesis and degradation pathways, ubiquitin ligases emerge as central regulators. Ubiquitination of ribosomal proteins has been described over three decades in a seminal paper showing that ubiquitination regulates ribosomal proteins abundance and that the assembly into the ribosome is facilitated by ubiquitin (Finley et al., 1989). More recent studies showed that K63-linked ubiquitination of ribosomal proteins and translation elongation factors promote translation in yeast (Silva et al., 2015). Similarly, in human cells ribosomal proteins were found to be ubiquitinated after the inhibition of translation (Higgins et al., 2015). Moreover, Culin-3, an E3 ubiquitin ligase previously implicated in NDDs (De Rubeis et al., 2014), has been involved in the formation of a ribosome modification platform that alters the translation of specific mRNAs (Werner et al., 2015). Ubiquitination can also regulate translation is by modulating translational surveillance pathways. When aberrant nascent polypeptides are stalled in ribosomes during translation and ribosomes collide, the ribosome quality control (RQC) surveillance pathway is activated, in which ubiquitinated ribosomal subunits are recognized to assist into the ribosome-splitting event (Matsuo et al., 2023). Although dysfunction of RQC is suggested to elicit neurological disorders, the molecular mechanisms involved remain poorly understood. Makorin ring finger protein (MRKN1), a ubiquitin ligase previously shown to control local translation in neurons during synaptic plasticity (Miroci et al., 2012), was recently implicated in the RQC pathway, promoting ribosome stalling at poly(A) sequences and starting RQC by ubiquitinating RPS10 and other RQC factors (Hildebrandt et al., 2019). Interestingly, MRKN1 is member of a family of ubiquitin ligases that also binds RNA, known as the RNA-binding ubiquitin ligases (RBULs). So far, over 30 RBULs have been identified (Thapa et al., 2020) but their function in the brain remains elusive.

Although previous studies demonstrate a direct link between ribosomal protein ubiquitination and changes in translation, the role of ribosomal protein ubiquitination in neurons hasn't been explored. The majority of ribosomal proteins is produced in the nucleus where ribosomes are assembled, but the enrichment of mRNAs of ribosomal proteins in dendrites and axons is a long-standing observation (Moccia et al., 2003). Proteomic studies show that over 80% of ribosomal proteins are ubiquitinated in neurons (Schreiber et al., 2015; Sun et al., 2023), 20 of those putatively ubiquitinated in synaptic fractions (Na et al., 2012; Table 1). Recent studies confirmed that ribosomal proteins are locally synthesized and incorporated into existing ribosomes in axons (Shigeoka et al., 2019) as well as in dendrites (Fusco et al., 2021). Both studies show that a subset of ribosomal proteins is more frequently incorporated or exchanged into mature ribosomes. Interestingly, a fraction of the exchanging ribosomal proteins is also ubiquitinated in neurons (Table 1; Na et al., 2012; Schreiber et al., 2015), suggesting an additional layer of regulation of ribosomal protein exchange in neurons that may be essential to regulate local protein synthesis in response to synaptic plasticity. Whether these processes are affected in NDDs is an open question, but since changes in ribosome abundance have been reported in several NDDs (Griesi-Oliveira et al., 2020; Seo et al., 2022), it would be interesting to investigate if their ubiquitination is aberrant in NDDs, and if that can be targeted to normalize ribosome levels and translation rates.

Alternative splicing regulation by ubiquitin and its dysfunction in NDDs

Most protein-coding genes in humans are transcribed as pre-mRNAs that contain a series of exons and introns. Following transcription, the removal of introns during the process of pre-mRNA splicing is required before the nascent transcript is translated into a protein. Alternative splicing generates multiple proteins from a single pre-mRNA by including and/or excluding alternative exons, thereby diversifying cellular proteomes (Han et al., 2011; Wang et al., 2015). This process is particularly important in neurons that rely on the function of heavily spliced genes such as Neurexins, n-Cadherins, and calcium-activated potassium channels that can produce hundreds of mRNA isoforms through alternative splicing. Indeed, some NDDs occur when alternative splicing goes awry. For example, extensive

TABLE 1 Several components of the splicing machinery as well as ribosomal proteins are ubiquitinated in the brain.

Cellular compartment	Protein names	References
Ribosome	RPL11, RPL12*, RPL13, RPL13A, RPL14*, RPL15, RPL17, RPL18, RPL18A, RPL24, RPL26, RPL27, RPL27A, RPL3, RPL32, RPL34, RPL35, RPL39, RPL4, RPL5, RPL6, RPL7, RPL7L1, RPL8*, RPL9, RPS10, RPS11, RPS14, RPS15, RPS15A, RPS16, RPS19*, RPS2, RPS23, RPS25*, RPS27, RPS27A, RPS29, RPS3*, RPS3A, RPS5, RPS6, RPS7, RPS8, RPS9	Sun et al., 2023
	RACK1*, RPL19, RPL23A, RPL30*, RPS20, RPS21, RPSA	Na et al., 2012
	RPL10A, RPL28*, RPL29, RPL31, RPL35A, RPL38*, RPL7A, RPS13*, RPS17, RPS18*, RPS24, RPS26, RPS4X	Schreiber et al., 2015
Spliceosome	CDC5L, DDX46, EIF4A3, LUC7L3, PRPF19, PRPF3, PRPF8, RPL11, RPL18A, RPL39, RPS23, RPS29, SF3A3, SF3B6, SMU1, SNRNP200, SNRNP70, SNRPA1, SNRPB2, SNRPF	Sun et al., 2023
	CWC22, CWC27, DDX23, IK, RBMXL2, SF3B1, SNRNP35, SNRPD3, SNRPE, SNRPN, SRRM2, USP39, YBX1	Schreiber et al., 2015

*Ubiquitination events found in synaptic fractions.

transcriptomics studies using post-mortem brain tissue from ASD patients have shown pervasive mis-regulation of microexon splicing (Irimia et al., 2014; Chanarat and Mishra, 2018; Su et al., 2018).

The molecular machinery responsible for pre-mRNA splicing is called the spliceosome complex. It is composed of five small nuclear RNAs (U1, U2, U4, U5, and U6) pre-assembled with proteins into small ribonucleoproteins (snRNPs), together with hundreds of auxiliary proteins that help the spliceosome recognize splice sites (Wassarman and Steitz, 1992; Zhou et al., 2002; Matlin and Moore, 2007). High-throughput genetic studies showed a possible link between ubiquitin ligases and the process of pre-mRNA splicing. For instance, ubiquitin binds to the highly conserved spliceosomal core protein PRPF8 via its C-terminal domain (Grainger and Beggs, 2005; Bellare et al., 2006, 2008). Additionally, the literature also suggests that ubiquitination of other splicing factors may modulate spliceosomal activity through reversible protein-protein interactions (Bellare et al., 2008). For example, PRPF3 and PRPF31 undergo K63-linked ubiquitination by an RBUL, PRPF19 (Chanarat and Mishra, 2018), an essential step for spliceosomal activation (Hogg et al., 2010). Ubiquitinated PRPF3 and PRPF31 then bind PRPF8 and stabilize the tri-snRNP complex (Park et al., 2016). As the splicing cycle progresses, PRPF3 and PRPF31 are deubiquitinated by USP4 and USP15, respectively (Song et al., 2010; Das et al., 2017). Altogether, this shows that the ubiquitination state of several components of the spliceosome tightly regulate its assembly and activation, therefore affecting splicing.

The regulation of the spliceosome by ubiquitination in neurons is less elucidated, but proteomic studies identify several ubiquitinated splicing factors such as PRPF3, PRP9, as well as the RBUL, PRPF19 (Table 1). Considering that neurons express highly spliced genes, dysregulated ubiquitination of the spliceosome could have major consequences in neuronal development and function and contribute to NDDs. Importantly, a recent study identified mutations in three spliceosome factors in NDDs, including six individuals who harbored mostly *de novo* heterozygous variants in *PRPF19*. This study demonstrated that these pathogenic variants lead to converging neurodevelopmental phenotypes, including, but not limited to developmental delay, ID and autism (Li et al., 2024).

Ubiquitination of RNA-binding proteins: contribution to NDDs

RNA metabolism is regulated at different stages by specific RNA-binding proteins (RBPs). RBPs are responsible for mRNA transport and translation regulation within dendrites and are required for long-lasting forms of synaptic plasticity (Glock et al., 2017). The loss of RBP function leads to numerous disorders, including ASD, Fragile X Syndrome (FXS; Bhakar et al., 2012; Zoghbi and Bear, 2012; Darnell and Klann, 2013; Lee et al., 2016; Popovitchenko et al., 2016) and epilepsy (Lee et al., 2016).

Due to its significant role in translation regulation and its impact on neuronal homeostasis, Fragile X messenger ribonucleoprotein (FMRP) stands out as one of the most extensively studied RBPs. Evidence suggests that FMRP is transported into dendrites and synapses where it acts as a central regulator of local translation (Darnell and Klann, 2013; Schieweck et al., 2021). Additionally, FMRP has a dual role in both RNA localization and translation; localizes to polyribosome complexes and is well-documented for its role as a translational repressor (Laggerbauer et al., 2001; Mazroui et al., 2002). Studies of *Fmr1* mutant models have revealed alterations in plasticity and excitability in several brain circuits, as a consequence of the excessive protein synthesis (Osterweil et al., 2013; Louros et al., 2023). Deficiency of FMRP, the underlying cause of Fragile X Syndrome, causes dysregulation of the translation of mRNAs that bind to FMRP. Interestingly, the majority of FMRP target mRNAs are less translated in the hippocampus (Ceolin et al., 2017; Thomson et al., 2017; Sawicka et al., 2019; Sharma et al., 2019; Seo et al., 2022) and this is reflected in the synapse-enriched proteome of *Fmr1* KO mouse (Louros et al., 2023).

FMRP undergoes degradation primarily through the ubiquitin-proteasome system (UPS), which is a major pathway for targeted protein degradation in cells (Chanarat and Mishra, 2018; Ebstein et al., 2021; Winden et al., 2023). Consistent with this, FMRP undergoes regulation by ubiquitination, a tightly controlled process that can be triggered by specific events such as dephosphorylation at key sites such as S499 (Wilkerson et al., 2023). Various factors contribute to this dephosphorylation, including activation of PP2A by the activation of metabotropic glutamate receptors

(Nalavadi et al., 2012). Additionally, developmental cues play a crucial role during specific stages of development by regulating dephosphorylation and subsequent ubiquitination of FMRP (Schieweck et al., 2021). Once dephosphorylated, FMRP becomes a target for specific E3 ubiquitin ligases such as APC/Cdh1, and once ubiquitinated, it is targeted for degradation (Nalavadi et al., 2012; Valdez-Sinon et al., 2020). FMRP is also known for its role in mRNA-protein interactions within ribonucleoprotein (RNP) granules, which are crucial for mRNA transport and localization (Valdez-Sinon et al., 2020). Ubiquitination-induced degradation of FMRP may disrupt these interactions, impairing the transport and proper localization of mRNAs, thereby affecting gene expression programs that are essential for normal cellular function (Valdez-Sinon et al., 2020; Wilkerson et al., 2023).

In addition to FMRP other RBPs related to NDDs are regulated by ubiquitination. One example is the ELAVL family, which undertakes essential functions across spatiotemporal windows in brain development to help regulate and specify transcriptomic programs for cell specialization (Mulligan and Bicknell, 2023). Different components of this family have been related to ASDs, behavioral abnormalities or seizures (Mulligan and Bicknell, 2023), with ELAV2 showing a clear role in neurodevelopment and listed by SFARI as a candidate gene for ASD. ELAV2 targets are also involved in synaptic function and neurodevelopmental disorders (Berto et al., 2016). Even though in the context of cancer, ELAV1 has been described to be ubiquitinated, facilitating its proteasome mediated degradation and leading to an increase in the survival rate of cells under heat-shock response (Daks et al., 2021), the ubiquitination of this RBP has not been demonstrated in neurons. RBFOX1 is another RBP strongly implicated in ASD. This protein regulates both splicing and transcriptional networks in human neuronal development (Fogel et al., 2012) and it has been found to be ubiquitinated in Alzheimer's disease post mortem human brain tissue, particularly in axons, tangles and neuropil threads, suggesting a role in axonal proteostasis (Fernandez et al., 2021).

Altogether, these evidences show that RNA binding proteins are regulated by ubiquitination in the brain and despite lacking detailed mechanisms it is possible that aberrant ubiquitination of RNA binding proteins contributes to the pathophysiology of NDDs.

Dysregulated proteostasis in NDDs: new therapeutic opportunities

Dysregulation of translational represents a common endpoint of familial and sporadic ASD-associated signaling pathways (De Rubeis et al., 2014; Krumm et al., 2014). The identification of this dysregulated pathway has been used to develop several therapeutic strategies for FXS and other NDDs, however, due to the limited success in clinical trials there is an urgent need for identification of new pathways amenable for therapeutic development.

A recent development was the discovery of upregulated protein degradation machinery in FXS, downstream of the increased protein translation rates that characterize this disorder (Louros et al., 2023). This study shows that the increase in protein degradation is primarily a consequence of excessive translation of proteasomal subunits and ubiquitin ligases in excitatory neurons from *Fmr1* mutant mice. Importantly, pharmacological reduction of proteasome activity and ubiquitin ligases was sufficient to normalize protein synthesis rates, demonstrating the intricate relationship between translation and degradation in FXS. This could be a consequence of modulating ribosomal subunits turnover since the authors found ribosomal subunits excessively targeted for degradation in synaptic enriched fractions, possibly through increased ubiquitination rates. Finally, this study found that increased proteasome activity contributes to hyperexcitability and audiogenic seizures in Fmr1 KO mice, and that this phenotype was corrected by pharmacological and genetic manipulation of the proteasome (Louros et al., 2023). This study opens the door to more investigations into the dysfunction of ubiquitin signaling and proteasomal degradation in other NDDs, and it demonstrates that targeting ubiquitin signaling could be a new pathway for therapeutic development.

One of the most studied ubiquitin ligases linked to neurodevelopmental disorders is UBE3A, with loss of function mutations causing Angelman syndrome (AS; Kalsner and Chamberlain, 2015). AS is characterized by intellectual disability, developmental delay, seizures, motor disruptions, and an unusually positive demeanor (LaSalle et al., 2015). Many studies have identified targets of Ube3a in mouse, rats and human AS samples (Pandya et al., 2022) including some regulators of protein synthesis. One interesting target of Ube3a is the mTOR suppressor protein TSC2 (Zheng et al., 2008), directly involved in the regulation of protein synthesis. Recent work suggests that degradation of TSC2 following ubiquitination by Ube3a may contribute to pathology, as treatment with the mTOR inhibitor rapamycin rescued motor deficits and abnormal dendritic branching in AS mutant mice (Sun et al., 2015). Furthermore, lovastatin, previously shown to correct excessive protein synthesis rates and seizures in FXS (Osterweil et al., 2013; Asiminas et al., 2019), was also shown to correct seizures in the AS mouse model (Chung et al., 2018), suggesting that protein synthesis rates could be increased in the AS mutant mouse. This was indeed confirmed in a recent study that found increased *de novo* protein synthesis in the hippocampus of the AS mutant mouse (Aria et al., 2023), as well as impaired autophagy that when enhanced was able to ameliorate cognitive impairments in AS mice.

Altogether, these findings show the intricate crosstalk between ubiquitin signaling and translation in NDDs. Targeted protein degradation technologies have emerged over 20 years ago with potential for targeting undruggable protein targets. PROTAC (proteolysis-targeting chimera) or molecular glue (MG)-driven ternary complex formation with an ubiquitin E3 ligase utilizes cells' UPS to degrade target proteins. Several such molecules have entered clinical development (Kong and Jones, 2023). Recent clinical proof-of-concept for PROTAC molecules against two cancer targets confirmed the successful clinical targeting of proteins previously considered "undruggable." There are currently over 20 new PROTACs under clinical development (Békés et al., 2022). The application of these strategies to brain disorders offers several advantages and

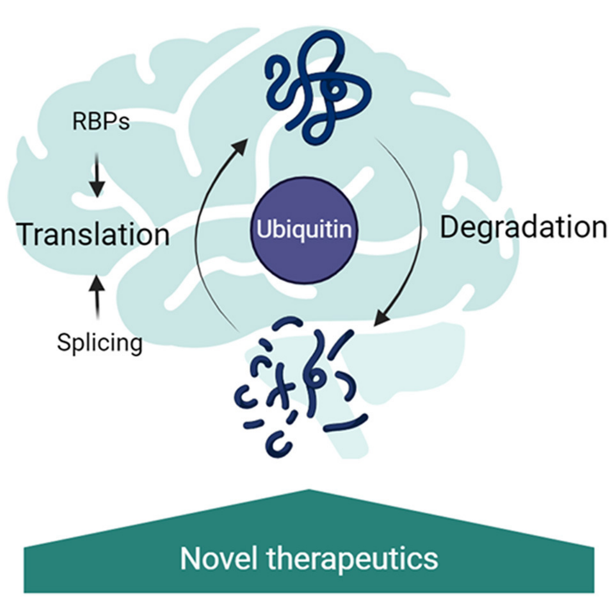


FIGURE 1
Ubiquitin signaling as a central regulator of RNA metabolism in the brain. Ubiquitination regulates splicing, through regulation of the spliceosome remodeling; RNA-binding protein abundance and binding partners; translation rates through ribosomal protein ubiquitination and the turnover rates of proteins in the brain. Disruption of these pathways has been implicated in neurodevelopmental disorders, therefore targeting the ubiquitin signaling is a promising new therapeutic strategy.

challenges but recent studies have shown promise in the context of neurodegenerative disorders [recently reviewed by Farrell and Jarome (2021)] suggesting that this new therapeutic avenue for NDDs could offer increased specificity and lower off-target effects.

Conclusions and perspectives

Molecular analysis of patient-derived tissues and mouse models of the monogenic ID has shown widespread changes at the epigenetic, transcriptional, and translational gene expression levels. The interplay between changes at multiple levels is essential to the pathophysiology of NDDs. Importantly, coordination between the translational machinery, RBPs and the ubiquitin proteasome system regulates dendritic proteostasis in response to neuronal activity (Hanus and Schuman, 2013). Indeed, mutations in components of these systems are associated with altered plasticity and may underlie the pathogenesis of NDDs. Considering that in several models of NDDs protein synthesis rates are affected (Auerbach et al., 2011; Barnes et al., 2015; Aria et al., 2023), ribosome abundance is increased and the ubiquitin proteasome system is overexpressed (Seo et al., 2022; Louros et al., 2023), it is pertinent to investigate the contributions of ubiquitin signaling dysfunction to ribosome quality control and alternative splicing. However, the isolation and identification of ubiquitinated proteins under physiological conditions from *in vivo* tissues is a challenging task, particularly in the brain, as the ubiquitinated proteins are generally found at very low levels within the cells. Besides, the fast kinetics at which some of the proteins conjugated with ubiquitin are degraded (Ronchi and Haas, 2012), the action of the deubiquitinating enzymes (Stegmeier et al., 2007) or the fact that proteins might be modified with ubiquitin only in well-defined temporal windows (Clute and Pines, 1999), make their analysis even more challenging. Considering that ubiquitin signaling modulates so many aspects of RNA biogenesis that are affected in NDDs (Figure 1), we believe it is vital to develop methods to improve the identification of dysregulated ubiquitination in the brain to accelerate the development of novel therapeutic options for NDDs.

Author contributions

NE: Writing – original draft, Writing – review & editing. SS: Writing – original draft. SRL: Conceptualization, Funding acquisition, Visualization, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the Simons Initiative for the Developing Brain and by the College of Medicine and Veterinary Medicine, University of Edinburgh.

Acknowledgments

The authors would like to acknowledge all the colleagues that discussed the manuscript prior to submission.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Aria, F., Pandey, K., and Alberini, C. M. (2023). Excessive protein accumulation and impaired autophagy in the hippocampus of angelman syndrome modeled in mice. *Biol. Psychiat.* 94, 68–83. doi: 10.1016/j.biopsych.2022.11.016

Asiminas, A., Jackson, A. D., Louros, S. R., Till, S. M., Spano, T., Dando, O., et al. (2019). Sustained correction of associative learning deficits after brief, early treatment in a rat model of Fragile X Syndrome. *Sci. Transl. Med.* 11:eaao0498. doi: 10.1126/scitranslmed.aao0498

Auerbach, B. D., Osterweil, E. K., and Bear, M. F. (2011). Mutations causing syndromic autism define an axis of synaptic pathophysiology. *Nature* 480, 63–68. doi: 10.1038/nature10658

Barnes, S. A., Wijetunge, L. S., Jackson, A. D., Katsanevaki, D., Osterweil, E. K., Komiyama, N. H., et al. (2015). Convergence of hippocampal pathophysiology in Syngap+/- and Fmr1-/y mice. *J. Neurosci.* 35, 15073–15081. doi: 10.1523/JNEUROSCI.1087-15.2015

Batool, S., Raza, H., Zaidi, J., Riaz, S., Hasan, S., and Syed, N. I. (2019). Synapse formation: from cellular and molecular mechanisms to neurodevelopmental and neurodegenerative disorders. *J. Neurophysiol.* 121, 1381–1397. doi: 10.1152/jn.00833.2018

Békés, M., Langley, D. R., and Crews, C. M. (2022). PROTAC targeted protein degraders: the past is prologue. *Nat. Rev. Drug Discov.* 21, 181–200. doi: 10.1038/s41573-021-00371-6

Bellare, P., Kutach, A. K., Rines, A. K., Guthrie, C., and Sontheimer, E. J. (2006). Ubiquitin binding by a variant Jab1/MPN domain in the essential pre-mRNA splicing factor Prp8p. *RNA* 12, 292–302. doi: 10.1261/rna.2152306

Bellare, P., Small, E. C., Huang, X., Wohlschlegel, J. A., Staley, J. P., and Sontheimer, E. J. (2008). A role for ubiquitin in the spliceosome assembly pathway. *Nat. Struct. Mol. Biol.* 15, 444–451. doi: 10.1038/nsmb.1401

Berto, S., Usui, N., Konopka, G., and Fogel, B. L. (2016). ELAVL2-regulated transcriptional and splicing networks in human neurons link neurodevelopment and autism. *Hum. Mol. Genet.* 2016:ddw110. doi: 10.1093/hmg/ddw110

Bhakar, A. L., Dölen, G., and Bear, M. F. (2012). The pathophysiology of fragile X (and what it teaches us about synapses). *Annu. Rev. Neurosci.* 35, 417–443. doi: 10.1146/annurev-neuro-060909-153138

Bingol, B., and Sheng, M. (2011). Deconstruction for reconstruction: the role of proteolysis in neural plasticity and disease. *Neuron* 69, 22–32. doi: 10.1016/j.neuron.2010.11.006

Ceolin, L., Bouquier, N., Vitre-Boubaker, J., Rialle, S., Severac, D., Valjent, E., et al. (2017). Cell type-specific mRNA dysregulation in hippocampal CA1 pyramidal neurons of the fragile X syndrome mouse model. *Front. Mol. Neurosci.* 10:340. doi: 10.3389/fnmol.2017.00340

Chanarat, S., and Mishra, S. K. (2018). Emerging roles of ubiquitin-like proteins in pre-mRNA splicing. *Trends Biochem. Sci.* 43, 896–907. doi: 10.1016/j.tibs.2018.09.001

Cho, S. K., Chaabane, S. B., Shah, P., Poulsen, C. P., and Yang, S. W. (2014). COP1 E3 ligase protects HYL1 to retain microRNA biogenesis. *Nat. Commun.* 5:5867. doi: 10.1038/ncomms6867

Chung, L., Bey, A. L., Towers, A. J., Cao, X., Kim, I. H., and Jiang, Y. (2018). Lovastatin suppresses hyperexcitability and seizure in Angelman syndrome model. *Neurobiol. Dis.* 110, 12–19. doi: 10.1016/j.nbd.2017.10.016

Clute, P., and Pines, J. (1999). Temporal and spatial control of cyclin B1 destruction in metaphase. *Nat. Cell Biol.* 1, 82–87. doi,: 10.1038/10049

Daks, A., Petukhov, A., Fedorova, O., Shuvalov, O., Kizenko, A., Tananykina, E., et al. (2021). The RNA-binding protein HuR is a novel target of Pirh2 E3 ubiquitin ligase. *Cell Death Dis.* 12:581. doi: 10.1038/s41419-021-03871-w

Darnell, J. C., and Klann, E. (2013). The translation of translational control by FMRP: therapeutic targets for fragile X syndrome. *Nat. Neurosci.* 16:1530. doi: 10.1038/nn.3379

Das, T., Park, J. K., Park, J., Kim, E., Rape, M., Kim, E. E. K., et al. (2017). USP15 regulates dynamic protein-protein interactions of the spliceosome through deubiquitination of PRP31. *Nucl. Acids Res.* 45, 4866–4880. doi: 10.1093/nar/gkw1365

de la Torre-Ubieta, L., Won, H., Stein, J. L., and Geschwind, D. H. (2016). Advancing the understanding of autism disease mechanisms through genetics. *Nat. Med.* 22, 345–361. doi: 10.1038/nm.4071

De Rubeis, S., He, X., Goldberg, A. P., Poultney, C. S., Samocha, K., Cicek, A. E., et al. (2014). Synaptic, transcriptional and chromatin genes disrupted in autism. *Nature* 515, 209–215. doi: 10.1038/nature13772

Dikic, I., and Schulman, B. A. (2023). An expanded lexicon for the ubiquitin code. *Nat. Rev. Mol. Cell Biol.* 24, 273–287. doi: 10.1038/s41580-022-00543-1

Ebstein, F., Küry, S., Papendorf, J. J., and Krüger, E. (2021). Neurodevelopmental disorders (NDD) caused by genomic alterations of the ubiquitin-proteasome system (UPS): the possible contribution of immune dysregulation to disease pathogenesis. *Front. Mol. Neurosci.* 14:733012. doi: 10.3389/fnmol.2021.733012

Farrell, K., and Jarome, T. J. (2021). Is PROTAC technology really a game changer for central nervous system drug discovery? *Expert Opin. Drug Discov.* 16, 833–840. doi: 10.1080/17460441.2021.1915979

Fernandez, W. R., Solopova, E., Shostak, A., Balotin, K. M., Mobley, B., Harmsen, H., et al. (2021). RBFOX1 is regulated by the adenosine 2a receptor and is ubiquitinated in tau tangles in Alzheimer's disease. *Alzheimer's Dement.* 17:e056367. doi: 10.1002/alz.056367

Finley, D., Bartel, B., and Varshavsky, A. (1989). The tails of ubiquitin precursors are ribosomal proteins whose fusion to ubiquitin facilitates ribosome biogenesis. *Nature* 338, 394–401. doi: 10.1038/338394a0

Fogel, B. L., Wexler, E., Wahnich, A., Friedrich, T., Vijayendran, C., Gao, F., et al. (2012). RBFOX1 regulates both splicing and transcriptional networks in human neuronal development. *Hum. Mol. Genet.* 21, 4171–4186. doi: 10.1093/hmg/dds240

Fukushima, T., Yoshihara, H., Furuta, H., Kamei, H., Hakuno, F., Luan, J., et al. (2015). Nedd4-induced monoubiquitination of IRS-2 enhances IGF signalling and mitogenic activity. *Nat. Commun.* 6:6780. doi: 10.1038/ncomms7780

Fusco, C. M., Desch, K., Dörrbaum, A. R., Wang, M., Staab, A., Chan, I. C. W., et al. (2021). Neuronal ribosomes exhibit dynamic and context-dependent exchange of ribosomal proteins. *Nat. Commun.* 12:6127. doi: 10.1038/s41467-021-26365-x

Glock, C., Heumüller, M., and Schuman, E. M. (2017). mRNA transport and local translation in neurons. *Curr. Opin. Neurobiol. Mol. Neurosci.* 45, 169–177. doi: 10.1016/j.conb.2017.05.005

Grainger, R. J., and Beggs, J. D. (2005). Prp8 protein: at the heart of the spliceosome. *RNA* 11, 533–557. doi: 10.1261/rna.2220705

Greer, S. F., Zika, E., Conti, B., Zhu, X.-S., and Ting, J. P.-Y. (2003). Enhancement of CIITA transcriptional function by ubiquitin. *Nat. Immunol.* 4, 1074–1082. doi: 10.1038/ni985

Griesi-Oliveira, K., Fogo, M. S., Pinto, B. G. G., Alves, A. Y., Suzuki, A. M., Morales, A. G., et al. (2020). Transcriptome of iPSC-derived neuronal cells reveals a module of co-expressed genes consistently associated with autism spectrum disorder. *Mol. Psychiat.* 26, 1589–1605. doi: 10.1038/s41380-020-0669-9

Haas, K. F., and Broadie, K. (2008). Roles of ubiquitination at the synapse. *Biochim. Biophys. Acta* 1779, 495–506. doi: 10.1016/j.bbagrm.2007.12.010

Han, J., Xiong, J., Wang, D., and Fu, X. D. (2011). Pre-mRNA splicing: where and when in the nucleus. *Trends Cell Biol.* 3:3. doi: 10.1016/j.tcb.2011.03.003

Hanus, C., and Schuman, E. M. (2013). Proteostasis in complex dendrites. *Nat. Rev. Neurosci.* 14, 638–648. doi: 10.1038/nrn3546

Higgins, R., Gendron, J. M., Rising, L., Mak, R., Webb, K., Kaiser, S. E., et al. (2015). The unfolded protein response triggers site-specific regulatory ubiquitylation of 40S ribosomal proteins. *Mol. Cell* 59, 35–49. doi: 10.1016/j.molcel.2015.04.026

Hildebrandt, A., Brüggemann, M., Rücklé, C., Boerner, S., Heidelberger, J. B., Busch, A., et al. (2019). The RNA-binding ubiquitin ligase MKRN1 functions in ribosome-associated quality control of poly(A) translation. *Genome Biol.* 20:216. doi: 10.1186/s13059-019-1814-0

Hogg, R., McGrail, J. C., and O'Keefe, R. T. (2010). The function of the NineTeen Complex (NTC) in regulating spliceosome conformations and fidelity during pre-mRNA splicing. *Biochem. Soc. Transact.* 38, 1110–1115. doi: 10.1042/BST0381110

Irimia, M., Weatheritt, R. J., Ellis, J. D., Parikshak, N. N., Gonatopoulos-Pournatzis, T., Babor, M., et al. (2014). A highly conserved program of neuronal microexons is misregulated in autistic brains. *Cell* 159, 1511–1523. doi: 10.1016/j.cell.2014.11.035

Ismail, F. Y., and Shapiro, B. K. (2019). What are neurodevelopmental disorders? *Curr. Opin. Neurol.* 32, 611–616. doi: 10.1097/WCO.0000000000000710

Iwai, K. (2021). Discovery of linear ubiquitination, a crucial regulator for immune signaling and cell death. *FEBS J.* 288, 1060–1069. doi: 10.1111/febs.15471

Jewell, J. L., Flores, F., and Guan, K.-L. (2015). Micro(RNA) managing by mTORC1. *Mol. Cell* 57, 575–576. doi: 10.1016/j.molcel.2015.02.006

Kalsner, L., and Chamberlain, S. J. (2015). Prader-Willi, Angelman, and 15q11-q13 duplication syndromes. *Pediatr. Clin. North Am.* 62, 587–606. doi: 10.1016/j.pcl.2015.03.004

Kong, N. R., and Jones, L. H. (2023). Clinical translation of targeted protein degraders. *Clin. Pharmacol. Therapeut.* 114, 558–568. doi: 10.1002/cpt.2985

Krumm, N., O'Roak, B. J., Shendure, J., and Eichler, E. E. (2014). A *de novo* convergence of autism genetics and molecular neuroscience. *Trends Neurosci.* 37, 95–105. doi: 10.1016/j.tins.2013.11.005

Laggerbauer, B., Ostareck, D., Keidel, E. M., Ostareck-Lederer, A., and Fischer, U. (2001). Evidence that fragile X mental retardation protein is a negative regulator of translation. *Hum. Mol. Genet.* 10, 329–338. doi: 10.1093/hmg/10.4.329

LaSalle, J. M., Reiter, L. T., and Chamberlain, S. J. (2015). Epigenetic regulation of UBE3A and roles in human neurodevelopmental disorders. *Epigenomics* 7, 1213–1228. doi: 10.2217/epi.15.70

Lee, J. A., Damianov, A., Lin, C. H., Fontes, M., Parikshak, N. N., Anderson, E. S., et al. (2016). Cytoplasmic Rbfox1 regulates the expression of synaptic and autism-related genes. *Neuron.* 89, 113–28. doi: 10.1016/j.neuron.2015.11.025

Lei, Z., Wang, J., Zhang, L., and Liu, C. H. (2021). Ubiquitination-dependent regulation of small GTPases in membrane trafficking: from cell biology to human diseases. *Front. Cell Dev. Biol.* 9:688352. doi: 10.3389/fcell.2021.688352

Li, D., Wang, Q., Bayat, A., Battig, M. R., Zhou, Y., Bosch, D. G., et al. (2024). Spliceosome malfunction causes neurodevelopmental disorders with overlapping features. *J. Clin. Invest.* 134:e171235. doi: 10.1172/JCI171235

Louros, S. R., and Osterweil, E. K. (2016). Perturbed proteostasis in autism spectrum disorders. *J. Neurochem.* 139, 1081–1092. doi: 10.1111/jnc.13723

Louros, S. R., Seo, S. S., Maio, B., Martinez-Gonzalez, C., Gonzalez-Lozano, M. A., Muscas, M., et al. (2023). Excessive proteostasis contributes to pathology in fragile X syndrome. *Neuron* 111, 508–525.e7. doi: 10.1016/j.neuron.2022.11.012

Madiraju, C., Novack, J. P., Reed, J. C., and Matsuzawa, S. (2022). K63 ubiquitination in immune signaling. *Trends Immunol.* 43, 148–162. doi: 10.1016/j.it.2021.12.005

Matlin, A. J., and Moore, M. J. (2007). Spliceosome assembly and composition. *Adv. Exp. Med. Biol.* 623, 14–35. doi: 10.1007/978-0-387-77374-2_2

Matsuo, Y., Uchihashi, T., and Inada, T. (2023). Decoding of the ubiquitin code for clearance of colliding ribosomes by the RQT complex. *Nat. Commun.* 14:79. doi: 10.1038/s41467-022-35608-4

Mazroui, R., Huot, M.-E., Tremblay, S., Filion, C., Labelle, Y., and Khandjian, E. W. (2002). Trapping of messenger RNA by Fragile X Mental Retardation protein into cytoplasmic granules induces translation repression. *Hum. Mol. Genet.* 11, 3007–3017. doi: 10.1093/hmg/11.24.3007

Mei, S., Ruan, H., Ma, Q., and Yao, W.-D. (2020). The ubiquitin-editing enzyme A20 regulates synapse remodeling and efficacy. *Brain Res.* 1727:146569. doi: 10.1016/j.brainres.2019.146569

Miroci, H., Schob, C., Kindler, S., Ölschläger-Schütt, J., Fehr, S., Jungenitz, T., et al. (2012). Makorin ring zinc finger protein 1 (MKRN1), a novel poly(A)-binding protein-interacting protein, stimulates translation in nerve cells. *J. Biol. Chem.* 287, 1322–1334. doi: 10.1074/jbc.M111.315291

Moccia, R., Chen, D., Lyles, V., Kapuya, E. E. Y., Kalachikov, S., Spahn, C. M. T., et al. (2003). An unbiased cDNA library prepared from isolated aplysia sensory neuron processes is enriched for cytoskeletal and translational mRNAs. *J. Neurosci.* 23, 9409–9417. doi: 10.1523/JNEUROSCI.23-28-09409.2003

Mulligan, M. R., and Bicknell, L. S. (2023). The molecular genetics of nELAVL in brain development and disease. *Eur. J. Hum. Genet.* 31, 1209–1217. doi: 10.1038/s41431-023-01456-z

Na, C.-H., Jones, D. R., Yang, Y., Wang, X., Xu, Y., and Peng, J. (2012). Synaptic protein ubiquitination in rat brain revealed by antibody-based ubiquitome analysis. *J. Proteome Res.* 11, 4722–4732. doi: 10.1021/pr300536k

Nalavadi, V. C., Muddashetty, R. S., Gross, C., and Bassell, G. J. (2012). Dephosphorylation-induced ubiquitination and degradation of FMRP in dendrites: a role in immediate early mGluR-stimulated translation. *J. Neurosci.* 32, 2582–2587. doi: 10.1523/JNEUROSCI.5057-11.2012

Osterweil, E. K., Chuang, S. C., Chubykin, A. A., Sidorov, M., Bianchi, R., Wong, R. K. S., et al. (2013). Lovastatin corrects excess protein synthesis and prevents epileptogenesis in a mouse model of fragile X syndrome. *Neuron* 77:243. doi: 10.1016/j.neuron.2012.01.034

Pandya, N. J., Meier, S., Tyanova, S., Terrigno, M., Wang, C., Punt, A. M., et al. (2022). A cross-species spatiotemporal proteomic analysis identifies UBE3A-dependent signaling pathways and targets. *Mol. Psychiat.* 27, 2590–2601. doi: 10.1038/s41380-022-01484-z

Park, J. K., Das, T., Song, E. J., and Kim, E. E. K. (2016). Structural basis for recruiting and shuttling of the spliceosomal deubiquitinase USP4 by SART3. *Nucl. Acids Res.* 44, 5424–5437. doi: 10.1093/nar/gkw218

Pelzer, C., Cabalzar, K., Wolf, A., Gonzalez, M., Lenz, G., and Thome, M. (2013). The protease activity of the paracaspase MALT1 is controlled by monoubiquitination. *Nat. Immunol.* 14, 337–345. doi: 10.1038/ni.2540

Pérez-Villegas, E. M., Ruiz, R., Bachiller, S., Ventura, F., Armengol, J. A., and Rosa, J. L. (2022). The HERC proteins and the nervous system. *Semin. Cell Dev. Biol.* 132, 5–15. doi: 10.1016/j.semcdb.2021.11.017

Pitts, S., Liu, H., Ibrahim, A., Garg, A., Felgueira, C. M., Begum, A., et al. (2022). Identification of an E3 ligase that targets the catalytic subunit of RNA Polymerase I upon transcription stress. *J. Biol. Chem.* 298:102690. doi: 10.1016/j.jbc.2022.102690

Popovitchenko, T., Thompson, K., Viljetic, B., Jiao, X., Kontonyiannis, D. L., Kiledjian, M., et al. (2016). The RNA binding protein HuR determines the differential translation of autism-associated FoxP subfamily members in the developing neocortex. *Sci. Rep.* 6:28998. doi: 10.1038/srep28998

Richard, T. J. C., Herzog, L. K., Vornberger, J., Rahmanto, A. S., Sangfelt, O., Salomons, F. A., et al. (2020). K63-linked ubiquitylation induces global sequestration of mitochondria. *Sci. Rep.* 10:22334. doi: 10.1038/s41598-020-78845-7

Romero-Ayuso, D. (2021). Future challenges in research in children with neurodevelopmental disorders. *Children* 8:328. doi: 10.3390/children8050328

Ronchi, V. P., and Haas, A. L. (2012). Measuring rates of ubiquitin chain formation as a functional readout of ligase activity. *Methods Mol. Biol.* 832, 197–218. doi: 10.1007/978-1-61779-474-2_14

Saeed, B., Deligne, F., Brillada, C., Dünser, K., Ditengou, F. A., Turek, I., et al. (2023). K63-linked ubiquitin chains are a global signal for endocytosis and contribute to selective autophagy in plants. *Curr. Biol.* 33, 1337–1345.e5. doi: 10.1016/j.cub.2023.02.024

Saez, I., Gerbracht, J. V., Koyuncu, S., Lee, H. J., Horn, M., Kroef, V., et al. (2020). The E3 ubiquitin ligase UBR5 interacts with the H/ACA ribonucleoprotein complex and regulates ribosomal RNA biogenesis in embryonic stem cells. *FEBS Lett.* 594, 175–188. doi: 10.1002/1873-3468.13559

Sawicka, K., Hale, C. R., Park, C. Y., Fak, J. J., Gresack, J. E., Driesche, S. J. V., et al. (2019). FMRP has a cell-type-specific role in CA1 pyramidal neurons to regulate autism-related transcripts and circadian memory. *eLife* 8:46919. doi: 10.7554/eLife.46919.sa2

Schieweck, R., Ninkovic, J., and Kiebler, M. A. (2021). RNA-binding proteins balance brain function in health and disease. *Physiol. Rev.* 47:2019. doi: 10.1152/physrev.00047.2019

Schreiber, J., Végh, M. J., Dawitz, J., Kroon, T., Loos, M., Labonté, D., et al. (2015). Ubiquitin ligase TRIM3 controls hippocampal plasticity and learning by regulating synaptic γ-actin levels. *J. Cell Biol.* 211, 569–586. doi: 10.1083/jcb.201506048

Seo, S. S., Louros, S. R., Anstey, N., Gonzalez-Lozano, M. A., Harper, C. B., Verity, N. C., et al. (2022). Excess ribosomal protein production unbalances translation in a model of Fragile X Syndrome. *Nat. Commun.* 13:30979. doi: 10.1038/s41467-022-30979-0

Sharma, S. D., Metz, J. B., Li, H., Hobson, B. D., Hornstein, N., Sulzer, D., et al. (2019). Widespread alterations in translation elongation in the brain of juvenile Fmr1 knockout mice. *Cell Rep.* 26, 3313–3322.e5. doi: 10.1016/j.celrep.2019.02.086

Shigeoka, T., Koppers, M., Wong, H. H. W., Lin, J. Q., Cagnetta, R., Dwivedy, A., et al. (2019). On-site ribosome remodeling by locally synthesized ribosomal proteins in axons. *Cell Rep.* 29, 3605–3619.e10. doi: 10.1016/j.celrep.2019.11.025

Silva, G. M., Finley, D., and Vogel, C. (2015). K63 polyubiquitination is a new modulator of the oxidative stress response. *Nat. Struct. Mol. Biol.* 22, 116–123. doi: 10.1038/nsmb.2955

Song, E. J., Werner, S. L., Neubauer, J., Stegmeier, F., Aspden, J., Rio, D., et al. (2010). The Prp19 complex and the Usp4Sart3 deubiquitinating enzyme control reversible ubiquitination at the spliceosome. *Genes Dev.* 24, 1434–1447. doi: 10.1101/gad.1925010

Stegmeier, F., Rape, M., Draviam, V. M., Nalepa, G., Sowa, M. E., Ang, X. L., et al. (2007). Anaphase initiation is regulated by antagonistic ubiquitination and deubiquitination activities. *Nature* 446, 876–881. doi: 10.1038/nature05694

Su, C. H., Dhananjaya, D., and Tarn, W. Y. (2018). Alternative splicing in neurogenesis and brain development. *Front. Mol. Biosci.* 2018:12. doi: 10.3389/fmolb.2018.00012

Sun, C., Desch, K., Nassim-Assir, B., Giandomenico, S. L., Nemcova, P., Langer, J. D., et al. (2023). An abundance of free regulatory (19S) proteasome particles regulates neuronal synapses. *Science* 380:eadf2018. doi: 10.1126/science.adf2018

Sun, J., Liu, Y., Moreno, S., Baudry, M., and Bi, X. (2015). Imbalanced mechanistic target of rapamycin C1 and C2 activity in the cerebellum of Angelman syndrome mice impairs motor function. *J. Neurosci.* 35, 4706–4718. doi: 10.1523/JNEUROSCI.4276-14.2015

Tai, H. C., and Schuman, E. M. (2008). Ubiquitin, the proteasome and protein degradation in neuronal function and dysfunction. *Nat. Rev. Neurosci.* 9, 826–838. doi: 10.1038/nrn2499

Thapa, P., Shanmugam, N., and Pokrzywa, W. (2020). Ubiquitin signaling regulates RNA biogenesis, processing, and metabolism. *BioEssays* 2020:171. doi: 10.1002/bies.201900171

Thomson, S. R., Seo, S. S., Barnes, S. A., Louros, S. R., Muscas, M., Dando, O., et al. (2017). Cell-type-specific translation profiling reveals a novel strategy for treating fragile X syndrome. *Neuron* 95, 550–563.e5. doi: 10.1016/j.neuron.2017.07.013

Thrower, J. S., Hoffman, L., Rechsteiner, M., and Pickart, C. M. (2000). Recognition of the polyubiquitin proteolytic signal. *EMBO J.* 19, 94–102. doi: 10.1093/emboj/19.1.94

Trost, B., Thiruvahindrapuram, B., Chan, A. J. S., Engchuan, W., Higginbotham, E. J., Howe, J. L., et al. (2022). Genomic architecture of autism from comprehensive whole-genome sequence annotation. *Cell* 185, 4409–4427.e18. doi: 10.1016/j.cell.2022.10.009

Valdez-Sinon, A. N., Lai, A., Shi, L., Lancaster, C. L., Gokhale, A., Faundez, V., et al. (2020). Cdh1-APC regulates protein synthesis and stress granules in neurons through an FMRP-dependent mechanism. *iScience* 23:101132. doi: 10.1016/j.isci.2020.101132

Wang, Y., Liu, J., Huang, B., Xu, Y. M., Li, J., Huang, L. F., et al. (2015). Mechanism of alternative splicing and its regulation. *Biomed. Rep.* 3:152. doi: 10.3892/br.2014.407

Wang, Y., Yang, L., Zhang, X., Cui, W., Liu, Y., Sun, Q., et al. (2019). Epigenetic regulation of ferroptosis by H2B monoubiquitination and p53. *EMBO Rep.* 20, e47563. doi: 10.15252/embr.201847563

Wassarman, D. A., and Steitz, J. A. (1992). Interactions of small nuclear RNA's with precursor messenger RNA during *in vitro* splicing. *Science* 257, 1918–1925. doi: 10.1126/science.1411506

Werner, A., Iwasaki, S., McGourty, C., Medina-Ruiz, S., Teerikorpi, N., Fedrigo, I., et al. (2015). Cell fate determination by ubiquitin-dependent regulation of translation. *Nature* 525, 523–527. doi: 10.1038/nature14978

Wilkerson, J. R., Ifrim, M. F., Valdez-Sinon, A. N., Hahn, P., Bowles, J. E., Molinaro, G., et al. (2023). FMRP phosphorylation and interactions with Cdh1 regulate association with dendritic RNA granules and MEF2-triggered synapse elimination. *Neurobiol. Dis.* 182:106136. doi: 10.1016/j.nbd.2023.106136

Winden, K. D., Pham, T. T., Teaney, N. A., Ruiz, J., Chen, R., Chen, C., et al. (2023). Increased degradation of FMRP contributes to neuronal hyperexcitability in tuberous sclerosis complex. *Cell Rep.* 42:112838. doi: 10.1016/j.celrep.2023.112838

Zheng, L., Ding, H., Lu, Z., Li, Y., Pan, Y., Ning, T., et al. (2008). E3 ubiquitin ligase E6AP-mediated TSC2 turnover in the presence and absence of HPV16 E6. *Genes Cells* 13, 285–294. doi: 10.1111/j.1365-2443.2008.01162.x

Zhou, F., Xie, F., Jin, K., Zhang, Z., Clerici, M., Gao, R., et al. (2017). USP4 inhibits SMAD4 monoubiquitination and promotes activin and BMP signaling. *EMBO J.* 36, 1623–1639. doi: 10.15252/embj.201695372

Zhou, Z., Licklider, L. J., Gygi, S. P., and Reed, R. (2002). Comprehensive proteomic analysis of the human spliceosome. *Nature* 419, 182–185. doi: 10.1038/nature01031

Zoghbi, H. Y., and Bear, M. F. (2012). Synaptic dysfunction in neurodevelopmental disorders associated with autism and intellectual disabilities. *Cold Spring Harb. Perspect. Biol.* 4:a009886. doi: 10.1101/cshperspect.a009886

Frontiers in Molecular Neuroscience

TYPE Review
PUBLISHED 18 September 2024
DOI 10.3389/fnmol.2024.1397378

OPEN ACCESS

EDITED BY
Clémence Bernard,
University of Exeter, United Kingdom

REVIEWED BY
Anne-Sophie Hafner,
Radboud University, Netherlands
Max Koppers,
VU Amsterdam, Netherlands

*CORRESPONDENCE
Richard Taylor
richard.taylor@kcl.ac.uk
Nikolas Nikolaou
nn456@bath.ac.uk

RECEIVED 07 March 2024
ACCEPTED 01 August 2024
PUBLISHED 18 September 2024

CITATION
Taylor R and Nikolaou N (2024) RNA in axons, dendrites, synapses and beyond. *Front. Mol. Neurosci.* 17:1397378. doi: 10.3389/fnmol.2024.1397378

RNA in axons, dendrites, synapses and beyond

Richard Taylor[1]* and Nikolas Nikolaou[2]*

[1]Centre for Developmental Neurobiology, Institute of Psychiatry, Psychology and Neuroscience, King's College London, London, United Kingdom, [2]Department of Life Sciences, University of Bath, Bath, United Kingdom

In neurons, a diverse range of coding and non-coding RNAs localize to axons, dendrites, and synapses, where they facilitate rapid responses to local needs, such as axon and dendrite extension and branching, synapse formation, and synaptic plasticity. Here, we review the extent of our current understanding of RNA subclass diversity in these functionally demanding subcellular compartments. We discuss the similarities and differences identified between axonal, dendritic and synaptic local transcriptomes, and discuss the reported and hypothesized fates and functions of localized RNAs. Furthermore, we outline the RNA composition of exosomes that bud off from neurites, and their implications for the biology of neighboring cells. Finally, we highlight recent advances in third-generation sequencing technologies that will likely provide transformative insights into splice isoform and RNA modification diversity in local transcriptomes.

KEYWORDS

messenger RNA (mRNA), local mRNA translation, cleavage and polyadenylation, stability and degradation, intron retaining RNA (IR RNA), microRNA (miRNA), long non coding RNA (lncRNA), circular RNA (circRNA)

Introduction

Neurons are highly polarized cells with often sophisticated morphologies, resulting in their axons, dendrites, and synapses (collectively termed neurites) being situated several millimeters from the soma. In some cases axons extend beyond a meter, and dendrites over a centimeter (Holt et al., 2019). As functionally and metabolically demanding cell compartments (Harris et al., 2012; Faria-Pereira and Morais, 2022; Yang et al., 2023), neurites require highly efficient protein production and cycling for their development and maintenance. This demand calls for elaborate mechanisms beyond centralized production in the soma and subsequent delivery to neurites (Hanus and Schuman, 2013). Exclusively somatic protein synthesis would delay any changes to local proteomes required for dynamic responses to locally received stimuli (Fonkeu et al., 2019). Furthermore, the short half-life of many neurite-localized proteins indicates they would not survive a journey centimeters in length, or not last long following their arrival (Piper and Holt, 2004; Sun and Schuman, 2022).

However, over the last few decades, extensive decentralization of these processes has been uncovered (Holt et al., 2019; Sun and Schuman, 2023). The delivery of ribonucleic acid (RNA) molecules to the far-flung limits of neurons enables agile, responsive, on-site production of proteins exactly when they are required. Early studies utilizing *in situ* hybridisation identified numerous messenger RNAs (mRNAs) localized to neurites. More recently, a plethora of high-throughput sequencing studies have more thoroughly characterized local transcriptomes, providing detailed global insight into the different types of RNAs enriched in neurites, revealing those that are common, as well as those specifically enriched in either axons, dendrites, or synapses. Such studies have utilized various mammalian and non-mammalian sample types, including compartmentalized culture of embryonic stem cell (ESC)- and induced pluripotent stem cell (iPSC)-derived neurons, embryonic and adult primary neurons, dorsal root ganglia (DRG) explants, or dissection of neuropil (axon- and dendrite-enriched tissue).

Whilst most studies characterizing axonal, dendritic, and synaptic transcriptomes thus far have focused on mRNA expression, this accounts for up to only 5% of total RNA in a cell, with the rest being non-coding RNAs (ncRNAs)—predominantly ribosomal RNA (rRNA) and transfer RNA (tRNA) (Wu et al., 2014; Deng et al., 2022). However, the proportions of each type of RNA specifically within axons, dendrites, and synapses is unknown. Indeed, more recently an increasing number of studies have turned their focus towards elucidating diversity amongst local ncRNAs.

In this review, we highlight the various classes of RNAs that localize to axons, dendrites and synapses, as well as exosomes, which enable the transfer of RNAs to neighbouring cells when secreted (Figure 1). We summarize the key datasets characterizing the classes found within each subcellular compartment across different sample types. We subsequently compare the transcriptomes for the different subcellular compartments. We go on to discuss the fates and functions of the different identified RNA classes, and their implications for the development and maintenance of each respective compartment. Finally, we outline recent advances in third-generation sequencing technologies, that hold the power to revolutionize our understanding of splice isoform diversity and RNA modifications in local transcriptomes.

Messenger RNAs (mRNAs)

mRNA diversity in axons, dendrites, and synapses

The most extensively studied RNAs in neurites are those that encode proteins. mRNAs were first identified within dendrites by *in situ* hybridization (Davis et al., 1987; Garner et al., 1988), and later in axons [reviewed in Steward (1997); **Figure 1**]. Before these findings, it was assumed that all neurite-localized proteins were trafficked from the soma (Alvarez et al., 2000). Our first insight into the notion of local translation was the observation of polysomes sitting immediately beneath post-synaptic sites within dendrites (Steward and Levy, 1982; Eberwine et al., 2001). Later, mRNAs were shown to associate with polysomes and undergo translation, underpinning plasticity (Holt and Schuman, 2013). More recently, monosomes were discovered to form the dominant ribosomal population within neurites (Biever et al., 2020).

Below, we outline the main findings from key studies characterizing the transcriptomes of axons, dendrites, and synapses. We compare datasets on a compartment-specific basis, before going on to compare axonal versus dendritic versus synaptic transcriptomes.

While early studies identified mRNAs for a small number of genes in neurites by *in situ* hybridisation, more recent studies have utilized high-throughput bulk RNA-Seq experiments to assay global populations of mRNAs. Datasets from 20 studies, most using high-throughput sequencing, were compiled and analyzed using a common pipeline allowing for their comparison, and the identification of a core neurite transcriptome (von Kügelgen and Chekulaeva, 2020). The datasets covered a range of sample types including neuroblastoma lines, primary neurons, ESC- and iPSC-derived neurons of various subtypes, and DRG explants, across mouse, rat, and human. In most cases, compartmentalized culture was performed using devices such as transwell inserts, where cells sit on a membrane containing tiny pores through which neurites extend and grow along the lower membrane surface (Taylor et al., 2022; Taylor and Houart, 2024). In this way, transwell inserts enable the separate isolation of neurite tissue, which is likely mostly axons, with dendrites contributing approximately only 10% of the neurite population (Rotem et al., 2017; Nijssen et al., 2018). Several of the included datasets were generated from neuropil dissection from tissue sections, however, where dendrites are well represented. The integrated analysis revealed a common set of transcripts as the most abundant, a core conserved neurite transcriptome, dominated by mRNAs encoding ribosomal and cytoskeletal proteins, with mitochondrial and synaptic proteins also well represented (**Table 1**; von Kügelgen and Chekulaeva, 2020). Another way to characterize the neurite transcriptome besides mRNA abundance is by focusing on transcripts enriched in neurites compared to the soma, indicative of active localisation, suggestive of neurite-specific functions. While mRNAs encoding common axonal and synaptic markers were often abundant in neurites, they were not typically enriched (von Kügelgen and Chekulaeva, 2020). 61 mRNAs were consistently neurite-enriched across datasets, mostly encoding ribosomal proteins. Many of these transcripts were shown by other studies to associate with ribosomes in neurites indicating their local translation (**Table 1**).

Transcriptomic variation in neurites owing to different sample types was unclear, possibly due to neurite populations comprising mixtures of axons and dendrites, and added heterogeneity, such as multiple neuron sub-types being represented (von Kügelgen and Chekulaeva, 2020). Also, primary neuron cultures likely contain some glia. Clear signatures were also unidentifiable for pre- or post-synaptic markers, possibly due to the maturity stage of neurites. Alternatively, the ratio of axons to dendrites present may not favor the formation of mature synapses in large quantities. Such findings highlight the importance of obtaining pure neuron and neurite type populations to explore questions of local transcriptomic diversity.

Other studies that have focused on characterizing specifically either axonal or dendritic transcriptomes have provided subcellular compartment-specific and temporal-related insights. Axons from embryonic and adult rat DRG sensory neurons assayed by microarray, identified significant differences in the pools of mRNAs between these stages (Gumy et al., 2011). Similar numbers of mRNAs were present with substantial overlap in mRNA identity. At both stages, axons were enriched for mRNAs encoding ribosomal and mitochondrial proteins. Those uniquely enriched in embryonic axons encoded proteins involved in axon guidance and growth, whilst those uniquely enriched in adult axons encoded those involved in inflammation and immunity. In a later study, RNA-Seq from embryonic mouse DRG sensory axons revealed a high degree of similarity across species, identifying 80% of genes detected in the embryonic rat DRG axons (Minis et al., 2014), as well as

TABLE 1 Most abundant transcripts within the core neurite transcriptome identified from analysis of many neurite datasets, and whether they have been reported to undergo local translation.

Gene name	Function	Reported neurite ribosome association
Actb	Cytoskeleton	Yes
Tpt1	Outgrowth formation, mitochondrial regulation	Yes
Rpl4	Ribosomal protein	Yes
Ybx1	RNA binding protein	No
Rps12	Ribosomal protein	Yes
Rps8	Ribosomal protein	Yes
Atp5b	Mitochondrial function	Yes
Ywhae	Outgrowth formation	Yes
Rpl6	Ribosomal protein	No
Npm1	Nuclear protein; ribosome associated	Yes
Map1b	Cytoskeleton	No
Fau	Ribosomal protein	Yes
Calm1	Calcium regulation	Yes
Rps3a1	Ribosomal protein	Yes
Kif5c	Synaptic function	Yes
Gap43	Outgrowth formation, synaptic function	No
Kif5a	Axonal transport	Yes
Park7	Oxidative stress protection	Yes
Arl3	Membrane trafficking	No
Vdac3	Mitochondrial regulation	No
Eef1a1	Translation machinery	Yes
Actg1	Cytoskeleton	Yes
Eef2	Translation machinery	Yes
Rplp1	Ribosomal protein	Yes
Rpl23	Ribosomal protein	Yes

Adapted from von Kügelgen and Chekulaeva (2020).

detecting many more. Gene ontology (GO) categories for mRNAs enriched in this dataset included translation, in line with the rat study, and other categories including sequence-specific DNA binding, extracellular matrix, and immune response (Minis et al., 2014). While DNA binding terms may be initially surprising, this reflects the known axonal localisation of transcripts encoding classically nuclear proteins, including transcription factors thought to mediate axon-to-nucleus signaling (Ji and Jaffrey, 2014; Twiss and Merianda, 2015). Such axonal localisation of nuclear proteins and their mRNAs has been reported by many studies since, including *in vivo* (Alon et al., 2021).

In line with the observations from adult rat DRG axons (Gumy et al., 2011), RNA-Seq on axoplasm from adult rat ventral root motor axons revealed enrichment in GO terms associated with translation, mitochondria, and the cytoskeleton (Farias et al., 2020). Mitochondrial and ribosomal genes also dominate enrichments in human iPSC (hiPSC)-derived motor neurons grown in transwell inserts, where axons strongly dominate the neurite population (Maciel et al., 2018), and in mouse embryonic motor axons following culture in microfluidic chambers (Briese et al., 2016), respectively.

Laser capture and microdissection of specifically the growth cones of retinal ganglion cell (RGC) axons in mouse and *Xenopus laevis* revealed a surprisingly large number of mRNAs belonging to similar functional categories including protein synthesis, oxidative phosphorylation, and signaling. Moreover, mRNA repertoire in growth cones was shown to be regulated dynamically with age and become increasingly complex with time as it advances along the retinotectal pathway (Zivraj et al., 2010). Another study identified enrichment primarily of transcripts containing the non-canonical 5′ TOP (5′ termini oligopyrimidine) motif in RNA-Seq from just the growth cones of axons *in vivo* (Poulopoulos et al., 2019). This motif is found specifically in transcripts encoding ribosomal proteins and translation initiation factors, and acts as an ON/OFF switch controlling translation through its direct responsiveness to mTOR. By this mechanism, the authors speculate that 5′ TOP transcripts enriched in the growth cone may be translated upon mTOR signaling in response to target-derived growth signals, driving axonal growth.

Studies focused on elucidating dendrite-specific transcriptomes have often taken single cell approaches owing to difficulties in isolating dendrite tissue from somas (Middleton et al., 2019; Perez et al., 2021). Single cell RNA-Seq (scRNA-Seq) analysis of mouse primary hippocampal neurons identified dendrite enrichment of GO terms related to the ribosome and mitochondria, including ribosomal subunits, mitochondrial membrane, and respiratory chain complex (Steward, 1997; Middleton et al., 2019).

Early *in situ* hybridisation studies indicated that dendrites of different neuronal sub-types contain distinct mRNAs (Steward, 1997; Eberwine et al., 2001). Comparison of glutamatergic and GABAergic rat hippocampal interneurons following scRNA-Seq identified easily discernible cell type-specific transcriptomic differences between somas (Perez et al., 2021). *Map1a* and *Calm1* were the most abundant mRNAs in dendrites of both glutamatergic and GABAergic neurons. While transcriptomic variation across dendrites was more subtle, some sub-type specificity was observed in those from different GABAergic neuron types,

At the sub-dendritic level, mouse hippocampal pyramidal neurons observed *in situ* following expansion microscopy, showed differential distribution of mRNAs in spines compared with adjacent dendrite (Alon et al., 2021). The most abundant transcripts in spines were *Shank1, Adenyl cyclase1* and *Kif5a,* specifically localized here along with *Map1a* and *Map2a*. *Camk2a* and *Ddn* were enriched in dendrites compared with spines and cell bodies. Such data indicates additional layers of compartmentalisation.

Numerous studies have focused on the isolation and dissection of the transcriptomes of pre- and post-synapses. Indeed, RNA-Seq on synaptoneurosomes purified from the forebrains of 10-week-old mice revealed dominance of mRNAs pertaining to cellular compartment ontology terms including membrane, synapse, neuronal projection, and post-synaptic density, with biological process ontology terms including transport, cell adhesion and long-term synaptic potentiation (Simbriger et al., 2020). Similarly, synaptosomes from 3-month-old mouse hippocampus revealed enrichment for synapse-related ontologies, with KEGG-pathway

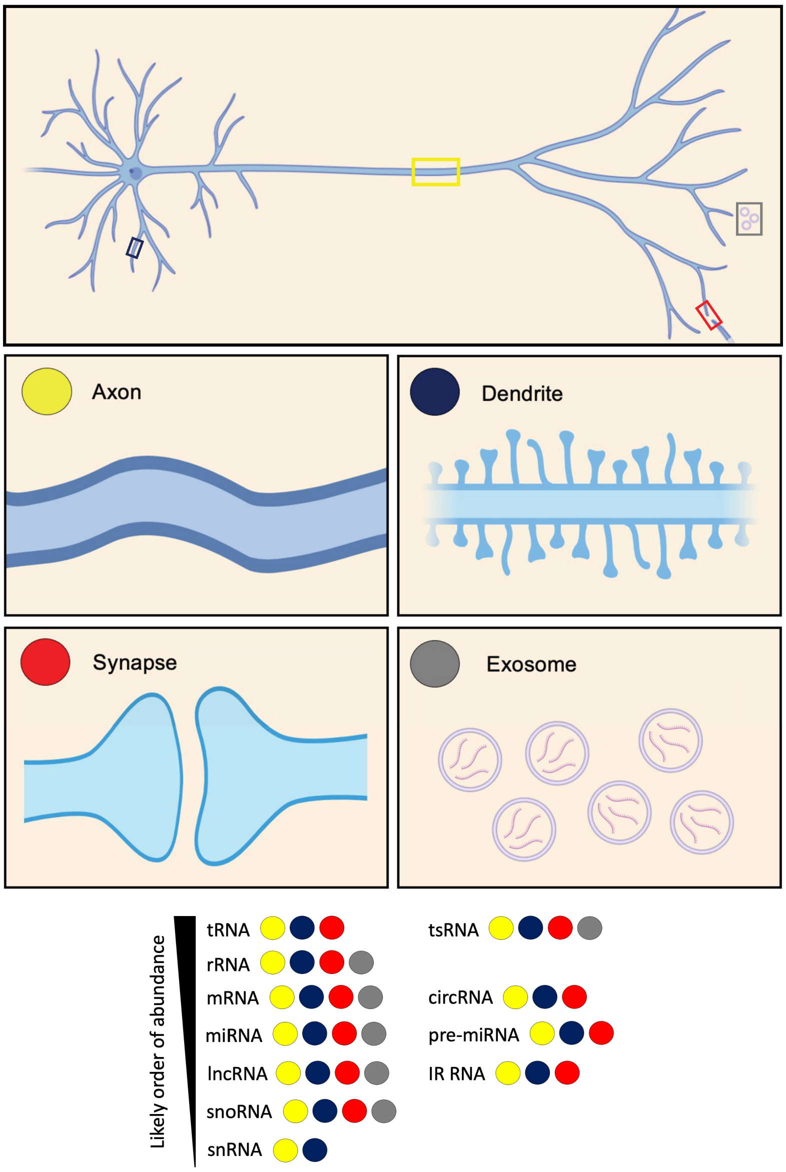


FIGURE 1
Diversity of RNA types present in neurites and exosomes. RNA types categorized based on their identification in axons, dendrites, synapses, and exosomes. These RNA types include: transfer RNA (tRNA); tRNA-derived small RNA (tsRNA); ribosomal RNA (rRNA); messenger RNA (mRNA); circular RNA (circRNA); microRNA (miRNA); pre-microRNA (pre-miRNA); long non-coding RNA (lncRNA); intron-retained RNA (IR-RNA); small nucleolar RNA (snoRNA); and small nuclear RNA (snRNA). RNA types are listed according to their likely order of abundance.

analysis identifying the strongest enrichments in glutamatergic synapses, cAMP signaling and long-term potentiation, as well as presence of terms linked to mitochondrial function (Epple et al., 2021). Mature mouse forebrain synaptosomes enriched for vGLUT1+ pre-synaptic terminals, reflecting excitatory synapses, versus a non-purified population of synaptosomes and neurite material, identified 468 enriched transcripts dominated by GO terms including pre-synaptic active zone and ribosomal proteins (Hafner et al., 2019). The most enriched transcripts within the group included known pre-synaptic (*Stx6*, *Bsn*, *Rims1-3*) and signaling molecules (*Sergef*, *Rapgef4*). Transcripts less well represented in the vGLUT1+ synaptosomes compared with the general population included many coding for GABA and AMPA families - post-synaptic and dendritic components.

In summary, the studies described characterizing the transcriptomes of either a neurite mix, or exclusively axons or dendrites, identify overwhelming enrichment of mRNAs encoding factors associated with translation. These include constituent ribosomal proteins, and translation initiation and elongation factors. Such findings are intriguing given that ribosome production classically occurs in the nucleolus. Indeed, recent studies, including some in neurites, have reported that ribosomes are locally remodeled through incorporation of newly synthesized proteins, facilitating specialization or repair (Mathis et al., 2017; Shigeoka et al., 2019; Fusco et al., 2021). Future studies aimed towards dissecting ribosomal specificity underlying mRNA translation, and local changes to ribosomal makeup, will likely shed new light on the mechanisms by which local transcriptomes replenish and shape the neurite proteome. Mitochondria-related ontologies are also well represented across neurite types, reflecting their high metabolic demand. It is perhaps surprising that membrane and signaling proteins are not more dominant, however mRNA copy number often does not directly correlate with the number of proteins produced (Edfors et al., 2016; Zappulo et al., 2017). The mRNAs found enriched in pre- and post-synapses are highly specialized based on the functions of these compartments and the proteins found within them. It will be intriguing to see if there are additional sub-compartments within axons and dendrites that serve as hubs for specific mRNA pools. Indeed, interaction with different subcellular organelles within neurites can be indicative of their fate or translational status (see below).

We will now discuss the fates of localized mRNAs in both axonal and dendritic arbors as well as synaptic compartments.

Fates and functions

Local translation

Most mRNAs are transported to neurites within RNA granules, which are dynamic, membrane-less cellular structures that contain mRNA molecules and various proteins (Dalla Costa et al., 2021). Interestingly, recent imaging experiments showed that the dynamics of endogenous RNA granules correlate with new branch emergence and branch stabilization (Wong et al., 2017), indicating that localized mRNAs play a role in the formation and stabilization of neural connections. Traditionally, protein synthesis was thought to occur exclusively in the soma cytoplasm. However, it has become increasingly evident that local mRNA translation can occur, and is widespread, at specific subcellular locations within neurons. During local protein synthesis, mRNA molecules are translated into proteins near the site they are required. Such local protein synthesis sites range from axonal and dendritic branch points to developing and mature pre- and post-synaptic compartments, as well as near cellular organelles (**Figure 2**). It is thought that up to half of the proteome in neurites has local protein synthesis being the predominant source (Zappulo et al., 2017; Glock et al., 2021).

Within axons, dendrites and synaptic compartments, an increasing number of studies have begun to reveal the importance of associations between organelles and ribonucleoprotein (RNP) complexes for local translation (Pushpalatha and Besse, 2019; Vargas et al., 2022). Indeed, RNA-bearing Rab7a late endosomes were found to pause on mitochondria along RGC axons, facilitating translation of mRNAs encoding mitochondrial proteins (Cioni et al., 2019). This mode of local translation was shown to be important for mitochondrial function and axonal viability. In another study, tethering of certain transcripts to axonal mitochondria has been shown to be important for their translation to maintain the mitophagy pathway (Harbauer et al., 2022). In a late-endosomme independent manner, *PINK1* mRNAs require tethering to the mitochondrial outer membrane by Synaptojanin2 (SYNJ2), for their transport and translation (Harbauer et al., 2022). Intriguingly, translation of the PINK1 mitochondrial targeting sequence was also required for such transport, suggesting a local translated peptide was essential for the localisation of its own transcript to neurites. Other studies have identified important roles for the endoplasmic reticulum (ER) in local translation. It was recently shown that ribosomes associate with ER upon activation of local translation in motor axonal growth cones following their stimulation with brain-derived neurotrophic factor (BDNF) (Deng et al., 2021). It is likely that these ribosomes translate membrane and secreted proteins, classically translated at the rough ER, which was not known to occupy axons prior. Another study also showed ribosomes contacting ER tubules in a translation-dependent manner, in a process facilitated by the axonal ribosome/mRNA receptor P180/RRBP1 (Koppers et al., 2022). Future studies will reveal the contribution of other organelles to local translation in neurites, and identify which mRNAs require specific organelles for the process.

Global pools of locally translated mRNAs in neurites have been revealed by studies using novel ribosome capturing and RNA sequencing techniques. One such technique was the development of axon-TRAP-RiboTag (Shigeoka et al., 2016), utilizing a mouse line harboring a modified allele of the ribosomal Rpl22 protein fused to a HA tag (Rpl22-HA), induced by the action of a Cre recombinase (Sanz et al., 2009). Using a RGC-specific Cre line, full-length mRNAs pulled-down with HA-tagged ribosomes revealed the local translatome within RGC axons at multiple stages (Shigeoka et al., 2016). This identified a dominance of mRNAs encoding proteins involved in vesicle-mediated transport and calcium-mediated signaling. Ribosome immunoprecipitation approaches have also been used to identify locally translated transcripts in dendrites isolated from adult mouse hippocampus, revealing a dominance of mRNAs encoding translation and cytoskeletal proteins (Ainsley et al., 2014). Transcripts encoding nuclear proteins, including histones, were also observed, as in axons (see above section on mRNA diversity).

An alternative method for determining which proteins are actively translated locally is ribosome footprinting/profiling (Ingolia et al., 2012). Also known as Ribo-Seq (ribosome sequencing) or ART-Seq (active mRNA translation sequencing), it provides a snapshot, revealing RNA fragments/"footprints" protected by ribosomes caught during active translation. To identify and quantify the transcriptome and translatome in cell bodies (somata) as well as dendrites and axons, a recent study performed simultaneous RNA-Seq and Ribo-Seq from micro-dissected hippocampal rodent brain slices (Glock et al., 2021). The study led to the identification of more than 800 mRNAs whose dominant source of translation is the neuropil, suggesting that many axonal/dendritic and synaptic proteins arise mostly from local translation (Glock et al., 2021). But how do these localized mRNAs undergo local protein synthesis? During translation in the soma, multiple ribosomes can occupy an individual mRNA (a complex called a polysome), resulting in the simultaneous generation of multiple copies of the encoded protein. A recent study showed that monosomes (single ribosomes), as opposed to polysomes, are the predominant ribosome population in neuronal processes (Biever et al., 2020). Indeed, measuring ribosome density on transcripts in synaptic neuropil, revealed monosomes predominantly elongate key synaptic transcripts in both dendritic and axon terminals (Biever et al., 2020). One possible explanation for the difference between somatic and local mRNA translation that could explain the high abundance of monosomes in the neuropil, is the production of a more diverse set of proteins from a limited pool of available ribosomes found at synapses (Ostroff et al., 2017).

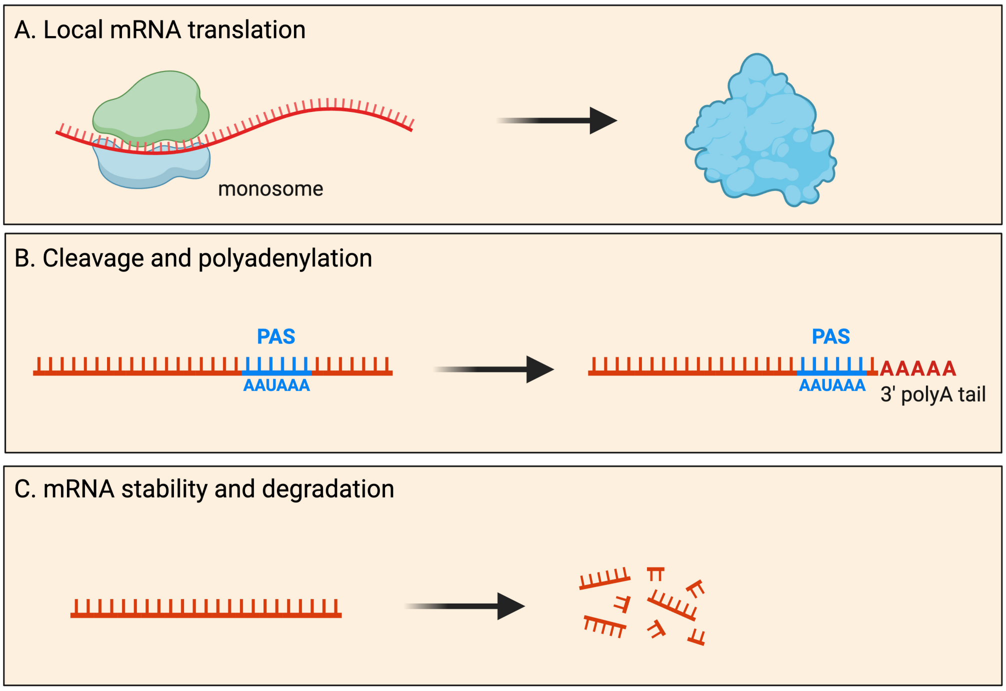


FIGURE 2
Fates of localized mRNAs in neurons. mRNA transcripts are transported into neurites within RNA granules. Within such local compartments mRNAs have been shown to undergo processing that includes: **(A)** mRNA translation for the local production of new proteins upon demand; **(B)** cleavage and polyadenylation of the 3′ UTR at the polyadenylation signal (PAS); and **(C)** mRNA stability as well as degradation.

Local protein synthesis is a highly regulated process, with most local transcripts not translated by default. Some of the most abundant transcripts in neurites seemingly do not associate with ribosomes (**Table 1**; von Kügelgen and Chekulaeva, 2020). Studies comparing the mRNA species constituting local transcriptomes and those associating with ribosomes, reveal that only specialized subsets of transcripts become translated, in a spatio-temporal fashion. Furthermore, ribosomal footprinting data from synaptoneurosomes reported that mRNAs undergoing translation were associated with different ontologies (mitochondrial and extracellular matrix and exosome proteins) to those generally dominant (see above in mRNA diversity section) (Simbriger et al., 2020).

How dynamic is the local translatome? Certain mRNAs encoding regulators of protein and energy homeostasis, and those associated with vesicle-mediated transport and calcium-mediated signaling are translated regardless of developmental stage (Shigeoka et al., 2016). Other mRNAs are dynamically regulated during development and maturation, suggesting that local translation plays an important role in the homeostasis of neurites. The translatome of younger axons was enriched for GO terms such as neuron projection morphogenesis (Shigeoka et al., 2016). Contrastingly, the adult axonal translatome was found to have strong links to axon survival, neurodegenerative disease, and neurotransmission, with key components of the trans-SNARE complex, which mediates neurotransmitter exocytosis, being highly translated in mature axons (Shigeoka et al., 2016). The findings indicating that axonal mRNA translation persists in adult CNS axons were intriguing because it has been controversial whether mature CNS axon terminals can synthesize proteins at all, partly because of early studies detecting few or no ribosomes in mature axons (Koenig et al., 2000). Therefore, these findings showed conclusively a unique adult local translatome is present in mature axons, whose main role is likely to be the regulation of synapse function. In contrast, local translation of transcripts involved in axonal and dendritic elongation, branching, pruning, synaptogenesis, and synaptic transmission occurs developmentally (Shigeoka et al., 2016; Biever et al., 2020), indicating the process has an equally crucial role in regulating neuronal connectivity and synaptic plasticity. Indeed, local translation is thought to enable neuronal cells to respond to signals from the environment. For instance, extracellular cues (e.g., Netrin-1, BDNF, Sema3A) were found to differentially influence axonal synthesis of multiple proteins in a cue-specific and temporally dynamic manner. Interestingly, the synthesis of proteasomal subunits (α and β type), some ribosomal proteins, histones, and methyltransferases is differentially regulated in response to such cues (Cagnetta et al., 2018). The significance of local mRNA translation in neurite growth is supported by functional experiments in *Xenopus laevis* RGC axons (Wong et al., 2017). Here, local protein synthesis was found to be essential for proper axon arbor formation *in vivo*, as inhibition of local translation or knockdown of local β-actin synthesis caused a marked reduction in axon branching dynamics and arbor complexity (Wong et al., 2017).

Local protein synthesis is also required for synaptic plasticity. At synapses, local protein synthesis was found to be differentially recruited to drive compartment-specific phenotypes that may underlie different forms of plasticity (Hafner et al., 2019). Evidence for a role of local translation in synaptic plasticity comes from a study utilizing dissociated rat hippocampal neuron cultures. During basal synaptic transmission, the amount of locally synthesized proteins detected at a synapse was correlated with its level of ongoing spontaneous activity. Plasticity induced by single-spine stimulations or by a global activity manipulation resulted in a significant increase in local protein synthesis (Sun et al., 2021). Similarly, depolarization of primary cortical neurons caused rapid reprogramming of dendritic protein expression (Hacisuleyman et al., 2024).

Many locally translated transcripts interact with RNA binding protein (RBPs) through sequences found within the non-coding untranslated regions (UTRs) (Andreassi et al., 2018). Such interactions have been shown to regulate local translation. A well-known negative regulator is Fragile X Messenger Ribonucleoprotein (FMRP), which has been shown to interact with the coding region and 3′ UTR of many mRNA transcripts encoding pre- and post-synaptic proteins, many of which were found to be linked to autism (Darnell et al., 2011; Ouwenga et al., 2017). These RNA-protein interactions repress the local translation of transcripts, with dendritic (Hale et al., 2021) and axonal (Jung et al., 2023) FMRP target mRNAs showing increased ribosome association in *Fmr1* knockout mice. RBFOX1, which regulates the splicing of many exons in neurons, binds to the 3′ UTR of cytoplasmic mRNA targets involved in cortical development and autism to increase their stability and local translation (Lee J. A. et al., 2016). Another positive regulator of local protein synthesis is PTBP2, which was shown to interact with the 3′ UTR of *Hnrnpr* mRNA, mediating the association of *Hnrnpr* with ribosomes in a translation factor eIF5A2-dependent manner (Salehi et al., 2023). Indeed, local synthesis of hnRNPR protein is strongly reduced when PTBP2 is depleted, leading to defective motor axon growth (Salehi et al., 2023).

It has been hypothesized that longer UTR sequences may permit a greater number of binding motifs for post-transcriptional regulation, including increased local protein synthesis (Andreassi and Riccio, 2009). Thus, an additional mechanism for regulating local protein synthesis could be through alternative splicing, such as the production of transcripts with alternative last exons (ALEs), and thus distinct 3′ UTRs. Indeed, transcripts with ALEs are disproportionately found in neurites (Taliaferro et al., 2016) undergoing local mRNA translation (Ouwenga et al., 2017). Moreover, cis-regulatory elements generated by alternative splicing at 5′ and 3′ UTRs have been shown to promote axonal mRNA translation (Shigeoka et al., 2016). Therefore, alternative splicing at the UTRs could influence the ability of transcripts to be locally translated. Control of mRNA translation in neuronal subcellular compartments is discussed in more detail elsewhere (Cagnetta et al., 2023).

Cleavage and polyadenylation

3′ UTRs are involved in many aspects of mRNA metabolism, including intracellular localisation and translation. Surprisingly, global mapping of 3′ end regions indicated that ∼75% of mammalian genes contain more than one polyadenylation (poly(A)) site (PAS), giving rise to multiple 3′ UTRs (Proudfoot, 2011; Tian and Manley, 2013; Gruber and Zavolan, 2019). There is remarkable variation in PAS and 3′ UTR length between tissues, with neurons characterized by significantly longer 3′ UTRs (Miura et al., 2013). During neuronal development, many genes are subjected to 3′ UTR and/or poly(A) lengthening (Miura et al., 2013; Kiltschewskij et al., 2023), suggesting this constitutes an important mechanism of post-transcriptional mRNA regulation associated with neuronal differentiation. The process is thought to be a mechanism that serves unique post-transcriptional regulatory needs of transcripts in neurons e.g., transcript localisation, stabilization, and local protein synthesis regulation (Miura et al., 2014).

Although 3′ end cleavage and polyadenylation predominantly occur in the soma, evidence for local processing of alternative 3′ UTR isoforms has also been observed in axons and dendrites (**Figure 2**). Within neurites, many local mRNA transcripts have long 3′ UTRs and have significantly longer half-lives than somata-enriched isoforms (see mRNA stability and degradation section below) (Tushev et al., 2018). Interestingly, these 3′ UTR isoforms can be significantly altered by neuronal activity, with elevated activity resulting in significant shortening of neuropil-localized 3′ UTR isoforms (Tushev et al., 2018). Although most 3′ UTR plasticity was found to be transcription-dependent, evidence for transcription-independent changes was also reported (Tushev et al., 2018), hypothesized to arise from altered stability, trafficking of 3′ UTR isoforms between soma and neuropil, or local remodeling of 3′ UTRs by shortening or lengthening. Direct evidence for local cleavage and polyadenylation comes from work on rat sympathetic neurons showing that axons and cell bodies express distinct pools of 3′ UTR isoforms (Andreassi et al., 2021). Axon-specific short 3′ UTR isoforms of *IMPA1*, *Maoa*, and *Sms* are generated through a process of 3′ UTR cleavage and polyadenylation in axons. This local processing generates translatable isoforms necessary for maintaining the integrity of sympathetic neuron axons (Andreassi et al., 2021). Local cleavage and polyadenylation are further supported by a recent study showing that exposure of sympathetic neurons to Nerve Growth Factor (NGF) or Neurotrophin 3 (NT-3) induces the localization of distinct 3′ UTR isoforms to axons, including short 3′ UTR isoforms found exclusively in axons (Luisier et al., 2023). These observations support a model whereby long 3′ UTR isoforms associate with RBP complexes in the nucleus and, upon reaching the axons, are remodeled locally into shorter isoforms.

A key factor controlling non-nuclear polyadenylation is cytoplasmic polyadenylation binding protein (CPEB), an RBP with strong association for the cis-acting cytoplasmic polyadenylation element (CPE) residing in 3′ UTRs of target mRNAs. CPEB regulates poly(A) tail length by interacting with deadenylating enzymes as well as noncanonical poly(A) polymerases. Many of the components of the cytoplasmic polyadenylation machinery have been found at post-synaptic sites of hippocampal neurons, including CPEB, the scaffold protein Symplekin, the deadenylase poly(A) ribonuclease (PARN), the noncanonical poly(A) polymerase germ line defective 2 (Gld2), and CPEB-interacting factor neuroguidin (Ngd) (Jung et al., 2006; Udagawa et al., 2012; Swanger et al., 2013). The decision whether CPEB binds a deadenylating enzyme (e.g., PARN) favoring short poly(A) tails and translational dormancy, or noncanonical poly(A) polymerases (e.g., Gld2) favoring elongated poly(A) tails and translation, depends on its phosphorylation (Barnard et al., 2004, 2005). Generally, synaptic stimulation promotes phosphorylation, which in turn stimulates poly(A) tail lengthening and local translation (Ivshina et al., 2014).

The cytoplasmic polyadenylation machinery locally acts to bidirectionally regulate mRNA-specific translation and plasticity at hippocampal synapses in response to synaptic transmission, with the poly(A) tail of 102 mRNAs shortened following depletion of Gld2 (Udagawa et al., 2012). One such local transcript is *NR2A* (or GluN2A) mRNA, encoding an NMDA receptor subunit, which contains CPEs in its 3′ UTR, has a short poly(A) tail and is translated inefficiently (Udagawa et al., 2012). *NR2A* RNA is bound by CPEB, which in turn is associated with PARN, Gld2, Symplekin, and Ngd. However, because Ngd is also bound to the cap binding factor, eIF4E, translation is blocked at initiation. NMDA receptor activation was found to promote phosphorylation of CPEB, expulsion of PARN from the RNP complex, and Gld2-catalyzed poly(A) lengthening of *NR2A* mRNA (Udagawa et al., 2012). This local polyadenylation is thought to displace Ngd from eIF4E, the binding of eIF4G to eIF4E, resulting in enhanced translation of NR2A mRNA and membrane insertion of NMDA receptors in dendrites (Swanger et al., 2013). These findings indicate that local polyadenylation has an important role in the activity-dependent synthesis, and NMDA receptor surface expression during synaptic plasticity. Indeed, depletion of CPEB or one of the noncanonical poly(A) polymerases from the mouse hippocampus results in a deficit in long term potentiation (LTP) and increase in long-term depression (LTD) (Zearfoss et al., 2008; Udagawa et al., 2012; Mansur et al., 2021).

Stability and degradation

Neurite-localized transcripts have longer half-lives than somata-enriched isoforms, with average half-lives of mRNAs recorded as 4.8 h and 3.7 h, in neurites and soma cytoplasm of primary cortical neurons, respectively (Tushev et al., 2018; Loedige et al., 2023). The stability and degradation of mRNAs in neurites are crucial for various neuronal functions, including neurite outgrowth and synaptic plasticity. Neurites are an integral part of neuronal communication, and the regulation of mRNA stability in these structures plays a key role in shaping neuronal responses (**Figure 2**). Several factors contribute to the regulation of mRNA stability and degradation in neurites. Below, we will review the evidence that supports a complex network of RNA-protein interactions underpinning the dynamics of mRNA stability and degradation in neurites.

How do longer 3′ UTRs link with increased stability of local mRNAs? It was postulated that alternative 3′ UTRs have novel and repeated regulatory motifs that might help establish localisation to distal regions of the dendrite or axon (Tushev et al., 2018). RBPs are increasingly found to be essential for transcript stability. Such RBPs, including FMRP, STAUFEN2 (STAU2), and TAR DNA-binding protein 43 (TDP-43), are often found to be associated with their mRNA targets in distal dendritic and axonal branches and synapses (Ortiz et al., 2017; Sharangdhar et al., 2017; Chu et al., 2019). Examples also include many RNA splicing regulators that localize in a bimodal fashion to both the nucleus and neurites, where they facilitate RNA metabolism. Such regulators include the Muscleblind proteins, which regulate alternative splicing in the nucleus (Pascual et al., 2006; Konieczny et al., 2014) and the correct localisation of mRNAs in neurons (Wang et al., 2012; Hildebrandt et al., 2023). Evidence from the nematode *Caenorhabditis elegans* (*C. elegans*) indicates that Muscleblind-1 (MBL-1) binds to mRNA transcripts encoding microtubule proteins to regulate their stability. Indeed, microtubule stability in sensory neuron axons is compromised in *mbl-1* mutants due to reduced levels of α-tubulin and β-tubulin (Puri et al., 2023). Another well-known splicing regulator also involved in RNA stability is SNRNP70, a core spliceosome protein. SNRNP70 was found to localize to cytoplasmic RNA granules and associate with mRNA transcripts, controlling their axonal trafficking and stability in zebrafish motor neurons, ultimately regulating neuromuscular connectivity (Nikolaou et al., 2022).

The longer half-lives of localized transcripts can also be explained by a lack of destabilization elements. Evidence suggests that neurite-localized mRNAs are depleted of destabilizing elements (Loedige et al., 2023). Such sequences include AU-rich elements (AREs), and those that promote m^6A (N^6-methyladenosine) modifications which induce mRNA degradation. It was shown that high mRNA stability is both necessary and sufficient for localisation to neurites, with depletion of mRNA-stabilizing proteins ELAVL and LARP1 interfering with transcript localisation to neurites. Also, alleviation of m^6A-dependent mRNA degradation by depletion of YTHDF, or removal of destabilizing AREs, were sufficient to increase the stability of transcripts and shift these toward neurites (Loedige et al., 2023).

The most extensively studied mechanism for RNA degradation is by nonsense-mediated RNA decay (NMD), a cellular surveillance mechanism that recognizes and degrades mRNAs containing premature termination codons (PTCs) or nonsense mutations. NMD is a crucial quality control mechanism in eukaryotic cells, ensuring the removal of faulty transcripts and maintaining the integrity of the cellular proteome. The NMD pathway involves a series of proteins and complexes that recognize PTCs and facilitate mRNA degradation. Key components include UPF1, UPF2, and UPF3, which form the core NMD complex. These proteins interact with the exon junction complex (EJC) and other factors to initiate mRNA degradation (Lykke-Andersen and Jensen, 2015). Although NMD is initiated as soon as a PTC is detected in the nucleus, evidence suggests that the pathway can also operate locally to regulate neurite outgrowth, axon guidance and synaptic plasticity through the degradation of selected mRNA isoforms containing NMD-inducing PTCs (see IR RNAs section).

In the hippocampus, the NMD pathway operates within dendrites to regulate synaptic function and plasticity by increasing Glutamate receptor, GLUR1, surface levels (Notaras et al., 2020). UPF2 was shown to promote local synthesis of GLUR1 in dendrites through local NMD-mediated degradation of *Arc* and *Prkag3* mRNAs, whose proteins negatively influence local translation (Notaras et al., 2020). This observation demonstrates that local translation is regulated by mechanisms that control mRNA degradation in dendrites. In addition to its canonical targets, NMD may also degrade mRNAs that do not carry identifiable NMD-inducing features (He and Jacobson, 2015), however, the mechanisms by which NMD recognizes its atypical targets remain unclear. It is also possible that NMD components could act independently of mRNA degradation to promote local protein synthesis. Indeed, UPF1 was found to regulate synaptic plasticity in hippocampal neurons by facilitating the transport and translation of mRNAs through its association with STAU2 (Graber et al., 2017).

Intron-retaining RNAs (IR RNAs)

Diversity in axons, dendrites, and synapses

Introns are sections of DNA within genes that intersperse exons. Generally considered non-coding sequences, they are typically spliced from pre-mRNAs co-transcriptionally. Sometimes, however, one or multiple introns may be retained in the mature transcript (Grabski et al., 2021). In recent years, the development of pipelines to identify intron retention events in high-throughput sequencing datasets, has revealed it to be a more common phenomenon than previously thought, and more widespread in neurons compared to other tissues (Braunschweig et al., 2014; Jacob and Smith, 2017; Middleton et al., 2017). Intron retention has mostly been considered in a nuclear context, either as a mechanism of inducing transcript degradation, thereby driving gene downregulation, or to detain transcripts in the nucleus, delaying their export until required. More recently, however, many intron-retaining (IR) mRNAs have been reported to localize and even become enriched in the cytoplasm and neurites (**Figure 1**), pointing towards functional roles for local IR isoforms. Below, we outline the key studies characterizing intron-retaining transcript populations in axons, dendrites, and synapses.

Early studies detected IR mRNAs in cultured embryonic rat hippocampal neuronal dendrites following reverse transcription of extracted mRNA and PCR amplification, and by microarray analysis and *in situ* hybridization (Bell et al., 2008; Buckley et al., 2011). Such IR transcripts pertained to genes encoding proteins such as synaptic proteins, ion channels, RBPs (inc. splicing factors), and translation factors (Buckley et al., 2014; Luisier et al., 2018). More recent studies have leveraged high-throughput sequencing approaches to more thoroughly identify and quantify IR transcripts in neurites. Primary rat hippocampal neurons cultured in transwell inserts, enabling the isolation of neurites, identified 428 neurite-enriched retained introns (Saini et al., 2019). In another study, mouse embryonic motor neurons cultured in microfluidic chambers revealed intronic sequences to be detected more strongly in axons compared to the somatodendritic compartment, likely representing IR transcripts (Briese et al., 2016). Many retained introns have also been reported in zebrafish neurites following primary culture of larvae-derived neurons in transwell inserts (Taylor et al., 2022). The same study also revealed dramatic neurite-specific increases in IR transcripts in absence of the neuronal-enriched splicing factor, SFPQ, identifying the protein as a key regulator of neurite intron retention.

Little is known regarding IR RNA localisation to synapses; partly due to a lack of RNA-Seq analyses mining for events from synapse-specific samples. However, *CamKIIa* intron-16-retaining RNAs were identified in synaptoneurosomes isolated from mouse primary cortical neurons and adult cortical tissue, and their levels were shown to decrease upon stimulation with BDNF or N-methyl-D-aspartate (NMDA) (Ortiz et al., 2017). These findings suggests a wider array of IR transcripts may be detected at synapses in future RNA-Seq analyses.

Thus far, most neurite-localized IR transcripts have been detected in cultured neurons. Recent data confirms localisation of such transcripts in tissue, in distal dendrites of hippocampal neurons imaged *in situ* following expansion microscopy combined with long-read sequencing (Alon et al., 2021). This includes *Grik2*, a glutamate ionotropic receptor kainate subunit implicated in excitatory glutamatergic neurotransmission.

In RNA-Seq datasets from neurite samples, often multiple introns within the same gene show reads mapping to them. However, it is unclear whether such introns are retained together in the same transcript isoform, or retained individually in distinct isoforms. This is due to the short-read lengths used in standard RNA-Seq experiments. The advent of third-generation long-read sequencing datasets will provide new insights that address this question. Multiple introns retained in a single isoform suggests even greater complexity in intron retention regulation, and the functions of IR mRNAs.

Fates and functions

Local translation and degradation

IR transcripts are thought to rarely serve a coding function. Retained introns frequently insert PTCs into transcripts, expected to activate transcript degradation by NMD upon the pioneer round of any translation. A well-known example of local translation of an IR mRNA occurs in the developing spinal cord (Chen et al., 2008; Colak et al., 2013). Here, commissural axons are initially attracted to the ventral midline and, upon crossing, become repulsed. Such axon guidance depends on the interaction between axon membrane receptors (Robo proteins) and proteins of the extracellular matrix (Slit proteins) (Jaworski et al., 2010). Following transcription, *Robo3* transcripts are processed into either of two isoforms—fully spliced *Robo3.1* (no IR), and intron-26-retaining *Robo3.2* containing a PTC (Chen et al., 2008; Colak et al., 2013). Prior to reaching the ventral midline, *Robo3.1* mRNAs are translated, preventing activation of ROBO1 and ROBO2 that are present at low levels on axons, while *Robo3.2* transcripts are translationally repressed. Once the axon has been exposed to floorplate signals in the spinal cord midline, rapid translation of *Robo3.2* mRNA is triggered, producing a peptide with a distinct C-terminus compared to the peptide produced from *Robo3.1*. ROBO3.2 protein increases the ability of ROBO1 and ROBO2 to bind to Slit proteins, which in turn repels the axon from the midline area, allowing appropriate axon positioning (Chen et al., 2008). The ROBO3.2 C-terminus is composed of intron-encoded amino acid residues up to the PTC. As expected, *Robo3.2* translation also activates NMD of the transcript, however, this was shown to be functionally important, limiting production of the protein to the correct quantity (**Figure 3**). Blocking NMD in commissural neurons caused accumulation of *Robo3.2* mRNA and ROBO3.2 protein and disproportionate axon repulsion from the midline, indicating the physiological importance of NMD to ensure functionally relevant amounts of protein are synthesized. Thus, NMD drives tight temporal and spatial control of the expression of the protein (Colak et al., 2013).

Other examples of proteins from IR transcripts have also been described. SMN1 functions in spliceosome assembly, implicating it in the splicing process. A specific isoform, aSMN, produced from an mRNA retaining intron-3, is found in axons and is important for axonogenesis (Setola et al., 2007). The specific function/s of

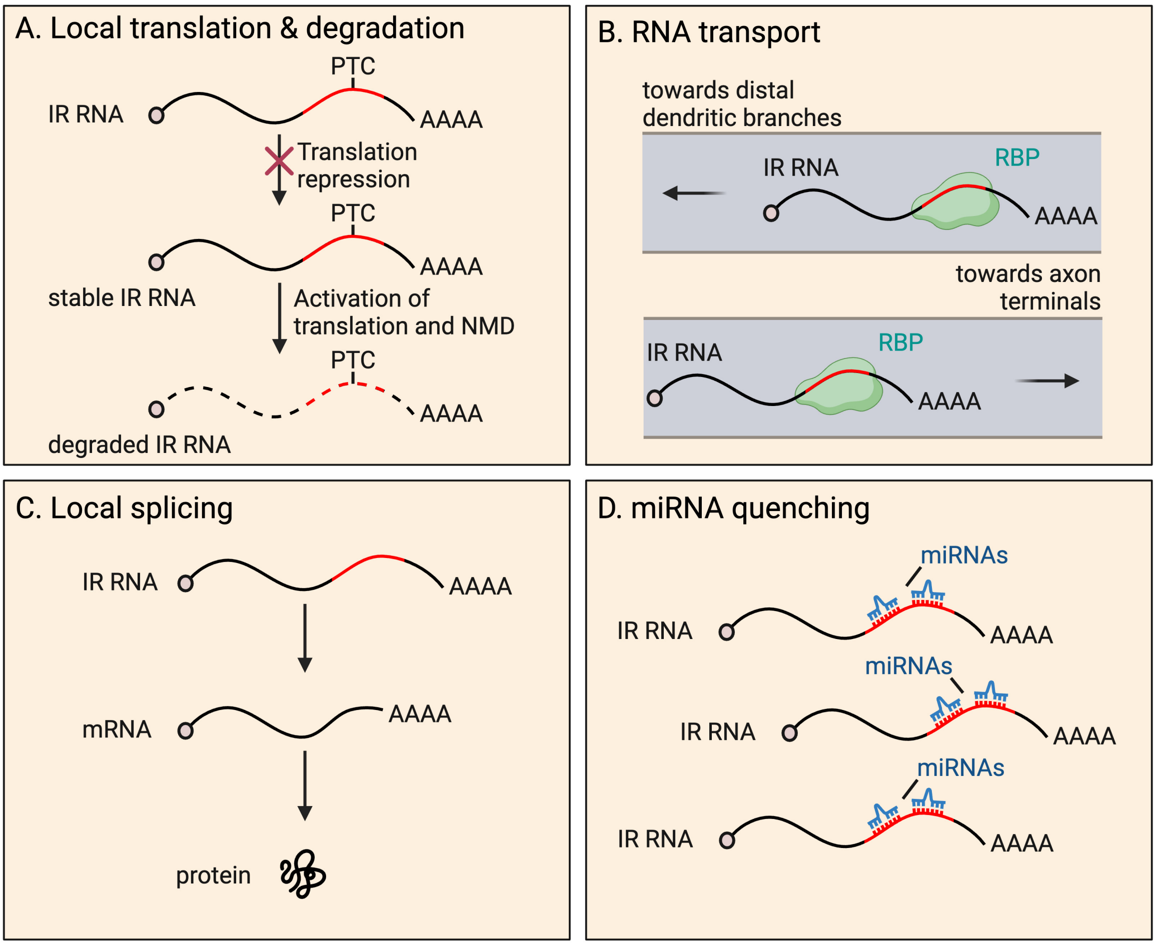


FIGURE 3
Fates and functions of intron-retaining (IR) RNAs in neurites. IR RNAs have several fates and functions within distal parts of neurons, including: **(A)** local translation and subsequent degradation due to presence of a premature termination codon (PTC), a process that provides tight temporal and spatial control of protein expression; **(B)** RNA granule organization and RNA transport toward distal dendritic and axonal regions; **(C)** local splicing to boost the pool of translatable fully spliced mRNAs; and **(D)** miRNA quenching through the harboring of miRNA recognition motifs.

the shortened peptide are unclear. In another study, *Nxf1*, which encodes the nuclear export factor NXF1, produces a transcript that retains intron-10 and undergoes translation to produce the shortened protein isoform, sNXF1, detected in dendrites of rodent cortex (Li et al., 2016). Intron-10 contains a constitutive transport element, which requires NXF1 for nuclear export of the IR mRNA (Li et al., 2006). The authors report a high level of expression of sNXF1 in endogenous adult rodent brain suggesting either the IR transcript does not undergo NMD, or that it is expressed at very high levels.

The extent to which translation occurs more widely from neurite-localized IR mRNAs is unclear. Analyses of neurite ribosome profiling/footprinting data have not explored the extent to which reads map to introns, likely because proportionally they represent very few, owing to NMD activation. Given that PTCs are introduced at some point within introns, reads would be expected to map specifically to the 5′ of introns. However, retained introns could alternatively produce novel peptides by introducing novel translational start sites. An example of this has not yet been reported in neurites. Translation from sequences outside canonical coding regions such as introns, typically produces unstable proteins with hydrophobic tails, either targeted for degradation by the proteasome, or to the membrane (Kesner et al., 2023). However, more stable proteins may be produced from IR transcripts, where retention status is often conserved (Sorek and Ast, 2003; Galante et al., 2004; Buckley et al., 2014), and introns are more GC rich than non-retained introns (Braunschweig et al., 2014).

Transport and granule organization

Retained introns have also been shown to be important for RNA transport to neurites (Buckley et al., 2011; Ortiz et al., 2017; **Figure 3**). Many retained introns, including *Fmr1* intron-1, were shown to contain ID elements with motifs that were previously shown to regulate BC1 ncRNA localisation to dendrites (Buckley et al., 2011). *Fmr1* encodes FMRP, which localizes to the soma and dendrites, and is important for proper synaptic plasticity (Richter and Zhao, 2021). Reporters expressing *Fmr1* intron-1 ID elements exhibit dendrite localisation, and compete with endogenous IR transcript populations, resulting in altered distribution of the overall population of FMRP protein (Buckley et al., 2011). Mutations in the ID element dramatically reduced dendritic targeting of the reporters, indicating the importance of the sequence to achieve localisation.

STAU2 binds to retained intron-16 of *CaMKIIα,* required for dendrite localisation of transcripts in mouse hippocampal neurons (Ortiz et al., 2017). Intron-16 retention is conserved in human (Braunschweig et al., 2014) and rat (Buckley et al., 2011), suggesting it may have a conserved role. The authors investigated the fate of IR transcripts under different conditions. Blocking protein synthesis by cycloheximide treatment does not increase intron-16-retaining transcript expression when not undergoing synaptic stimulation, indicating the transcripts are not NMD targets under these conditions. Stimulation with BDNF or NMDA results in decreases in intron-16-retaining *CaMKIIα* transcripts, which was prevented by cycloheximide treatment, suggestive of translation-dependent degradation by NMD. However, given that overall transcript levels are unaffected by stimulation, one could also hypothesize that the intron-16-retaining portion are instead locally spliced (see Local splicing section below). CaMKIIα protein levels or isoform differences following stimulation were not investigated.

STAU2 has also been shown to be required for the transport of an IR *Calm3* mRNA, in dendrites of mature rat hippocampal neurons (Sharangdhar et al., 2017). In this case, the 5′ and 3′ exons flanking the intron are 3′ UTR, sequence classically associated with mRNA transport. Overall, STAU2 was found to strongly bind retained introns within the 3′ UTRs of 28 mRNAs, suggesting the protein similarly regulates the localisation of other transcripts.

Many questions remain regarding the nature of transport granules containing IR RNAs. One hypothesis is that IR transcripts act as the means of transport of fully spliced counterparts occupying the same granule. Such retained introns could also act as a scaffold/platform, binding relevant RBPs, facilitating time or activity sensitive RNA processing of neighboring spliced transcript counterparts. Alternatively, retained introns could act as a scaffold, binding RBPs to catalyze granule organization. Similar roles have been reported for 3′ UTR sequences (Ma and Mayr, 2018; Mayr, 2019).

Local splicing

The local splicing of IR mRNAs could provide a powerful means for the rapid expansion of the pool of translatable mRNAs when needed, or for local decisions to be made on whether to excise introns alone or with a neighboring exon to generate alternative protein isoforms on demand. Direct mechanistic evidence for endogenous local splicing has yet to be shown, and its possibility remains a controversial hypothesis in the field. Despite studies finding an increasing number of splicing factors localized to neurites, spliceosomes are huge and complex structures, and only a small portion of the snRNA and protein components have been detected at substantial levels (Poulopoulos et al., 2019). Below, we discuss the studies that have shown evidence supporting the possibility of local mRNA splicing (**Figure 3**).

One early study focused on the 6000-nucleotide long retained intron-16 in *Kcnma1* transcripts (Bell et al., 2008). Intron-16-retaining transcripts were estimated to form 10% of the total population of *Kcnma1* transcripts in rat hippocampal neuron dendrites. Targeting specifically the IR isoform with siRNAs was able to specifically reduce their pools. Significantly lower levels of KCNMA1, a calcium-activated BK channel protein, and perturbed neuronal firing properties were also observed. The authors hypothesized that intron-16 may be locally spliced in dendrites to increase the pool of translatable mRNAs. In a subsequent study by the same group, intron-17 of *Kcnma1* was also shown to be retained (Bell et al., 2010). STREX (stress axis regulated exon) is an alternative exon sitting immediately downstream of intron-17. The intron contains regulatory elements controlling the splicing of STREX in response to activity. Inclusion of the exon alters the activity of the channel the protein sits in. In the study, intron-17-retaining mRNAs were detected in dendrites, with the intron either retained alone or in combination with STREX. Knockdown of intron-17-retaining isoforms downregulates STREX-containing isoforms of KCNMA1, most prominently in dendrites, and also disrupts the burst firing abilities of hippocampal neurons. The authors suggested intron-17-retaining isoforms become spliced within dendrites, facilitating the production of STREX-containing KCNM1A. However, the mechanism by which any splicing event would occur is unclear and was not addressed in either study.

Intriguingly, an earlier study by the group indicated canonical splicing capabilities in dendrites of primary cultured rat hippocampal neurons, a process widely accepted as exclusively nuclear (Glanzer et al., 2005). U1 snRNA and splicing factors required for spliceosome assembly were detected by *in situ* hybridisation and immunohistochemistry, respectively. Dendrites were isolated from somas and transfected with the pre-mRNA splicing construct, chicken δ-*crystallin (cdc)* mRNA, consisting of a 257-nucleotide intron flanked by exons-14 and -15, with a FLAG sequence in-frame with exon-15. Spliced transfected mRNA was detected in 50% of experiments, with multiple splice junction variants clustering around the canonical donor and acceptor splice sites suggesting classic pre-mRNA splicing. FLAG epitope was also detected in dendrites, which was not possible without local splicing. Another more recent study suggesting canonical splicing occurring outside of the nucleus in neurons, identified that cytoplasmic pools of spliceosomal protein, SNRNP70, a core U1 snRNP component, rescue defects in alternative splicing events in *snrnp70* null zebrafish embryos (Nikolaou et al., 2022). Rescued events were enriched in genes associated with neuronal ontologies such as synaptic vesicle recycling proteins.

Although studies thus far have explored the possibility of canonical local splicing, the mechanism may be non-canonical, such as that described at the endoplasmic reticulum (ER) membrane during the unfolded protein response (UPR) (Back et al., 2006; Uemura et al., 2009). The accumulation of incorrectly folded proteins in cells causes ER stress and subsequent activation of the UPR to resolve the situation. This involves upregulated transcription of *XBP1*, mRNAs of which localize to the ER surface where a 26-nucleotide intron is excised by IRE1, inositol requiring kinase-1, which has endoribonuclease activity. The exposed mRNA 5′ and 3′ fragments are then ligated. Following non-canonical splicing the transcript undergoes translation producing a transcriptional activator of genes involved in the UPR. ER extends into axons and dendrites and could therefore similarly act as a platform for neurite splicing events (Öztürk et al., 2020).

miRNA quenching

A role in microRNA (miRNA) regulation has been suggested for retained introns in the cytoplasm of motor neurons (**Figure 3**). A recent study identified that a specific set of introns become transiently retained in the cytoplasm of neural precursor cells during lineage restriction of human iPSC-derived motor neurons (Petrić Howe et al., 2022). Intriguingly, these introns were enriched for 14 miRNA motifs. The authors showed that the IR transcripts are not targets for downregulation by miRNA binding. Conversely, reduced IR transcript expression led to increased expression of predicted miRNA target genes (a readout of miRNA activity). Such findings were not explained by changes in miRNA levels. The authors suggest the retained introns act as sponges, quenching miRNA binding and action on target mRNAs. Intriguingly, the reported retained introns were also enriched for binding capacity of miRNA regulatory proteins, including DROSHA and PUM2. However, loss of DROSHA did not affect levels of IR transcripts suggesting the protein does not process miRNAs from the introns. Regardless, it remains intriguing to hypothesize in other cases that miRNAs could be synthesized locally from introns. Thus far, processing of miRNAs from introns has only been observed in the nucleus (Westholm and Lai, 2011). While the study did not focus on neurite-localized IR transcripts, many miRNAs are known to localize to axons and dendrites (see ncRNAs section below), suggesting similar regulation could be present in neurites.

Non-coding RNAs (ncRNAs)

ncRNAs are diverse, and often loosely categorized either by size as short or long, or functionally based on whether they are housekeeping (tRNA, rRNA, snRNA, snoRNA) or regulatory (lncRNA, sncRNA including miRNA, circRNA) (**Figure 4**; Li et al., 2021; Mattick et al., 2023). Comparatively little is known regarding the true diversity amongst local ncRNAs at subcellular resolution in axons, dendrites, and synapses, including their relative abundance. However, data from motor axons identified that some of the most abundant localized transcripts are ncRNAs (**Figure 1**), including the rRNA, *Gm26924*, and *7SK* and *7SL* ncRNAs (Briese et al., 2016).

Post-transcriptional regulation of mRNAs in neurites by various classes of ncRNAs drive processes such as neurite outgrowth and synaptic plasticity. Such studies have tended to focus on regulatory RNAs, and thus these form the focus of discussion below.

Short ncRNAs (sncRNAs)

Early studies investigating the subcellular distribution of sncRNAs in neurons focused on miRNAs. Canonically, transcribed primary miRNAs are processed into precursor miRNAs in the nucleus before being exported to the cytoplasm (O'Brien et al., 2018). Here, they form mature miRNAs around 22-nucleotides in length that can bind complementarily to mRNA targets to suppress their expression. Microarray studies have identified over 100 miRNAs in axons and growth cones, some enriched, and subsequent studies have revealed their importance in different aspects of axonal development and function (Natera-Naranjo et al., 2010; Han et al., 2011; Dajas-Bailador et al., 2012; Kaplan et al., 2013; Sasaki et al., 2014; Zhang et al., 2015). Microarray and RT-qPCR studies have also identified many miRNAs and their precursors, pre-miRNAs, in dendrites and synapses, along with Dicer and other proteins involved in miRNA biogenesis (Lugli et al., 2008, 2012). Enrichment of such precursors in synaptic fractions suggests additional compartmentalisation of local processing into functional miRNAs (Lugli et al., 2005, 2008).

Pre-miRNAs were found to associate with CD63-labelled vesicles, thought to represent late endosomes, for transport into axons (Vargas et al., 2016; Corradi et al., 2020). Intriguingly, the RNA-induced silencing complex (RISC), which is needed for miRNA processing, has also been shown to localise to axonal branch points and growth cones, a process that is facilitated by mitochondria (Gershoni-Emek et al., 2018). However, it is not clear whether the RISC functions directly on or adjacent to the vesicle to process co-trafficked pre-miRNAs, or whether it acts on different pre-miRNAs that already reside in the axon. Nevertheless, the presence of pre-miRNAs in distal regions of neurons suggests that these RNA precursors are processed locally to exert their function in response to environmental stimuli. Indeed, evidence has shown that pre-miRNAs are processed in axons and dendrites in response to injury (Kim et al., 2015) or neuronal excitation (Sambandan et al., 2017), respectively.

Recently, unbiased total RNA-Seq approaches have assayed the range of small ncRNAs in axons, dendrites, and synapses more globally. RNA-Seq performed following mouse embryonic spinal cord compartmentalized culture identified 401 miRNAs, with 34 enriched in neurites (Rotem et al., 2017). Several of the neurite-localized miRNAs were up- or down-regulated in neurons containing mutations causing the neurodegenerative disease, Amyotrophic lateral sclerosis (ALS), suggesting that perturbations in miRNA regulation may play a central role in driving neurodegeneration.

In another study investigating sncRNAs in mouse cortical neuron axons following compartmentalized primary culture, identified tRNA-derived small RNAs (tsRNAs) as the most enriched class (Mesquita-Ribeiro et al., 2021). Derived from tRNA genes, tsRNAs are cleavage fragments of around 14-50-nucleotides. The functions of such axonal tsRNAs were not addressed in the study, but generally they are reported to bind specific RBPs and mRNAs, proposed to act as regulators of translation and degradation (Zong et al., 2021; Tian et al., 2022). The second most abundant group was rRNA, reflective of ribosome localisation to axons. miRNAs represented $<$ 10% of the small ncRNAs in axons, with just over 35 miRNAs making up 80% of the miRNA reads. Fragment RNAs derived from snRNA genes, particularly U1 and U2, were also detected. Subsequent sncRNA-Seq on axoplasm from rat dorsal and ventral root nerves *in vivo* revealed rRNA and miRNA as dominant, with tsRNAs well-represented and snRNAs also identified (Mesquita-Ribeiro et al., 2021). The same miRNAs were the most abundant in both the mouse cortical axon and rat axoplasm datasets.

A study investigating the non-coding transcriptome in synaptosomes purified from mouse hippocampus, identified 65 miRNAs and 37 snoRNAs (Epple et al., 2021). Intersecting the list of miRNAs with mRNAs that localize to synapses, revealed 98% of the mRNAs would be targeted, suggesting a high degree of local regulation by miRNAs at synapses. Compartmentalized culture of hippocampal neurons also allowed for the isolation of synapses for sequencing. These samples contain more neurite tissue compared to the synaptosomes sample, but are less prone to contamination by RNAs from other neural cell types. 57 miRNAs were identified,

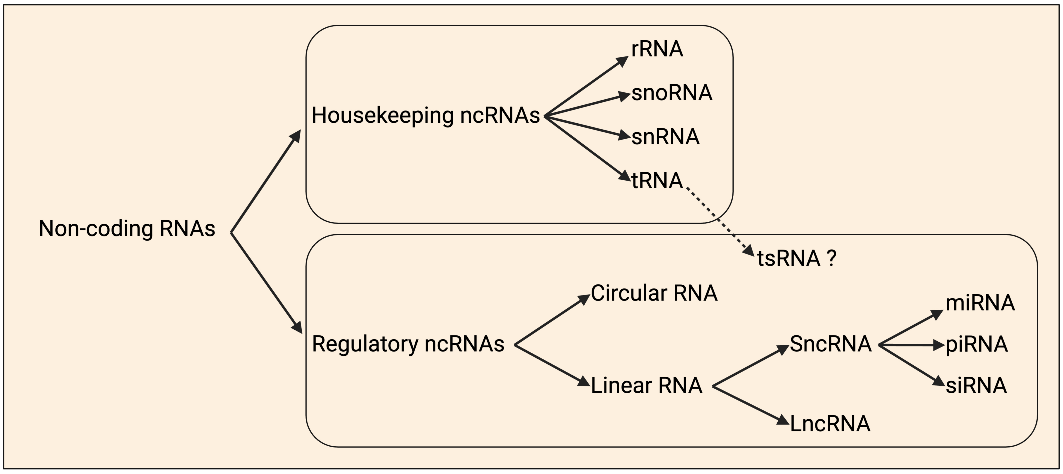


FIGURE 4
Schematic classification of non-coding RNAs. Non-coding RNAs (ncRNAs) are organized as housekeeping and regulatory ncRNAs. Housekeeping ncRNAs are divided into ribosomal (rRNA), small nucleolar (snoRNAs), small nuclear (snRNA), and transfer (tRNA). tRNA-derived small RNAs (tsRNAs) are a group of ncRNAs that are hypothesized to have regulatory roles. Regulatory ncRNAs include the circular and linear RNAs, and within the latter class there are the short ncRNAs (sncRNAs) and long ncRNAs (lncRNAs). The sncRNAs group is divided into the microRNAs (miRNAs), small interfering RNAs (siRNAs), and piwi-associated RNAs (piRNAs). Adapted from Baptista et al. (2021).

17 of which were conserved with those in synaptosomes. This conserved group regulate 80% of synaptic mRNAs. Many of the other 48 miRNAs specific to synaptosomes have been previously reported to be released by exosomes from astrocytes, suggesting this may be their source in that dataset.

Thus far, functional studies of sncRNAs have mostly focused on miRNAs, which regulate mRNA targets by two mechanisms: translational repression and/or mRNA degradation (Baek et al., 2008; Bartel, 2009; **Figure 5**). Recent years have seen significant progress in our understanding of how miRNAs induce translational repression of local mRNAs. *miR-181d* was shown to mediate axon elongation in DRG neurons by repressing the local synthesis of MAP1B and CALM1 in response to NGF (Wang et al., 2015). Acting along similar lines, *miR-26a* and *miR-132* were shown to promote axon growth by repressing local protein synthesis of GSK3β and Rasa1, respectively (Hancock et al., 2014; Lucci et al., 2020). Moreover, *miR-181a* and *miR-182*, two highly abundant miRNAs in RGC axons, were shown to regulate the responsiveness of RGC axons to guidance cues by silencing the local translation of specific mRNA targets (Bellon et al., 2017; Corradi et al., 2020). Interestingly, recent work has also shown that upon exposure to axon guidance cues, pre-miRNAs are processed to miRNAs within RGC axons, silencing the basal translation of tubulin beta 3 class III (TUBB3) to enable accurate growth cone steering (Corradi et al., 2020). These findings support a model in which pre-miRNAs are stored within growth cones and synapses in an inactive form. Upon stimulation, rapid processing into active miRNAs for local translational repression ensures fast neuronal responses.

Together, these results provide experimental support for a model in which translational repression may be preferable over mRNA degradation in axons (Vo et al., 2010). Constitutive degradation of localized mRNAs that have been transported over long distances into axons would be inefficient or counterproductive. Moreover, while mRNA degradation is a terminal event, translational repression is reversible and can be employed for rapid response to internal or external cues.

In summary, functional studies of short ncRNAs have mostly focused on miRNAs thus far, which are particularly enriched in synaptic fractions and have the capability to target the entire local mRNA pool. Future studies could address miRNA and mRNA combinations occupying individual neurons to better understand the dynamics of such regulation. The functional impacts of tsRNAs, snoRNAs, and snRNAs in neurites and synapses is yet to be revealed and will likely form an important focus of future studies.

Long ncRNAs (lncRNAs)

LncRNAs are generally defined as ncRNAs more than 200 nucleotides in length. They are enriched in the brain, where 40% of the tens of thousands that mammals possess are expressed (Briggs et al., 2015). Many are derived from protein-coding genes, being antisense, intronic, or intergenic in origin, while many others are pseudogenes (Mattick et al., 2023). LncRNAs are often spliced like mRNAs, and can be polyadenylated or not. *BC1/BC200* was the first lncRNA identified to localize to neurites, present in dendrites where it binds to various proteins and regulates local translation at synapses (Tiedge et al., 1991; Muslimov et al., 1997; Eom et al., 2011; Smalheiser, 2014; Briggs et al., 2015). *MALAT1* lncRNA also plays roles in synapse function, and both transcripts have been reported *in situ* in dendrites of mouse hippocampal pyramidal neurons (Alon et al., 2021).

Although many high-throughput sequencing datasets have globally characterized the transcriptomes of specifically neurites, most have focused on protein-coding transcripts. Typically, only handfuls of lncRNAs are highlighted, suggesting these datasets are untapped resources for identifying lncRNAs and aspects of mRNA regulation.

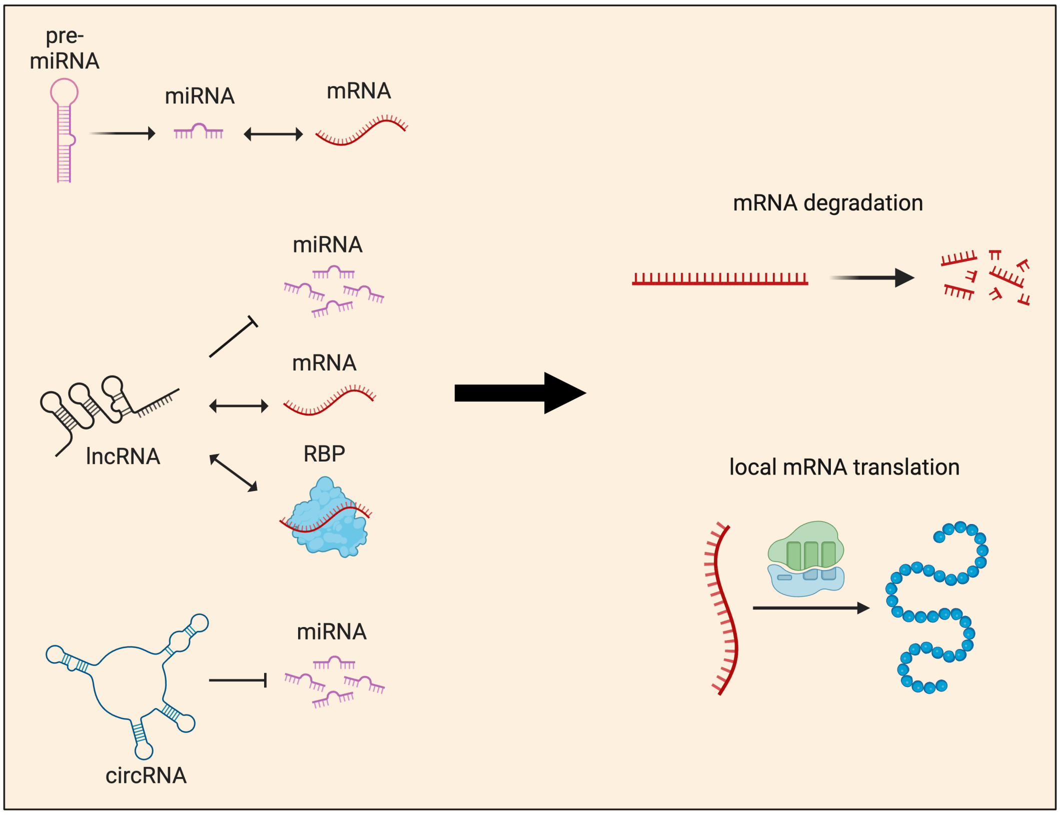


FIGURE 5
Local functions of non-coding RNAs in neurites. Non-coding RNAs (ncRNAs) influence gene expression at the post-transcriptional level by regulating either local mRNA translation and/or degradation. These two major outcomes are achieved through multiple mechanisms. miRNAs, which can be produced locally from pre-miRNAs, are known to interact directly with mRNAs to regulate their abundance and local protein synthesis. lncRNAs act as guides or scaffolds, interacting with both mRNAs and protein, but also compete with miRNAs to regulate local protein synthesis. circRNAs' main mode of function is through acting as miRNA sponges, sequestering and preventing miRNAs from binding to their target mRNAs.

One study focusing on revealing lncRNAs more extensively in the rat spinal cord, though not at subcellular resolution, identified 772 transcripts differentially regulated following contusive injury, the majority (68%) upregulated (Zhou et al., 2018). This suggests that lncRNA functions are implicated in pathogenesis and limited repair capacity associated with spinal cord damage. Numerous specific neurite-localized lncRNAs have now been identified in various RNA-Seq datasets. In mouse embryonic motor axons, this includes the well reported on *MALAT1*, as well as *XIST, MIAT, RMST*, and *7SL* RNA, a component of the signal recognition particle, important for ER localisation of proteins (Briese et al., 2016). High-throughput sequencing of rat DRG neurons identified 3103 lncRNAs, the 20 most abundant of which were subsequently investigated for axonal enrichment (Wei et al., 2021). *ALAE* was shown to be the top candidate, important in axon growth through the regulation of *Gap43* local translation.

Studies focused on characterizing synaptic transcriptomes have typically covered lncRNAs in more detail. In one study, 6 high-confidence lncRNAs were identified in synaptosomes purified from mouse hippocampus (Epple et al., 2021). Strikingly, sequencing of synapses following a compartmentalized culture protocol where the tissue isolated includes more neurite material, identified 199 lncRNAs. This expanded group are associated with regulating oxidative phosphorylation and synaptic plasticity. Thus, this data suggests a wider range of lncRNAs localize to neurites than synapses than is currently understood. Another study characterizing lncRNAs from synaptoneurosomes of activated hippocampal neurons identified *Gm38257/ADEPTR* as the most enriched transcript compared to whole hippocampal neurons (Grinman et al., 2021). Derived from intron-1 of *Arl5b*, *Gm38257/ADEPTR* lncRNA is upregulated and trafficked to synapses upon activation, independent of Arl5b mRNA. The transcript acts as a scaffold, binding to ANKB and SPTN1 proteins for their transport to dendrites, and such transport is KIF2A-dependent.

Natural antisense transcripts are lncRNAs important for neurite development (Modarresi et al., 2012), and have been detected sitting alongside their complementary protein-coding sense transcripts in synaptoneurosomes isolated from adult mouse forebrain (Smalheiser et al., 2008). In some cases, the two transcripts are expressed at similar levels, while others exhibited significant differences in expression. The degree of interaction between these complementary transcripts in synaptoneurosomes is unclear.

Functionally, lncRNAs can act via several mechanisms to influence gene expression at the post-transcriptional level (**Figure 5**), and while their expression levels are often relatively low, they can exert great influence (Wu et al., 2021). They are increasingly found to be associated with RNA granules in axons and dendrites, indicating they may provide key functions to such membrane-less organelles. For instance, it is known that RNA granules with distinct RNPs can contribute to translational repression (Vessey et al., 2006). LncRNAs may associate with RNPs to form these granules (Khong et al., 2017) as *BC1* is known to associate with poly(A) binding protein (PABP), translation initiation factors and components of the ribosome at the synapse (Tiedge et al., 1991; Muddashetty et al., 2002; Lin et al., 2008). Indeed, RNA granules have been shown to play a role in synaptic plasticity and long-term memory formation (Solomon et al., 2007; Nakayama et al., 2017) by silencing translation and promoting RNA stability (Hubstenberger et al., 2017; Khong et al., 2017). Alternatively, lncRNAs within RNA granules can also rapidly facilitate local protein synthesis when translation is in high demand (Mazroui et al., 2007; Baez et al., 2011). Indeed, it was recently shown that an m^6A-modified lncRNA *Dubr* binds YTHDF1/3 complex through its m^6A modification, thereby preventing YTHDF1/3 complex from degradation via the proteasome pathway, facilitating translation of *Tau* and *Calmodulin*. Although it is not yet known whether *Dubr* acts in the cytoplasm or axons, this process was found to be essential for DRG axon elongation (Huang et al., 2022).

In distal parts of neurons, lncRNAs have been shown to work as guides or scaffolds. For example, *BC1* mediates translation silencing at the synapse by bridging the repressor FMRP and its target mRNAs (Zalfa et al., 2005; Lacoux et al., 2012; Briz et al., 2017). At the synapse, *BC1* can also bind to translation initiation factor, eIF4A, and PABP, preventing their interaction with target mRNAs to initiate translation (Muddashetty et al., 2002; Lin et al., 2008). Acting along similar lines, the lncRNA *NORAD* has been hypothesized to act as a decoy for dendrite-localized PUMILIO to prevent it from repressing translation (Vessey et al., 2010; Lee S. et al., 2016). Finally, the lncRNA *Meg3* was found to regulate AMPA receptor insertion to the plasma membrane, a process that has been hypothesized to be partly due to *Meg3* competition with miRNAs regulating PTEN/PI3K/AKT signaling pathway during synaptic plasticity in neurons (Tan et al., 2017). Despite these interesting lines of evidence, the functional relevance of lncRNAs in neurites and at the synapse is not fully understood, and future studies will likely provide new insight into the role of such localized lncRNAs.

Circular RNAs (circRNAs)

CircRNAs are a highly stable class of RNAs formed from non-canonical back-splicing, where a downstream/3′ splice donor fuses with an upstream/5′ splice acceptor (Kristensen et al., 2022). They can contain exonic sequences only or include introns too. Also, intron lariats resulting from canonical pre-mRNA splicing can remain present as circRNAs if they evade linearisation by debranching enzymes (Kristensen et al., 2022). Both canonical splicing and back-splicing depend upon the spliceosome, and often, the two types of reaction are in competition on pre-mRNAs.

Investigations of circRNAs across various mouse tissues revealed their enrichment in the brain, and formation associated with neuronal differentiation (Rybak-Wolf et al., 2015; You et al., 2015; Dong et al., 2023). Such findings were observed across mammalian species. Comparing circRNAs in mouse and human brain samples, identified 15,849 and 65,731, respectively—the discrepancy likely in part due to deeper sequencing of human samples (Rybak-Wolf et al., 2015). Strikingly, 2,338 of the genes giving rise to circRNAs produce 10 or more circularized isoforms, which are frequently expressed at higher levels than linear mRNA counterparts (Rybak-Wolf et al., 2015; You et al., 2015).

CircRNAs are derived particularly from genes encoding synaptic proteins (Rybak-Wolf et al., 2015; You et al., 2015; Watts et al., 2023). Indeed, comparing expression between cell soma and neuropil in mouse, revealed that circRNAs are often enriched in neuropil more than linear mRNAs from the same genes. Similar results were also observed in rat samples, and a 23.6% overlap in the circRNAs in neuropil of the two species was observed (You et al., 2015; Saini et al., 2019). Furthermore, circRNAs were shown to be especially enriched in synaptosomes (Rybak-Wolf et al., 2015; You et al., 2015). Shifts in circRNA expression have been reported to occur with synaptogenesis, independent of overall host gene expression (You et al., 2015). Their levels can also be modulated by changes in neuronal activity and plasticity (You et al., 2015). CircRNAs derived from synaptic genes bind and are regulated by the neuronal-enriched splicing factor, SFPQ (Watts et al., 2023). The nature of such regulation is unclear, including where in the neuron it occurs given that in addition to its nuclear expression, SFPQ was recently reported to also localise to axons and dendrites (Cosker et al., 2016; Thomas-Jinu et al., 2017; Watts et al., 2023).

Functionally, ribosomal profiling data supports the consensus that while circRNAs may have roles in regulating local translation (**Figure 5**), they themselves are not translated (You et al., 2015). A circRNA from the gene encoding the nuclear lncRNA, *Rmst,* was highly enriched in dendrites and synapses, suggesting very distinct non-coding roles for circRNAs than the non-coding roles of linear isoforms (Rybak-Wolf et al., 2015). It has been demonstrated that circRNAs can functionally act as miRNA sponges, sequestering and preventing them from binding to their target mRNAs (Hansen et al., 2013; Memczak et al., 2013). For instance, *ciRS-7*, also known as *circCdr1as*, has more than 70 putative binding sites for the dendritically enriched *miR-7*, allowing multiple interactions (Hansen et al., 2013). Knockout of *ciRS-7* downregulated *miR-7* expression, whereas knockdown of *ciRS-7* decreased the expression of *miR-7* target genes (Hansen et al., 2013; Memczak et al., 2013; Piwecka et al., 2017). Although the specific function of circRNAs in neurites has not yet been addressed, these ncRNAs could similarly participate in the regulation of local protein synthesis.

Neuron-to-neuron RNA transfer

Exosomes are small secretory extracellular vesicles (EVs) that play a role in intercellular communication by transporting a collection of biomolecules, including proteins, nucleic acids and lipids, between adjacent cells or over longer distances. RNAs in exosomes include mRNAs and ncRNAs like miRNAs (Valadi et al., 2007; Crescitelli et al., 2013; Xia et al., 2019). A recent investigation of sncRNAs in mouse primary cortical neurons identified exosomes were dramatically enriched for tsRNAs, with rRNAs also highly abundant, while miRNAs represent < 10% of their contents (Mesquita-Ribeiro et al., 2021). snoRNA-derived fragments were also present. The identification of coding and non-coding RNAs in exosomes (**Figure 1**) suggests such vesicles have the potential to influence the functional and molecular characteristics of recipient cells.

How RNAs are sorted into exosomes is not well understood. Some evidence for a passive sorting mechanism of RNAs into exosomes exists, however, recent literature has demonstrated that soluble RBPs could serve as key players, forming complexes with RNAs and transporting them into extracellular vesicles during the biosynthesis of exosomes (Villarroya-Beltri et al., 2013; McKenzie et al., 2016; Santangelo et al., 2016; Statello et al., 2018). Neuronal exosomes can also package mRNAs in association with proteins, such as the activity-regulated cytoskeleton-associated protein (ARC). As a master regulator of synaptic plasticity, ARC protein in exosomes encapsulates its own mRNA or other highly abundant mRNAs and traffics them between cells (Ashley et al., 2018).

The transfer of exosomes at synapses has long been proposed as a potential mechanism of cell-to-cell communication within the nervous system (Smalheiser, 2007). Studies on both developing and mature neurons have suggested that glutamatergic stimulation can induce exosome release (Fauré et al., 2006; Lachenal et al., 2011), demonstrating the involvement of synaptic activities in the process. Mounting evidence has revealed exosomes are a key modulator of synaptic activity under physiological conditions, as they contain neurite-associated miRNAs and mRNAs that modulate circuit formation and synaptic function after being internalized by local neurons (Morel et al., 2013; Goldie et al., 2014). For example, during circuit formation, BDNF mediates the sorting of specific miRNAs in neuron-derived exosomes (Antoniou et al., 2023). BDNF-induced exosomes in turn increase excitatory synapse formation in recipient hippocampal neurons, a mechanism dependent on inter-neuronal delivery of miRNAs (Antoniou et al., 2023). Depolarisation of differentiated human SH-SY5Y neuroblastoma cells was shown to be associated with an increase in exosomes enriched with primate specific miRNAs, whose mRNA targets are related to synaptic function (Goldie et al., 2014). These observations point to a mechanism where miRNA transfer across the synaptic cleft could influence local mRNA translation and degradation. Finally, blocking the trafficking of exosomes containing activity-regulated cytoskeleton-associated (*Arc*) mRNA from pre-synaptic terminals to post-synaptic muscle has been shown to result in dysregulation of synapse maturation and activity-dependent plasticity (Ashley et al., 2018).

Future perspectives: revealing the scope of local splice isoform diversity using third-generation sequencing technologies

Huge strides have been made in understanding the genes whose RNAs (often of multiple RNA types) reside in axons, dendrites, and at synapses. However, relatively little is known regarding splice isoform diversity at such subcellular resolution. This is largely due to the nature of next-generation RNA sequencing technologies that have been the gold standard thus far, relying on short reads typically covering a single exon or single exon-exon junction. These datasets enable robust comparison of gene expression values across samples, and enable individual alternative splicing events comparison. However, analyses of the same dataset using different bioinformatic tools, has been reported to identify little overlap in splicing events identified, owing to varying requirements in mapped read distribution to detect events, emphasizing the need for new approaches (David et al., 2022). Furthermore, short-read splicing analyses are insufficient for providing insight regarding full-length splice isoform diversity. This requires a sequencing approach where RNA is not fragmented prior to reverse transcription, and hence does not utilize short reads.

Recent advancement in the development of third-generation sequencing technologies, producing long reads, are paving the way to revolutionize our understanding in this area. Identification of full-length transcriptomes with depth and breadth can now be achieved, with two techniques dominating. PacBio Iso-Seq involves sequencing cDNA following 3′ poly(A) tail primed reverse transcription, while Oxford Nanopore Technologies (ONT) sequencing can occur directly from RNA. Each technique offers its own advantages. PacBio Iso-Seq achieves $> 99\%$ accuracy, as each cDNA is sequenced many times to produce consensus HiFi reads (Wenger et al., 2019). ONT can sequence RNAs without poly(A) tails and can detect RNA modifications (e.g., methylation), as well as infer RNA structure (Wang et al., 2021). Both technologies are effective for sequencing transcripts < 10 kb in length, however, for especially long transcripts, reads are better detected by ONT sequencing, likely due to limitations in reverse transcription during PacBio sequencing library prep (Udaondo et al., 2021).

Transcripts from 95% human genes are prone to alternative splicing (Pan et al., 2008; Wang et al., 2008), and the process is particularly elaborate in the nervous system (Yeo et al., 2004; Barbosa-Morais et al., 2012; Raj and Blencowe, 2015). So far, long-read transcriptomic sequencing has been applied to developing and adult cortices in mouse and human (Leung et al., 2021; Patowary et al., 2023), revealing huge swathes of transcript isoforms that were not characterized by short-read RNA-Seq analyses. Given the broad nature of the samples used in these studies (sub-regions of cortical tissue), and limited depth of sequencing, it is highly likely that many more transcript isoforms remain uncovered.

PacBio long-read RNA sequencing has also been harnessed to reveal more accurately the extent of mRNA diversity for 30 genes encoding CNS cell-surface molecules in the mouse retina and brain (Ray et al., 2020). Some of the genes were known to generate many isoforms, but their full repertoires were not well characterized. The study identified hundreds of isoforms for some molecules, with *Nrxn3* showing over 750. In some cases, novel transcript isoforms showed far greater abundance than the canonical isoform. Expectedly, a higher number of transcript isoforms correlated with more protein isoforms, however, open reading frame (ORF) prediction identified that genes often have many more transcript isoforms than the number of ORFs, potentially indicating the presence of many uncharacterised lncRNAs. Inputting assembled transcripts from such datasets into tools such as CPAT (Wang et al., 2013), CPC2 (Kang et al., 2017) and Pfam (El-Gebali et al., 2019), may be used to determine the coding potential of transcripts on a greater scale. While the study examined splice isoform diversity in detail amongst this small subset of 30 genes, diversity amongst other classes of genes whose mRNAs are expressed locally such as those encoding ribosomal and mitochondrial proteins, remains largely uncovered.

Housekeeping ncRNAs reflect a huge amount of the total RNA in cells, with around 80% being rRNA and up to 15% tRNA (Deng et al., 2022). Indeed, local translation points towards an abundance of rRNA and tRNAs in axons, dendrites, and synapses, however, the specific proportions of each type of RNA within these subcellular compartments is largely unknown. Axonal ribosomes have been suggested to exhibit heterogeneity and undergo local remodeling (Shigeoka et al., 2019; Fusco et al., 2021). It is possible that housekeeping ncRNAs, including rRNAs, may also exhibit layers of cell type functional specificity (Ferretti and Karbstein, 2019). Although rRNA is not thought to undergo exchange in ribosomes (Mathis et al., 2017), with pre-rRNAs restricted to the nucleolus (Shigeoka et al., 2019), cell type- or even subcellular-specific differences in rRNAs could be exhibited in other ways (Ferretti and Karbstein, 2019). Changes in rRNA distribution, and chemical modifications affecting their stability or interaction with specific ribosomal proteins remain to be addressed by future studies.

Regarding regulatory ncRNAs, circRNAs pose a particularly intriguing, diverse class of underexplored highly abundant RNAs in neurites, with isoforms often more enriched in the periphery than linearised coding isoforms and understanding of their functions limited (Rybak-Wolf et al., 2015; You et al., 2015). The full extent of their diversity can be elucidated by long-read sequencing (Rahimi et al., 2021).

In conclusion, third-generation sequencing holds the power to provide significant advances towards revealing the true range of full-length mRNA and ncRNA splice isoforms present within far-flung neuronal subcellular compartments. This will enable the identification of alternative isoforms specific to axons versus dendrites versus synapses at new resolution. Single-cell based long-read sequencing will provide true insight into cell-specific isoform diversity. Altogether, such information will likely transform our understanding of the variety of ways by which individual genes are able to regulate their own expression, and that of other genes, to assert regulatory influence on local transcriptomes and proteomes.

Author contributions

RT: Writing – original draft, Writing – review and editing. NN: Writing – original draft, Writing – review and editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of the article. NN is supported by a Springboard award from the Academy of Medical Sciences (SBF008\ 1073). RT is supported by a Wellcome Trust Investigator Award, WT 220861/Z/20/Z, and MRC grant, MR/T033126/1, awarded to Corinne Houart.

Acknowledgments

We thank Corinne Houart and Tilly Baldacchino for helpful suggestions. We apologies to authors whose work could not be cited because of space limitations.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

Ainsley, J. A., Drane, L., Jacobs, J., Kittelberger, K. A., and Reijmers, L. G. (2014). Functionally diverse dendritic mRNAs rapidly associate with ribosomes following a novel experience. *Nat. Commun.* 5:4510. doi: 10.1038/ncomms5510

Alon, S., Goodwin, D. R., Sinha, A., Wassie, A. T., Chen, F., Daugharthy, E. R., et al. (2021). Expansion sequencing: Spatially precise in situ transcriptomics in intact biological systems. *Science* 371:2656. doi: 10.1126/science.aax2656

Alvarez, J., Giuditta, A., and Koenig, E. (2000). Protein synthesis in axons and terminals: Significance for maintenance, plasticity and regulation of phenotype. *Prog. Neurobiol.* 62, 1–62. doi: 10.1016/S0301-0082(99)00062-3

Andreassi, C., and Riccio, A. (2009). To localize or not to localize: mRNA fate is in 3′UTR ends. *Trends Cell Biol.* 19, 465–474. doi: 10.1016/j.tcb.2009.06.001

Andreassi, C., Crerar, H., and Riccio, A. (2018). Post-transcriptional processing of mRNA in neurons: The vestiges of the RNA world drive transcriptome diversity. *Front. Mol. Neurosci.* 11:304. doi: 10.3389/fnmol.2018.00304

Andreassi, C., Luisier, R., Crerar, H., Darsinou, M., Blokzijl-Franke, S., Lenn, T., et al. (2021). Cytoplasmic cleavage of IMPA1 3′ UTR is necessary for maintaining axon integrity. *Cell Rep.* 34:8778. doi: 10.1016/j.celrep.2021.108778

Antoniou, A., Auderset, L., Kaurani, L., Sebastian, E., Zeng, Y., Allahham, M., et al. (2023). Neuronal extracellular vesicles and associated microRNAs induce circuit connectivity downstream BDNF. *Cell Rep.* 42:2063. doi: 10.1016/j.celrep.2023.112063

Ashley, J., Cordy, B., Lucia, D., Fradkin, L. G., Budnik, V., and Thomson, T. (2018). Retrovirus-like gag protein Arc1 binds RNA and traffics across synaptic boutons. *Cell* 172:262–274.e11. doi: 10.1016/j.cell.2017.12.022.

Back, S. H., Lee, K., Vink, E., and Kaufman, R. J. (2006). Cytoplasmic IRE1α-mediated XBP1 mRNA splicing in the absence of nuclear processing and endoplasmic reticulum stress. *J. Biol. Chem.* 281, 18691–18706. doi: 10.1074/jbc.M602030200

Baek, D., Villén, J., Shin, C., Camargo, F. D., Gygi, S. P., and Bartel, D. P. (2008). The impact of microRNAs on protein output. *Nature* 455, 64–71. doi: 10.1038/nature07242

Baez, M. V., Luchelli, L., Maschi, D., Habif, M., Pascual, M., Thomas, M. G., et al. (2011). Smaug1 mRNA-silencing foci respond to NMDA and modulate synapse formation. *J. Cell Biol.* 195, 1141–1157. doi: 10.1083/jcb.201108159

Baptista, B., Riscado, M., Queiroz, J. A., Pichon, C., and Sousa, F. (2021). Non-coding RNAs: Emerging from the discovery to therapeutic applications. *Biochem. Pharmacol.* 189:4469. doi: 10.1016/j.bcp.2021.114469

Barbosa-Morais, N. L., Irimia, M., Pan, Q., Xiong, H. Y., Gueroussov, S., Lee, L. J., et al. (2012). The evolutionary landscape of alternative splicing in vertebrate species. *Science (1979)* 338, 1587–1593. doi: 10.1126/science.1230612

Barnard, D. C., Cao, Q., and Richter, J. D. (2005). Differential phosphorylation controls maskin association with eukaryotic translation initiation factor 4e and localization on the mitotic apparatus. *Mol. Cell. Biol.* 25, 7605–7615. doi: 10.1128/mcb.25.17.7605-7615.2005

Barnard, D. C., Ryan, K., Manley, J. L., and Richter, J. D. (2004). Symplekin and xGLD-2 are required for CPEB-mediated cytoplasmic polyadenylation. *Cell* 119, 641–651. doi: 10.1016/j.cell.2004.10.029

Bartel, D. P. (2009). MicroRNAs: Target recognition and regulatory functions. *Cell* 136, 215–233. doi: 10.1016/j.cell.2009.01.002

Bell, T. J., Miyashiro, K. Y., Sul, J.-Y., Buckley, P. T., Lee, M. T., McCullough, R., et al. (2010). Intron retention facilitates splice variant diversity in calcium-activated big potassium channel populations. *Proc. Natl. Acad. Sci. U.S.A.* 107, 21152–21157. doi: 10.1073/pnas.1015264107

Bell, T. J., Miyashiro, K. Y., Sul, J.-Y., McCullough, R., Buckley, P. T., Jochems, J., et al. (2008). Cytoplasmic BK Ca channel intron-containing mRNAs contribute to the intrinsic excitability of hippocampal neurons. *Proc. Natl. Acad. Sci. U.S.A.* 105, 1901–1906. doi: 10.1073/pnas.0711796105

Bellon, A., Iyer, A., Bridi, S., Lee, F. C. Y., Ovando-Vázquez, C., Corradi, E., et al. (2017). miR-182 regulates Slit2-mediated axon guidance by modulating the local translation of a specific mRNA. *Cell Rep.* 18, 1171–1186. doi: 10.1016/j.celrep.2016.12.093

Biever, A., Glock, C., Tushev, G., Ciirdaeva, E., Dalmay, T., Langer, J. D., et al. (2020). Monosomes actively translate synaptic mRNAs in neuronal processes. *Science (1979)* 367:aay4991. doi: 10.1126/science.aay4991

Braunschweig, U., Barbosa-Morais, N. L., Pan, Q., Nachman, E. N., Alipanahi, B., Gonatopoulos-Pournatzis, T., et al. (2014). Widespread intron retention in mammals functionally tunes transcriptomes. *Genome Res.* 24, 1774–1786. doi: 10.1101/gr.177790.114

Briese, M., Saal, L., Appenzeller, S., Moradi, M., Baluapuri, A., and Sendtner, M. (2016). Whole transcriptome profiling reveals the RNA content of motor axons. *Nucleic Acids Res.* 44:e33. doi: 10.1093/nar/gkv1027

Briggs, J. A., Wolvetang, E. J., Mattick, J. S., Rinn, J. L., and Barry, G. (2015). Mechanisms of long non-coding RNAs in mammalian nervous system development, plasticity, disease, and evolution. *Neuron* 88, 861–877. doi: 10.1016/j.neuron.2015.09.045

Briz, V., Restivo, L., Pasciuto, E., Juczewski, K., Mercaldo, V., Lo, A. C., et al. (2017). The non-coding RNA BC1 regulates experience-dependent structural plasticity and learning. *Nat. Commun.* 8:293. doi: 10.1038/s41467-017-00311-2

Buckley, P. T., Khaladkar, M., Kim, J., and Eberwine, J. (2014). Cytoplasmic intron retention, function, splicing, and the sentinel RNA hypothesis. *Wiley Interdiscip. Rev. RNA* 5, 223–230. doi: 10.1002/wrna.1203

Buckley, P. T., Lee, M. T., Sul, J.-Y., Miyashiro, K. Y., Bell, T. J., Fisher, S. A., et al. (2011). Cytoplasmic intron sequence-retaining transcripts can be dendritically targeted via ID element retrotransposons. *Neuron* 69, 877–884. doi: 10.1016/j.neuron.2011.02.028

Cagnetta, R., Flanagan, J. G., and Soneberg, N. (2023). Control of selective mRNA translation in neuronal subcellular compartments in health and disease. *J. Neurosci.* 43, 7247–7263. doi: 10.1523/JNEUROSCI.2240-22.2023

Cagnetta, R., Frese, C. K., Shigeoka, T., Krijgsveld, J., and Holt, C. E. (2018). Rapid cue-specific remodeling of the nascent axonal proteome. *Neuron* 99:29–46.e4. doi: 10.1016/j.neuron.2018.06.004

Chen, Z., Gore, B. B., Long, H., Ma, L., and Tessier-Lavigne, M. (2008). Alternative splicing of the Robo3 axon guidance receptor governs the midline switch from attraction to repulsion. *Neuron* 58, 325–332. doi: 10.1016/j.neuron.2008.02.016

Chu, J. F., Majumder, P., Chatterjee, B., Huang, S. L., and Shen, C. K. J. (2019). TDP-43 regulates coupled dendritic mRNA transport-translation processes in co-operation with FMRP and staufen1. *Cell Rep.* 29:3118–3133.e6. doi: 10.1016/j.celrep.2019.10.061

Cioni, J. M., Lin, J. Q., Holtermann, A. V., Koppers, M., Jakobs, M. A. H., Azizi, A., et al. (2019). Late endosomes act as mRNA translation platforms and sustain mitochondria in axons. *Cell* 176:56–72.e15. doi: 10.1016/j.cell.2018.11.030

Colak, D., Ji, S. J., Porse, B. T., and Jaffrey, S. R. (2013). XRegulation of axon guidance by compartmentalized nonsense-mediated mRNA decay. *Cell* 153:1252. doi: 10.1016/j.cell.2013.04.056

Corradi, E., Dalla Costa, I., Gavoci, A., Iyer, A., Roccuzzo, M., Otto, T. A., et al. (2020). Axonal precursor mi RNA s hitchhike on endosomes and locally regulate the development of neural circuits. *EMBO J.* 39, 1–24. doi: 10.15252/embj.2019102513

Cosker, K. E., Fenstermacher, S. J., Pazyra-Murphy, M. F., Elliott, H. L., and Segal, R. A. (2016). The RNA-binding protein SFPQ orchestrates an RNA regulon to promote axon viability. *Nat. Neurosci.* 19, 690–696. doi: 10.1038/nn.4280

Crescitelli, R., Lässer, C., Szabó, T. G., Kittel, A., Eldh, M., Dianzani, I., et al. (2013). Distinct RNA profiles in subpopulations of extracellular vesicles: Apoptotic bodies, microvesicles and exosomes. *J. Extracell. Vesicles* 2:677. doi: 10.3402/jev.v2i0.20677

Dajas-Bailador, F., Bonev, B., Garcez, P., Stanley, P., Guillemot, F., and Papalopulu, N. (2012). microRNA-9 regulates axon extension and branching by targeting Map1b in mouse cortical neurons. *Nat. Neurosci.* 15, 697–699. doi: 10.1038/nn.3082

Dalla Costa, I., Buchanan, C. N., Zdradzinski, M. D., Sahoo, P. K., Smith, T. P., Thames, E., et al. (2021). The functional organization of axonal mRNA transport and translation. *Nat. Rev. Neurosci.* 22, 77–91. doi: 10.1038/s41583-020-00407-7

Darnell, J. C., Van Driesche, S. J., Zhang, C., Hung, K. Y. S., Mele, A., Fraser, C. E., et al. (2011). FMRP stalls ribosomal translocation on mRNAs linked to synaptic function and autism. *Cell* 146, 247–261. doi: 10.1016/j.cell.2011.06.013

David, J. K., Maden, S. K., Wood, M. A., Thompson, R. F., and Nellore, A. (2022). Retained introns in long RNA-seq reads are not reliably detected in sample-matched short reads. *Genome Biol.* 23:240. doi: 10.1186/s13059-022-02789-6

Davis, L., Banker, G. A., and Steward, O. (1987). Selective dendritic transport of RNA in hippocampal neurons in culture. *Nature* 330, 477–479. doi: 10.1038/330477a0

Deng, C., Moradi, M., Reinhard, S., Ji, C., Jablonka, S., Hennlein, L., et al. (2021). Dynamic remodeling of ribosomes and endoplasmic reticulum in axon terminals of motoneurons. *J. Cell Sci.* 134:8785. doi: 10.1242/jcs.258785

Deng, Z.-L., Münch, P. C., Mreches, R., and McHardy, A. C. (2022). Rapid and accurate identification of ribosomal RNA sequences via deep learning. *Nucleic Acids Res.* 50:e60. doi: 10.1093/nar/gkac112

Dong, X., Bai, Y., Liao, Z., Gritsch, D., Liu, X., Wang, T., et al. (2023). Circular RNAs in the human brain are tailored to neuron identity and neuropsychiatric disease. *Nat. Commun.* 14:5327. doi: 10.1038/s41467-023-40348-0

Eberwine, J., Miyashiro, K., Kacharmina, J. E., and Job, C. (2001). Local translation of classes of mRNAs that are targeted to neuronal dendrites. *Proc. Natl. Acad. Sci. U.S.A.* 98, 7080–7085. doi: 10.1073/pnas.121146698

Edfors, F., Danielsson, F., Hallström, B. M., Käll, L., Lundberg, E., Pontén, F., et al. (2016). Gene-specific correlation of RNA and protein levels in human cells and tissues. *Mol. Syst. Biol.* 12:7144. doi: 10.15252/msb.20167144

El-Gebali, S., Mistry, J., Bateman, A., Eddy, S. R., Luciani, A., Potter, S. C., et al. (2019). The Pfam protein families database in 2019. *Nucleic Acids Res.* 47, D427–D432. doi: 10.1093/nar/gky995

Eom, T., Berardi, V., Zhong, J., Risuleo, G., and Tiedge, H. (2011). Dual nature of translational control by regulatory BC RNAs. *Mol. Cell Biol.* 31, 4538–4549. doi: 10.1128/MCB.05885-11

Epple, R., Krüger, D., Berulava, T., Brehm, G., Ninov, M., Islam, R., et al. (2021). The coding and small non-coding hippocampal synaptic RNAome. *Mol. Neurobiol.* 58, 2940–2953. doi: 10.1007/s12035-021-02296-y

Faria-Pereira, A., and Morais, V. A. (2022). Synapses: The brain's energy-demanding sites. *Int. J. Mol. Sci.* 23:3627. doi: 10.3390/ijms23073627

Farias, J., Holt, C. E., Sotelo, J. R., and Sotelo-Silveira, J. R. (2020). Axon microdissection and transcriptome profiling reveals the in vivo RNA content of fully differentiated myelinated motor axons. *RNA* 26, 595–612. doi: 10.1261/rna.073700.119

Fauré, J., Lachenal, G., Court, M., Hirrlinger, J., Chatellard-Causse, C., Blot, B., et al. (2006). Exosomes are released by cultured cortical neurones. *Mol. Cell. Neurosci.* 31, 642–648. doi: 10.1016/j.mcn.2005.12.003

Ferretti, M. B., and Karbstein, K. (2019). Does functional specialization of ribosomes really exist? *RNA* 25, 521–538. doi: 10.1261/rna.069823.118

Fonkeu, Y., Kraynyukova, N., Hafner, A.-S., Kochen, L., Sartori, F., Schuman, E. M., et al. (2019). How mRNA localization and protein synthesis sites influence dendritic protein distribution and dynamics. *Neuron* 103:1109–1122.e7. doi: 10.1016/j.neuron.2019.06.022

Fusco, C. M., Desch, K., Dörrbaum, A. R., Wang, M., Staab, A., Chan, I. C. W., et al. (2021). Neuronal ribosomes exhibit dynamic and context-dependent exchange of ribosomal proteins. *Nat. Commun.* 12:6127. doi: 10.1038/s41467-021-26365-x

Galante, P. A. F., Sakabe, N. J., Kirschbaum-Slager, N., and De, S. (2004). Detection and evaluation of intron retention events in the human transcriptome. *RNA* 10, 757–765. doi: 10.1261/rna.5123504

Garner, C. C., Tucker, R. P., and Matus, A. (1988). Selective localization of messenger RNA for cytoskeletal protein MAP2 in dendrites. *Nature* 336, 674–677. doi: 10.1038/336674a0

Gershoni-Emek, N., Altman, T., Ionescu, A., Costa, C. J., Gradus-Pery, T., Willis, D. E., et al. (2018). Localization of RNAi machinery to axonal branch points and growth cones is facilitated by mitochondria and is disrupted in ALS. *Front. Mol. Neurosci.* 11:311. doi: 10.3389/fnmol.2018.00311

Glanzer, J., Miyashiro, K. Y., Sul, J.-Y., Barrett, L., Belt, B., Haydon, P., et al. (2005). RNA splicing capability of live neuronal dendrites. *Proc. Natl. Acad. Sci. U.S.A.* 102, 16859–16864. doi: 10.1073/pnas.0503783102

Glock, C., Biever, A., Tushev, G., Nassim-Assir, B., Kao, A., Bartnik, I., et al. (2021). The translatome of neuronal cell bodies, dendrites, and axons. *Proc. Natl. Acad. Sci. U.S.A.* 118, 1–11. doi: 10.1073/pnas.2113929118

Goldie, B. J., Dun, M. D., Lin, M., Smith, N. D., Verrills, N. M., Dayas, C. V., et al. (2014). Activity-associated miRNA are packaged in Map1b-enriched exosomes released from depolarized neurons. *Nucleic Acids Res* 42, 9195–9208. doi: 10.1093/nar/gku594

Graber, T. E., Freemantle, E., Anadolu, M. N., Hébert-Seropian, S., Macadam, R. L., Shin, U., et al. (2017). UPF1 governs synaptic plasticity through association with a STAU2 RNA granule. *J. Neurosci.* 37, 9116–9131. doi: 10.1523/JNEUROSCI.0088-17.2017

Grabski, D. F., Broseus, L., Kumari, B., Rekosh, D., Hammarskjold, M., and Ritchie, W. (2021). Intron retention and its impact on gene expression and protein diversity: A review and a practical guide. *WIREs RNA* 12:e1631. doi: 10.1002/wrna.1631

Grinman, E., Nakahata, Y., Avchalumov, Y., Espadas, I., Swarnkar, S., Yasuda, R., et al. (2021). Activity-regulated synaptic targeting of lncRNA ADEPTR mediates structural plasticity by localizing Sptn1 and AnkB in dendrites. *Sci. Adv.* 7:605. doi: 10.1126/sciadv.abf0605

Gruber, A. J., and Zavolan, M. (2019). Alternative cleavage and polyadenylation in health and disease. *Nat. Rev. Genet.* 20, 599–614. doi: 10.1038/s41576-019-0145-z

Gumy, L. F., Yeo, G. S. H., Tung, Y.-C. L., Zivraj, K. H., Willis, D., Coppola, G., et al. (2011). Transcriptome analysis of embryonic and adult sensory axons reveals changes in mRNA repertoire localization. *RNA* 17, 85–98. doi: 10.1261/rna.2386111

Hacisuleyman, E., Hale, C. R., Noble, N., Luo, J., Fak, J. J., Saito, M., et al. (2024). Neuronal activity rapidly reprograms dendritic translation via eIF4G2:uORF binding. *Nat. Neurosci.* 27, 822–835. doi: 10.1038/s41593-024-01615-5

Hafner, A. S., Donlin-Asp, P. G., Leitch, B., Herzog, E., and Schuman, E. M. (2019). Local protein synthesis is a ubiquitous feature of neuronal pre- And postsynaptic compartments. *Science (1979)* 364:aau3644. doi: 10.1126/science.aau3644

Hale, C. R., Sawicka, K., Mora, K., Fak, J., Kang, J. J., Cutrim, P., et al. (2021). FMRP regulates mRNAs encoding distinct functions in the cell body and dendrites of CA1 pyramidal neurons. *Elife* 10:1892. doi: 10.7554/eLife.71892

Han, L., Wen, Z., Lynn, R. C., Baudet, M.-L., Holt, C. E., Sasaki, Y., et al. (2011). Regulation of chemotropic guidance of nerve growth cones by microRNA. *Mol. Brain* 4:40. doi: 10.1186/1756-6606-4-40

Hancock, M. L., Preitner, N., Quan, J., and Flanagan, J. G. (2014). MicroRNA-132 is enriched in developing axons, locally regulates Rasa1 mRNA, and promotes axon extension. *J. Neurosci.* 34, 66–78. doi: 10.1523/JNEUROSCI.3371-13.2014

Hansen, T. B., Jensen, T. I., Clausen, B. H., Bramsen, J. B., Finsen, B., Damgaard, C. K., et al. (2013). Natural RNA circles function as efficient microRNA sponges. *Nature* 495, 384–388. doi: 10.1038/nature11993

Hanus, C., and Schuman, E. M. (2013). Proteostasis in complex dendrites. *Nat. Rev. Neurosci.* 14, 638–648. doi: 10.1038/nrn3546

Harbauer, A. B., Hees, J. T., Wanderoy, S., Segura, I., Gibbs, W., Cheng, Y., et al. (2022). Neuronal mitochondria transport Pink1 mRNA via synaptojanin 2 to support local mitophagy. *Neuron* 110:1516–1531.e9. doi: 10.1016/j.neuron.2022.01.035

Harris, J. J., Jolivet, R., and Attwell, D. (2012). Synaptic energy use and supply. *Neuron* 75, 762–777. doi: 10.1016/j.neuron.2012.08.019

He, F., and Jacobson, A. (2015). Nonsense-mediated mRNA decay: Degradation of defective transcripts is only part of the story. *Annu. Rev. Genet.* 49, 339–366. doi: 10.1146/annurev-genet-112414-054639

Hildebrandt, R. P., Moss, K. R., Janusz-Kaminska, A., Knudson, L. A., Denes, L. T., Saxena, T., et al. (2023). Muscleblind-like proteins use modular domains to localize RNAs by riding kinesins and docking to membranes. *Nat. Commun.* 14:3427. doi: 10.1038/s41467-023-38923-6

Holt, C. E., and Schuman, E. M. (2013). The central dogma decentralized: New perspectives on RNA function and local translation in neurons. *Neuron* 80, 648–657. doi: 10.1016/j.neuron.2013.10.036

Holt, C. E., Martin, K. C., and Schuman, E. M. (2019). Local translation in neurons: Visualization and function. *Nat. Struct. Mol. Biol.* 26, 557–566. doi: 10.1038/s41594-019-0263-5

Huang, J., Jiang, B., Li, G. W., Zheng, D., Li, M., Xie, X., et al. (2022). m6A-modified lincRNA Dubr is required for neuronal development by stabilizing YTHDF1/3 and facilitating mRNA translation. *Cell Rep.* 41:111693. doi: 10.1016/j.celrep.2022.111693

Hubstenberger, A., Courel, M., Bénard, M., Souquere, S., Ernoult-Lange, M., Chouaib, R., et al. (2017). P-body purification reveals the condensation of repressed mRNA regulons. *Mol Cell* 68:144–157.e5. doi: 10.1016/j.molcel.2017.09.003

Ingolia, N. T., Brar, G. A., Rouskin, S., McGeachy, A. M., and Weissman, J. S. (2012). The ribosome profiling strategy for monitoring translation in vivo by deep sequencing of ribosome-protected mRNA fragments. *Nat. Protoc.* 7, 1534–1550. doi: 10.1038/nprot.2012.086

Ivshina, M., Lasko, P., and Richter, J. D. (2014). Cytoplasmic polyadenylation element binding proteins in development, health, and disease. *Annu. Rev. Cell Dev. Biol.* 30, 393–415. doi: 10.1146/annurev-cellbio-101011-155831

Jacob, A. G., and Smith, C. W. J. (2017). Intron retention as a component of regulated gene expression programs. *Hum. Genet.* 136, 1043–1057. doi: 10.1007/s00439-017-1791-x

Jaworski, A., Long, H., and Tessier-Lavigne, M. (2010). Collaborative and specialized functions of Robo1 and Robo2 in spinal commissural axon guidance. *J. Neurosci.* 30, 9445–9453. doi: 10.1523/JNEUROSCI.6290-09.2010

Ji, S., and Jaffrey, S. R. (2014). Axonal transcription factors: Novel regulators of growth cone-to-nucleus signaling. *Dev. Neurobiol.* 74, 245–258. doi: 10.1002/dneu.22112

Jung, J., Ohk, J., Kim, H., Holt, C. E., Park, H. J., and Jung, H. (2023). mRNA transport, translation, and decay in adult mammalian central nervous system axons. *Neuron* 111:650–668.e4. doi: 10.1016/j.neuron.2022.12.015

Jung, M.-Y., Lorenz, L., and Richter, J. D. (2006). Translational control by neuroguidin, a eukaryotic initiation factor 4E and CPEB binding protein. *Mol. Cell Biol.* 26, 4277–4287. doi: 10.1128/mcb.02470-05

Kang, Y.-J., Yang, D.-C., Kong, L., Hou, M., Meng, Y.-Q., Wei, L., et al. (2017). CPC2: A fast and accurate coding potential calculator based on sequence intrinsic features. *Nucleic Acids Res.* 45, W12–W16. doi: 10.1093/nar/gkx428

Kaplan, B. B., Kar, A. N., Gioio, A. E., and Aschrafi, A. (2013). MicroRNAs in the axon and presynaptic nerve terminal. *Front. Cell. Neurosci.* 7:126. doi: 10.3389/fncel.2013.00126

Kesner, J. S., Chen, Z., Shi, P., Aparicio, A. O., Murphy, M. R., Guo, Y., et al. (2023). Noncoding translation mitigation. *Nature* 617, 395–402. doi: 10.1038/s41586-023-05946-4

Khong, A., Matheny, T., Jain, S., Mitchell, S. F., Wheeler, J. R., and Parker, R. (2017). The stress granule transcriptome reveals principles of mRNA accumulation in stress granules. *Mol. Cell* 68:808–820.e5. doi: 10.1016/j.molcel.2017.10.015

Kiltschewskij, D. J., Harrison, P. F., Fitzsimmons, C., Beilharz, T. H., and Cairns, M. J. (2023). Extension of mRNA poly(A) tails and 3′UTRs during neuronal differentiation exhibits variable association with post-transcriptional dynamics. *Nucleic Acids Res.* 51, 8181–8198. doi: 10.1093/nar/gkad499

Kim, H. H., Kim, P., Phay, M., and Yoo, S. (2015). Identification of precursor microRNAs within distal axons of sensory neuron. *J. Neurochem.* 134, 193–199. doi: 10.1111/jnc.13140

Koenig, E., Martin, R., Titmus, M., and Sotelo-Silveira, J. R. (2000). Cryptic peripheral ribosomal domains distributed intermittently along mammalian myelinated axons. *J. Neurosci.* 20, 8390–8400.

Konieczny, P., Stepniak-Konieczna, E., and Sobczak, K. (2014). MBNL proteins and their target RNAs, interaction and splicing regulation. *Nucleic Acids Res.* 42, 10873–10887. doi: 10.1093/nar/gku767

Koppers, M., Özkan, N., Nguyen, H. H., Jurriens, D., McCaughey, J., Stucchi, R., et al. (2022). Axonal ER tubules regulate local translation via P180/RRBP1-mediated ribosome interactions. *bioRxiv* [Preprint]. doi: 10.1101/2022.11.30.518484

Kristensen, L. S., Jakobsen, T., Hager, H., and Kjems, J. (2022). The emerging roles of circRNAs in cancer and oncology. *Nat. Rev. Clin. Oncol.* 19, 188–206. doi: 10.1038/s41571-021-00585-y

Lachenal, G., Pernet-Gallay, K., Chivet, M., Hemming, F. J., Belly, A., Bodon, G., et al. (2011). Release of exosomes from differentiated neurons and its regulation by synaptic glutamatergic activity. *Mol. Cell. Neurosci.* 46, 409–418. doi: 10.1016/j.mcn.2010.11.004

Lacoux, C., Di Marino, D., Boyl, P. P., Zalfa, F., Yan, B., Ciotti, M. T., et al. (2012). BC1-FMRP interaction is modulated by 2′-O-methylation: RNA-binding activity of the tudor domain and translational regulation at synapses. *Nucleic Acids Res.* 40, 4086–4096. doi: 10.1093/nar/gkr1254

Lee, J. A., Damianov, A., Lin, C. H., Fontes, M., Parikshak, N. N., Anderson, E. S., et al. (2016). Cytoplasmic Rbfox1 regulates the expression of synaptic and autism-related genes. *Neuron* 89, 113–128. doi: 10.1016/j.neuron.2015.11.025

Lee, S., Kopp, F., Chang, T. C., Sataluri, A., Chen, B., Sivakumar, S., et al. (2016). Noncoding RNA NORAD regulates genomic stability by sequestering PUMILIO proteins. *Cell* 164, 69–80. doi: 10.1016/j.cell.2015.12.017

Leung, S. K., Jeffries, A. R., Castanho, I., Jordan, B. T., Moore, K., Davies, J. P., et al. (2021). Full-length transcript sequencing of human and mouse cerebral cortex identifies widespread isoform diversity and alternative splicing. *Cell Rep.* 37:110022. doi: 10.1016/j.celrep.2021.110022

Li, P., Jia, Y., Tang, W., Cui, Q., Liu, M., and Jiang, J. (2021). Roles of non-coding RNAs in central nervous system axon regeneration. *Front. Neurosci.* 15:630633. doi: 10.3389/fnins.2021.630633

Li, Y., Bor, Y., Fitzgerald, M. P., Lee, K. S., Rekosh, D., and Hammarskjold, M.-L. (2016). An NXF1 mRNA with a retained intron is expressed in hippocampal and neocortical neurons and is translated into a protein that functions as an Nxf1 cofactor. *Mol. Biol. Cell* 27, 3903–3912. doi: 10.1091/mbc.E16-07-0515

Li, Y., Bor, Y., Misawa, Y., Xue, Y., Rekosh, D., and Hammarskjöld, M.-L. (2006). An intron with a constitutive transport element is retained in a Tap messenger RNA. *Nature* 443, 234–237. doi: 10.1038/nature05107

Lin, D., Pestova, T. V., Hellen, C. U. T., and Tiedge, H. (2008). Translational control by a small RNA: Dendritic BC1 RNA targets the eukaryotic initiation factor 4A helicase mechanism. *Mol. Cell Biol.* 28, 3008–3019. doi: 10.1128/mcb.01800-07

Loedige, I., Baranovskii, A., Mendonsa, S., Dantsuji, S., Popitsch, N., Breimann, L., et al. (2023). mRNA stability and m6A are major determinants of subcellular mRNA localization in neurons. *Mol. Cell* 83:2709–2725.e10. doi: 10.1016/j.molcel.2023.06.021

Lucci, C., Mesquita-Ribeiro, R., Rathbone, A., and Dajas-Bailador, F. (2020). Spatiotemporal regulation of GSK3β levels by miRNA-26a controls axon development in cortical neurons. *Development (Cambridge)* 147:232. doi: 10.1242/dev.180232

Lugli, G., Larson, J., Demars, M. P., and Smalheiser, N. R. (2012). Primary micro RNA precursor transcripts are localized at post-synaptic densities in adult mouse forebrain. *J. Neurochem.* 123, 459–466. doi: 10.1111/j.1471-4159.2012.07921.x

Lugli, G., Larson, J., Martone, M. E., Jones, Y., and Smalheiser, N. R. (2005). Dicer and eIF2c are enriched at postsynaptic densities in adult mouse brain and are modified by neuronal activity in a calpain-dependent manner. *J. Neurochem.* 94, 896–905. doi: 10.1111/j.1471-4159.2005.03224.x

Lugli, G., Torvik, V. I., Larson, J., and Smalheiser, N. R. (2008). Expression of microRNAs and their precursors in synaptic fractions of adult mouse forebrain. *J. Neurochem.* 106, 650–661. doi: 10.1111/j.1471-4159.2008.05413.x

Luisier, R., Andreassi, C., Fournier, L., and Riccio, A. (2023). The predicted RNA-binding protein regulome of axonal mRNAs. *Genome Res.* 33, 1497–1512. doi: 10.1101/gr.277804.123

Luisier, R., Tyzack, G. E., Hall, C. E., Mitchell, J. S., Devine, H., Taha, D. M., et al. (2018). Intron retention and nuclear loss of SFPQ are molecular hallmarks of ALS. *Nat. Commun.* 9:2010. doi: 10.1038/s41467-018-04373-8

Lykke-Andersen, S., and Jensen, T. H. (2015). Nonsense-mediated mRNA decay: An intricate machinery that shapes transcriptomes. *Nat. Rev. Mol. Cell Biol.* 16, 665–677. doi: 10.1038/nrm4063

Ma, W., and Mayr, C. (2018). A membraneless organelle associated with the endoplasmic reticulum enables 3′UTR-mediated protein-protein interactions. *Cell* 175, 1492–1506. doi: 10.1016/j.cell.2018.10.007

Maciel, R., Bis, D. M., Rebelo, A. P., Saghira, C., Züchner, S., and Saporta, M. A. (2018). The human motor neuron axonal transcriptome is enriched for transcripts related to mitochondrial function and microtubule-based axonal transport. *Exp. Neurol.* 307, 155–163. doi: 10.1016/j.expneurol.2018.06.008

Mansur, F., Alarcon, J. M., Stackpole, E. E., Wang, R., and Richter, J. D. (2021). Noncanonical cytoplasmic poly(A) polymerases regulate RNA levels, alternative RNA processing, and synaptic plasticity but not hippocampal-dependent behaviours. *RNA Biol.* 18, 962–971. doi: 10.1080/15476286.2020.1824061

Mathis, A. D., Naylor, B. C., Carson, R. H., Evans, E., Harwell, J., Knecht, J., et al. (2017). Mechanisms of in vivo ribosome maintenance change in response to nutrient signals. *Mol. Cell. Proteomics* 16, 243–254. doi: 10.1074/mcp.M116.063255

Mattick, J. S., Amaral, P. P., Carninci, P., Carpenter, S., Chang, H. Y., Chen, L.-L., et al. (2023). Long non-coding RNAs: Definitions, functions, challenges and recommendations. *Nat. Rev. Mol. Cell Biol.* 24, 430–447. doi: 10.1038/s41580-022-00566-8

Mayr, C. (2019). What are 3′ UTRs doing? *Cold Spring Harb. Perspect. Biol.* 11:a034728. doi: 10.1101/cshperspect.a034728

Mazroui, R., Marco, S., Di, Kaufman, R. J., and Gallouzi, I.-E. (2007). Inhibition of the ubiquitin-proteasome system induces stress granule formation. *Mol. Biol. Cell* 18, 2603–2618. doi: 10.1091/mbc.E06

McKenzie, A. J., Hoshino, D., Hong, N. H., Cha, D. J., Franklin, J. L., Coffey, R. J., et al. (2016). KRAS-MEK signaling controls Ago2 sorting into exosomes. *Cell Rep.* 15, 978–987. doi: 10.1016/j.celrep.2016.03.085

Memczak, S., Jens, M., Elefsinioti, A., Torti, F., Krueger, J., Rybak, A., et al. (2013). Circular RNAs are a large class of animal RNAs with regulatory potency. *Nature* 495, 333–338. doi: 10.1038/nature11928

Mesquita-Ribeiro, R., Fort, R. S., Rathbone, A., Farias, J., Lucci, C., James, V., et al. (2021). Distinct small non-coding RNA landscape in the axons and released extracellular vesicles of developing primary cortical neurons and the axoplasm of adult nerves. *RNA Biol.* 18, 832–855. doi: 10.1080/15476286.2021.2000792

Middleton, R., Gao, D., Thomas, A., Singh, B., Au, A., Wong, J. J.-L., et al. (2017). IRFinder: Assessing the impact of intron retention on mammalian gene expression. *Genome Biol.* 18:51. doi: 10.1186/s13059-017-1184-4

Middleton, S. A., Eberwine, J., and Kim, J. (2019). Comprehensive catalog of dendritically localized mRNA isoforms from sub-cellular sequencing of single mouse neurons. *BMC Biol.* 17:5. doi: 10.1186/s12915-019-0630-z

Minis, A., Dahary, D., Manor, O., Leshkowitz, D., Pilpel, Y., and Yaron, A. (2014). Subcellular transcriptomics—Dissection of the mRNA composition in the axonal compartment of sensory neurons. *Dev. Neurobiol.* 74, 365–381. doi: 10.1002/dneu.22140

Miura, P., Sanfilippo, P., Shenker, S., and Lai, E. C. (2014). Alternative polyadenylation in the nervous system: To what lengths will 3′ UTR extensions take us? *BioEssays* 36, 766–777. doi: 10.1002/bies.201300174

Miura, P., Shenker, S., Andreu-Agullo, C., Westholm, J. O., and Lai, E. C. (2013). Widespread and extensive lengthening of 39 UTRs in the mammalian brain. *Genome Res.* 23, 812–825. doi: 10.1101/gr.146886.112

Modarresi, F., Faghihi, M. A., Lopez-Toledano, M. A., Fatemi, R. P., Magistri, M., Brothers, S. P., et al. (2012). Inhibition of natural antisense transcripts in vivo results in gene-specific transcriptional upregulation. *Nat. Biotechnol.* 30, 453–459. doi: 10.1038/nbt.2158

Morel, L., Regan, M., Higashimori, H., Ng, S. K., Esau, C., Vidensky, S., et al. (2013). Neuronal exosomal mirna-dependent translational regulation of astroglial glutamate transporter glt1. *J. Biol. Chem.* 288, 7105–7116. doi: 10.1074/jbc.M112.410944

Muddashetty, R. S., Khanam, T., Kondrashov, A., Bundman, M., Iacoangeli, A., Kremerskothen, J., et al. (2002). Poly(A)-binding protein is associated with neuronal BC1 and BC200 ribonucleoprotein particles. *J. Mol. Biol.* 321, 433–445. doi: 10.1016/S0022-2836(02)00655-1

Muslimov, I. A., Santi, E., Homel, P., Perini, S., Higgins, D., and Tiedge, H. (1997). RNA transport in dendrites: A cis -acting targeting element is contained within neuronal BC1 RNA. *J. Neurosci.* 17, 4722–4733. doi: 10.1523/JNEUROSCI.17-12-04722.1997

Nakayama, K., Ohashi, R., Shinoda, Y., Yamazaki, M., Abe, M., Fujikawa, A., et al. (2017). RNG105/caprin1, an RNA granule protein for dendritic mRNA localization, is essential for long-term memory formation. *Elife* 21:e29677. doi: 10.7554/eLife.29677.001

Natera-Naranjo, O., Aschrafi, A., Gioio, A. E., and Kaplan, B. B. (2010). Identification and quantitative analyses of microRNAs located in the distal axons of sympathetic neurons. *RNA* 16, 1516–1529. doi: 10.1261/rna.1833310

Nijssen, J., Aguila, J., Hoogstraaten, R., Kee, N., and Hedlund, E. (2018). Axon-Seq decodes the motor axon transcriptome and its modulation in response to ALS. *Stem Cell Rep.* 11, 1565–1578. doi: 10.1016/j.stemcr.2018.11.005

Nikolaou, N., Gordon, P. M., Hamid, F., Taylor, R., Lloyd-Jones, J., Makeyev, E. V., et al. (2022). Cytoplasmic pool of U1 spliceosome protein SNRNP70 shapes the axonal transcriptome and regulates motor connectivity. *Curr. Biol.* 32, 5099–5115. doi: 10.1016/j.cub.2022.10.048

Notaras, M., Allen, M., Longo, F., Volk, N., Toth, M., Li Jeon, N., et al. (2020). UPF2 leads to degradation of dendritically targeted mRNAs to regulate synaptic plasticity and cognitive function. *Mol. Psychiatry* 25, 3360–3379. doi: 10.1038/s41380-019-0547-5

O'Brien, J., Hayder, H., Zayed, Y., and Peng, C. (2018). Overview of MicroRNA biogenesis, mechanisms of actions, and circulation. *Front. Endocrinol.* 9:402. doi: 10.3389/fendo.2018.00402

Ortiz, R., Georgieva, M. V., Gutiérrez, S., Pedraza, N., Fernández-Moya, S. M., and Gallego, C. (2017). Recruitment of staufen2 enhances dendritic localization of an intron-containing CaMKIIα mRNA. *Cell Rep.* 20, 13–20. doi: 10.1016/j.celrep.2017.06.026

Ostroff, L. E., Botsford, B., Gindina, S., Cowansage, K. K., Ledoux, J. E., Klann, E., et al. (2017). Accumulation of polyribosomes in dendritic spine heads, but not bases and necks, during memory consolidation depends on cap-dependent translation initiation. *J. Neurosci.* 37, 1862–1872. doi: 10.1523/JNEUROSCI.3301-16.2017

Ouwenga, R., Lake, A. M., O'Brien, D., Mogha, A., Dani, A., and Dougherty, J. D. (2017). Transcriptomic analysis of ribosome-bound mRNA in cortical neurites in vivo. *J. Neurosci.* 37, 8688–8705. doi: 10.1523/JNEUROSCI.3044-16.2017

Öztürk, Z., O'Kane, C. J., and Pérez-Moreno, J. J. (2020). Axonal Endoplasmic Reticulum Dynamics and Its Roles in Neurodegeneration. *Front. Neurosci.* 14:48. doi: 10.3389/fnins.2020.00048

Pan, Q., Shai, O., Lee, L. J., Frey, B. J., and Blencowe, B. J. (2008). Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. *Nat. Genet.* 40, 1413–1415. doi: 10.1038/ng.259

Pascual, M., Vicente, M., Monferrer, L., and Artero, R. (2006). The Muscleblind family of proteins: An emerging class of regulators of developmentally programmed alternative splicing. *Differentiation* 74, 65–80. doi: 10.1111/j.1432-0436.2006.00060.x

Patowary, A., Zhang, P., Jops, C., Vuong, C., Ge, X., Hou, K., et al. (2023). Developmental isoform diversity in the human neocortex informs neuropsychiatric risk mechanisms. *bioRxiv* [Preprint]. doi: 10.1101/2023.03.25.534016

Perez, J. D., Dieck, S., Alvarez-Castelao, B., Tushev, G., Chan, I. C., and Schuman, E. M. (2021). Subcellular sequencing of single neurons reveals the dendritic transcriptome of GABAergic interneurons. *Elife* 10:63092. doi: 10.7554/eLife.63092

Petrić Howe, M., Crerar, H., Neeves, J., Harley, J., Tyzack, G. E., Klein, P., et al. (2022). Physiological intron retaining transcripts in the cytoplasm abound during human motor neurogenesis. *Genome Res.* 32, 1808–1825. doi: 10.1101/gr.276898.122

Piper, M., and Holt, C. (2004). RNA TRANSLATION IN AXONS. *Annu. Rev. Cell Dev. Biol.* 20, 505–523. doi: 10.1146/annurev.cellbio.20.010403.111746

Piwecka, M., Glažar, P., Hernandez-Miranda, L. R., Memczak, S., Wolf, S. A., Rybak-Wolf, A., et al. (2017). Loss of a mammalian circular RNA locus causes miRNA deregulation and affects brain function. *Science (1979)* 357:aam8526. doi: 10.1126/science.aam8526

Poulopoulos, A., Murphy, A. J., Ozkan, A., Davis, P., Hatch, J., Kirchner, R., et al. (2019). Subcellular transcriptomes and proteomes of developing axon projections in the cerebral cortex. *Nature* 565, 356–360. doi: 10.1038/s41586-018-0847-y

Proudfoot, N. J. (2011). Ending the message: Poly(A) signals then and now. *Genes Dev.* 25, 1770–1782. doi: 10.1101/gad.17268411

Puri, D., Sharma, S., Samaddar, S., Ravivarma, S., Banerjee, S., and Ghosh-Roy, A. (2023). Muscleblind-1 interacts with tubulin mRNAs to regulate the microtubule cytoskeleton in *C. elegans* mechanosensory neurons. *PLoS Genet.* 19:e1010885. doi: 10.1371/journal.pgen.1010885

Pushpalatha, K. V., and Besse, F. (2019). Local translation in axons: When membraneless RNP granules meet membrane-bound organelles. *Front. Mol. Biosci.* 6:129. doi: 10.3389/fmolb.2019.00129

Rahimi, K., Venø, M. T., Dupont, D. M., and Kjems, J. (2021). Nanopore sequencing of brain-derived full-length circRNAs reveals circRNA-specific exon usage, intron retention and microexons. *Nat. Commun.* 12:4825. doi: 10.1038/s41467-021-24975-z

Raj, B., and Blencowe, B. J. (2015). Alternative splicing in the mammalian nervous system: Recent insights into mechanisms and functional roles. *Neuron* 87, 14–27. doi: 10.1016/j.neuron.2015.05.004

Ray, T. A., Cochran, K., Kozlowski, C., Wang, J., Alexander, G., Cady, M. A., et al. (2020). Comprehensive identification of mRNA isoforms reveals the diversity of neural cell-surface molecules with roles in retinal development and disease. *Nat. Commun.* 11:3328. doi: 10.1038/s41467-020-17009-7

Richter, J. D., and Zhao, X. (2021). The molecular biology of FMRP: New insights into fragile X syndrome. *Nat. Rev. Neurosci.* 22, 209–222. doi: 10.1038/s41583-021-00432-0

Rotem, N., Magen, I., Ionescu, A., Gershoni-Emek, N., Altman, T., Costa, C. J., et al. (2017). ALS along the axons – expression of coding and noncoding RNA differs in axons of ALS models. *Sci. Rep.* 7:44500. doi: 10.1038/srep44500

Rybak-Wolf, A., Stottmeister, C., Glažar, P., Jens, M., Pino, N., Giusti, S., et al. (2015). Circular RNAs in the mammalian brain are highly abundant, conserved, and dynamically expressed. *Mol. Cell* 58, 870–885. doi: 10.1016/j.molcel.2015.03.027

Saini, H., Bicknell, A. A., Eddy, S. R., and Moore, M. J. (2019). Free circular introns with an unusual branchpoint in neuronal projections. *Elife* 8:47809. doi: 10.7554/eLife.47809

Salehi, S., Zare, A., Prezza, G., Bader, J., Schneider, C., Fischer, U., et al. (2023). Cytosolic Ptbp2 modulates axon growth in motoneurons through axonal localization and translation of Hnrnpr. *Nat. Commun.* 14:4158. doi: 10.1038/s41467-023-39787-6

Sambandan, S., Akbalik, G., Kochen, L., Rinne, J., Kahlstatt, J., Glock, C., et al. (2017). Activity-dependent spatially localized miRNA maturation in neuronal dendrites. *Science* 355, 634–637.

Santangelo, L., Giurato, G., Cicchini, C., Montaldo, C., Mancone, C., Tarallo, R., et al. (2016). The RNA-binding protein SYNCRIP is a component of the hepatocyte exosomal machinery controlling MicroRNA sorting. *Cell Rep.* 17, 799–808. doi: 10.1016/j.celrep.2016.09.031

Sanz, E., Yang, L., Su, T., Morris, D. R., McKnight, G. S., and Amieux, P. S. (2009). Cell-type-specific isolation of ribosome-associated mRNA from complex tissues. *Proc. Natl. Acad. Sci. U.S.A.* 106, 13939–13944. doi: 10.1073/pnas.0907143106

Sasaki, Y., Gross, C., Xing, L., Goshima, Y., and Bassell, G. J. (2014). Identification of axon-enriched MicroRNAs localized to growth cones of cortical neurons. *Dev. Neurobiol.* 74, 397–406. doi: 10.1002/dneu.22113

Setola, V., Terao, M., Locatelli, D., Bassanini, S., Garattini, E., and Battaglia, G. (2007). Axonal-SMN (a-SMN), a protein isoform of the survival motor neuron gene, is specifically involved in axonogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 104, 1959–1964. doi: 10.1073/pnas.0610660104

Sharangdhar, T., Sugimoto, Y., Heraud-Farlow, J., Fernández-Moya, S. M., Ehses, J., Ruiz de los Mozos, I., et al. (2017). A retained intron in the 3′- UTR of Calm3 mRNA mediates its Staufen2- and activity-dependent localization to neuronal dendrites. *EMBO Rep.* 18, 1762–1774. doi: 10.15252/embr.201744334

Shigeoka, T., Jung, H., Jung, J., Turner-Bridger, B., Ohk, J., Lin, J., et al. (2016). Dynamic axonal translation in developing and mature visual circuits. *Cell* 166, 181–192. doi: 10.1016/j.cell.2016.05.029

Shigeoka, T., Koppers, M., Wong, H. H.-W., Lin, J. Q., Cagnetta, R., Dwivedy, A., et al. (2019). On-site ribosome remodeling by locally synthesized ribosomal proteins in axons. *Cell Rep.* 29, 3605–3619. doi: 10.1016/j.celrep.2019.11.025

Simbriger, K., Amorim, I. S., Chalkiadaki, K., Lach, G., Jafarnejad, S. M., Khoutorsky, A., et al. (2020). Monitoring translation in synaptic fractions using a ribosome profiling strategy. *J. Neurosci. Methods* 329:108456. doi: 10.1016/j.jneumeth.2019.108456

Smalheiser, N. R. (2007). Exosomal transfer of proteins and RNAs at synapses in the nervous system. *Biol. Direct.* 2:35. doi: 10.1186/1745-6150-2-35

Smalheiser, N. R. (2014). The RNA-centred view of the synapse: Non-coding RNAs and synaptic plasticity. *Philos. Trans. R. Soc. B Biol. Sci.* 369:20130504. doi: 10.1098/rstb.2013.0504

Smalheiser, N. R., Lugli, G., Torvik, V. I., Mise, N., Ikeda, R., and Abe, K. (2008). Natural antisense transcripts are co-expressed with sense mRNAs in synaptoneurosomes of adult mouse forebrain. *Neurosci. Res.* 62, 236–239. doi: 10.1016/j.neures.2008.08.010

Solomon, S., Xu, Y., Wang, B., David, M. D., Schubert, P., Kennedy, D., et al. (2007). Distinct structural features of caprin-1 mediate its interaction with G3BP-1 and its induction of phosphorylation of eukaryotic translation initiation factor 2 α, entry to cytoplasmic stress granules, and selective interaction with a subset of mRNAs. *Mol. Cell. Biol.* 27, 2324–2342. doi: 10.1128/mcb.02300-06

Sorek, R., and Ast, G. (2003). Intronic sequences flanking alternatively spliced exons are conserved between human and mouse. *Genome Res.* 13, 1631–1637. doi: 10.1101/gr.1208803

Statello, L., Maugeri, M., Garre, E., Nawaz, M., Wahlgren, J., Papadimitriou, A., et al. (2018). Identification of RNA-binding proteins in exosomes capable of interacting with different types of RNA: RBP-facilitated transport of RNAs into exosomes. *PLoS One* 13:e0195969. doi: 10.1371/journal.pone.0195969

Steward, O. (1997). mRNA localization in neurons: A multipurpose mechanism? *Neuron* 18, 9–12. doi: 10.1016/S0896-6273(01)80041-6

Steward, O., and Levy, W. (1982). Preferential localization of polyribosomes under the base of dendritic spines in granule cells of the dentate gyrus. *J. Neurosci.* 2, 284–291. doi: 10.1523/JNEUROSCI.02-03-00284.1982

Sun, C., and Schuman, E. (2023). A multi-omics view of neuronal subcellular protein synthesis. *Curr. Opin. Neurobiol.* 80:102705. doi: 10.1016/j.conb.2023.102705

Sun, C., and Schuman, E. M. (2022). Logistics of neuronal protein turnover: Numbers and mechanisms. *Mol. Cell. Neurosci.* 123:103793. doi: 10.1016/j.mcn.2022.103793

Sun, C., Nold, A., Fusco, C. M., Rangaraju, V., Tchumatchenko, T., Heilemann, M., et al. (2021). The prevalence and specificity of local protein synthesis during neuronal synaptic plasticity. *Sci. Adv.* 7:abj0790. doi: 10.1126/sciadv.abj0790

Swanger, S. A., He, Y. A., Richter, J. D., and Bassell, G. J. (2013). Dendritic GluN2A synthesis mediates activity-induced NMDA receptor insertion. *J. Neurosci.* 33, 8898–8908. doi: 10.1523/JNEUROSCI.0289-13.2013

Taliaferro, J. M., Vidaki, M., Oliveira, R., Olson, S., Zhan, L., Saxena, T., et al. (2016). Distal alternative last exons localize mRNAs to neural projections. *Mol. Cell* 61, 821–833. doi: 10.1016/j.molcel.2016.01.020

Tan, M. C., Widagdo, J., Chau, Y. Q., Zhu, T., Wong, J. J. L., Cheung, A., et al. (2017). The activity-induced long non-coding RNA Meg3 modulates AMPA receptor surface expression in primary cortical neurons. *Front. Cell. Neurosci.* 11:124. doi: 10.3389/fncel.2017.00124

Taylor, R., Hamid, F., Fielding, T., Gordon, P. M., Maloney, M., Makeyev, E. V., et al. (2022). Prematurely terminated intron-retaining mRNAs invade axons in SFPQ null-driven neurodegeneration and are a hallmark of ALS. *Nat. Commun.* 13:6994. doi: 10.1038/s41467-022-34331-4

Taylor, R., and Houart, C. (2024). Optimized primary culture of neuronal populations for subcellular omics applications. *Methods Mol. Biol.* 2707, 113–124. doi: 10.1007/978-1-0716-3401-1_7

Thomas-Jinu, S., Gordon, P. M., Fielding, T., Taylor, R., Smith, B. N., Snowden, V., et al. (2017). Non-nuclear pool of splicing factor SFPQ regulates axonal transcripts required for normal motor development. *Neuron* 94, 322–336. doi: 10.1016/j.neuron.2017.03.026

Tian, B., and Manley, J. L. (2013). Alternative cleavage and polyadenylation: The long and short of it. *Trends Biochem. Sci.* 38, 312–320. doi: 10.1016/j.tibs.2013.03.005

Tian, H., Hu, Z., and Wang, C. (2022). The therapeutic potential of tRNA-derived Small RNAs in neurodegenerative disorders. *Aging Dis.* 13:389. doi: 10.14336/AD.2021.0903

Tiedge, H., Fremeau, R. T., Weinstock, P. H., Arancio, O., and Brosius, J. (1991). Dendritic location of neural BC1 RNA. *Proc. Natl. Acad. Sci. U.S.A.* 88, 2093–2097. doi: 10.1073/pnas.88.6.2093

Tushev, G., Glock, C., Heumüller, M., Biever, A., Jovanovic, M., and Schuman, E. M. (2018). Alternative 3′ UTRs modify the localization, regulatory potential, stability, and plasticity of mRNAs in neuronal compartments. *Neuron* 98, 495–511. doi: 10.1016/j.neuron.2018.03.030

Twiss, J., and Merianda, T. (2015). Old dogs with new tricks: Intra-axonal translation of nuclear proteins. *Neural Regen. Res.* 10:1560. doi: 10.4103/1673-5374.165264

Udagawa, T., Swanger, S. A., Takeuchi, K., Kim, J. H., Nalavadi, V., Shin, J., et al. (2012). Bidirectional control of mRNA translation and synaptic plasticity by the cytoplasmic polyadenylation complex. *Mol. Cell* 47, 253–266. doi: 10.1016/j.molcel.2012.05.016

Udaondo, Z., Sittikankaew, K., Uengwetwanit, T., Wongsurawat, T., Sonthirod, C., Jenjaroenpun, P., et al. (2021). Comparative analysis of PacBio and Oxford nanopore sequencing technologies for transcriptomic landscape identification of *Penaeus monodon*. *Life* 11:862. doi: 10.3390/life11080862

Uemura, A., Oku, M., Mori, K., and Yoshida, H. (2009). Unconventional splicing of XBP1 mRNA occurs in the cytoplasm during the mammalian unfolded protein response. *J. Cell Sci.* 122, 2877–2886. doi: 10.1242/jcs.040584

Valadi, H., Ekström, K., Bossios, A., Sjöstrand, M., Lee, J. J., and Lötvall, J. O. (2007). Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat. Cell Biol.* 9, 654–659. doi: 10.1038/ncb1596

Vargas, J. N. S., Kar, A. N., Kowalak, J. A., Gale, J. R., Aschrafi, A., Chen, C. Y., et al. (2016). Axonal localization and mitochondrial association of precursor microRNA 338. *Cell. Mol. Life Sci.* 73, 4327–4340. doi: 10.1007/s00018-016-2270-6

Vargas, J. N. S., Sleigh, J. N., and Schiavo, G. (2022). Coupling axonal mRNA transport and local translation to organelle maintenance and function. *Curr. Opin. Cell Biol.* 74, 97–103. doi: 10.1016/j.ceb.2022.01.008

Vessey, J. P., Vaccani, A., Xie, Y., Dahm, R., Karra, D., Kiebler, M. A., et al. (2006). Dendritic localization of the translational repressor Pumilio 2 and its contribution to dendritic stress granules. *J. Neurosci.* 26, 6496–6508. doi: 10.1523/JNEUROSCI.0649-06.2006

Vessey, J., Schoderboeck, L., Gingl, E., Luzi, E., Riefler, J., Di Leva, F., et al. (2010). Mammalian Pumilio 2 regulates dendrite morphogenesis and synaptic function. *Proc. Natl. Acad. Sci. U.S.A.* 107, 3222–3227. doi: 10.1073/pnas.0907128107

Villarroya-Beltri, C., Gutiérrez-Vázquez, C., Sánchez-Cabo, F., Pérez-Hernández, D., Vázquez, J., Martin-Cofreces, N., et al. (2013). Sumoylated hnRNPA2B1 controls the sorting of miRNAs into exosomes through binding to specific motifs. *Nat. Commun.* 4:2980. doi: 10.1038/ncomms3980

Vo, N. K., Cambronne, X. A., and Goodman, R. H. (2010). MicroRNA pathways in neural development and plasticity. *Curr. Opin. Neurobiol.* 20, 457–465. doi: 10.1016/j.conb.2010.04.002

von Kügelgen, N., and Chekulaeva, M. (2020). Conservation of a core neurite transcriptome across neuronal types and species. *WIREs RNA* 11:e1590. doi: 10.1002/wrna.1590

Wang, B., Pan, L., Wei, M., Wang, Q., Liu, W. W., Wang, N., et al. (2015). FMRP-mediated axonal delivery of miR-181d regulates axon elongation by locally targeting Map1b and Calm1. *Cell Rep.* 13, 2794–2807. doi: 10.1016/j.celrep.2015.11.057

Wang, E. T., Cody, N. A. L., Jog, S., Biancolella, M., Wang, T. T., Treacy, D. J., et al. (2012). Transcriptome-wide regulation of pre-mRNA splicing and mRNA localization by muscleblind proteins. *Cell* 150, 710–724. doi: 10.1016/j.cell.2012.06.041

Wang, E. T., Sandberg, R., Luo, S., Khrebtukova, I., Zhang, L., Mayr, C., et al. (2008). Alternative isoform regulation in human tissue transcriptomes. *Nature* 456, 470–476. doi: 10.1038/nature07509

Wang, L., Park, H. J., Dasari, S., Wang, S., Kocher, J.-P., and Li, W. (2013). CPAT: Coding-potential assessment tool using an alignment-free logistic regression model. *Nucleic Acids Res.* 41:e74. doi: 10.1093/nar/gkt006

Wang, Y., Zhao, Y., Bollas, A., Wang, Y., and Au, K. F. (2021). Nanopore sequencing technology, bioinformatics and applications. *Nat. Biotechnol.* 39, 1348–1365. doi: 10.1038/s41587-021-01108-x

Watts, M. E., Oksanen, M., Lejerkrans, S., Mastropasqua, F., Gorospe, M., and Tammimies, K. (2023). Circular RNAs arising from synaptic host genes during human neuronal differentiation are modulated by SFPQ RNA-binding protein. *BMC Biol.* 21:127. doi: 10.1186/s12915-023-01627-w

Wei, M., Huang, J., Li, G.-W., Jiang, B., Cheng, H., Liu, X., et al. (2021). Axon-enriched lincRNA ALAE is required for axon elongation via regulation of local mRNA translation. *Cell Rep.* 35:109053. doi: 10.1016/j.celrep.2021.109053

Wenger, A. M., Peluso, P., Rowell, W. J., Chang, P.-C., Hall, R. J., Concepcion, G. T., et al. (2019). Accurate circular consensus long-read sequencing improves variant detection and assembly of a human genome. *Nat. Biotechnol.* 37, 1155–1162. doi: 10.1038/s41587-019-0217-9

Westholm, J. O., and Lai, E. C. (2011). Mirtrons: MicroRNA biogenesis via splicing. *Biochimie* 93, 1897–1904. doi: 10.1016/j.biochi.2011.06.017

Wong, H. H. W., Lin, J. Q., Ströhl, F., Roque, C. G., Cioni, J. M., Cagnetta, R., et al. (2017). RNA docking and local translation regulate site-specific axon remodeling in vivo. *Neuron* 95, 852–868. doi: 10.1016/j.neuron.2017.07.016

Wu, J., Xiao, J., Zhang, Z., Wang, X., Hu, S., and Yu, J. (2014). Ribogenomics: The science and knowledge of RNA. *Genom. Proteomics Bioinform.* 12, 57–63. doi: 10.1016/j.gpb.2014.04.002

Wu, M., Yang, L.-Z., and Chen, L.-L. (2021). Long noncoding RNA and protein abundance in lncRNPs. *RNA* 27, 1427–1440.

Xia, X., Wang, Y., Huang, Y., Zhang, H., Lu, H., and Zheng, J. C. (2019). Exosomal miRNAs in central nervous system diseases: Biomarkers, pathological mediators, protective factors and therapeutic agents. *Prog. Neurobiol.* 183:1694. doi: 10.1016/j.pneurobio.2019.101694

Yang, S., Park, J. H., and Lu, H.-C. (2023). Axonal energy metabolism, and the effects in aging and neurodegenerative diseases. *Mol. Neurodegener.* 18:49. doi: 10.1186/s13024-023-00634-3

Yeo, G., Holste, D., Kreiman, G., and Burge, C. B. (2004). Variation in alternative splicing across human tissues. *Genome Biol.* 5:R74. doi: 10.1186/gb-2004-5-10-r74

You, X., Vlatkovic, I., Babic, A., Will, T., Epstein, I., Tushev, G., et al. (2015). Neural circular RNAs are derived from synaptic genes and regulated by development and plasticity. *Nat. Neurosci.* 18, 603–610. doi: 10.1038/nn.3975

Zalfa, F., Adinolfi, S., Napoli, I., Kühn-Hölsken, E., Urlaub, H., Achsel, T., et al. (2005). Fragile X mental retardation protein (FMRP) binds specifically to the brain cytoplasmic RNAs BC1/BC200 via a novel RNA-binding motif. *J. Biol. Chem.* 280, 33403–33410. doi: 10.1074/jbc.M504286200

Zappulo, A., Van Den Bruck, D., Ciolli Mattioli, C., Franke, V., Imami, K., McShane, E., et al. (2017). RNA localization is a key determinant of neurite-enriched proteome. *Nat. Commun.* 8:583. doi: 10.1038/s41467-017-00690-6

Zearfoss, N. R., Alarcon, J. M., Trifilieff, P., Kandel, E., and Richter, J. D. (2008). A molecular circuit composed of CPEB-1 and c-Jun controls growth hormone-mediated synaptic plasticity in the mouse hippocampus. *J. Neurosci.* 28, 8502–8509. doi: 10.1523/JNEUROSCI.1756-08.2008

Zhang, Y., Chopp, M., Liu, X. S., Kassis, H., Wang, X., Li, C., et al. (2015). MicroRNAs in the axon locally mediate the effects of chondroitin sulfate proteoglycans and cGMP on axonal growth. *Dev. Neurobiol.* 75, 1402–1419. doi: 10.1002/dneu.22292

Zhou, H., Shi, Z., Kang, Y., Wang, Y., Lu, L., Pan, B., et al. (2018). Investigation of candidate long noncoding RNAs and messenger RNAs in the immediate phase of spinal cord injury based on gene expression profiles. *Gene* 661, 119–125. doi: 10.1016/j.gene.2018.03.074

Zivraj, K. H., Tung, Y. C. L., Piper, M., Gumy, L., Fawcett, J. W., Yeo, G. S. H., et al. (2010). Subcellular profiling reveals distinct and developmentally regulated repertoire of growth cone mRNAs. *J. Neurosci.* 30, 15464–15478. doi: 10.1523/JNEUROSCI.1800-10.2010

Zong, T., Yang, Y., Zhao, H., Li, L., Liu, M., Fu, X., et al. (2021). tsRNAs: Novel small molecules from cell function and regulatory mechanism to therapeutic targets. *Cell Prolif.* 54:e12977. doi: 10.1111/cpr.12977

frontiers | Frontiers in Molecular Neuroscience

TYPE Review
PUBLISHED 23 December 2024
DOI 10.3389/fnmol.2024.1483901

OPEN ACCESS

EDITED BY
Oriane Mauger,
Max Planck Institute of Psychiatry, Germany

REVIEWED BY
Jonathan Enriquez,
UMR5242 Institut de Génomique Fonctionnelle de Lyon (IGFL), France
Mathieu Quesnel-Vallières,
Université de Sherbrooke, Canada

*CORRESPONDENCE
Mohammad Nazim
✉ nazim30@g.ucla.edu

RECEIVED 20 August 2024
ACCEPTED 09 December 2024
PUBLISHED 23 December 2024

CITATION
Nazim M (2024) Post-transcriptional regulation of the transcriptional apparatus in neuronal development.
Front. Mol. Neurosci. 17:1483901.
doi: 10.3389/fnmol.2024.1483901

Post-transcriptional regulation of the transcriptional apparatus in neuronal development

Mohammad Nazim*

Department of Microbiology, Immunology, and Molecular Genetics, University of California, Los Angeles, Los Angeles, CA, United States

Post-transcriptional mechanisms, such as alternative splicing and polyadenylation, are recognized as critical regulatory processes that increase transcriptomic and proteomic diversity. The advent of next-generation sequencing and whole-genome analyses has revealed that numerous transcription and epigenetic regulators, including transcription factors and histone-modifying enzymes, undergo alternative splicing, most notably in the nervous system. Given the complexity of regulatory processes in the brain, it is conceivable that many of these splice variants control different aspects of neuronal development. Mutations or dysregulation of splicing and transcription regulatory proteins are frequently linked to various neurodevelopmental disorders, highlighting the importance of understanding the role of neuron-specific alternative splicing in maintaining proper transcriptional regulation in the brain. This review consolidates current insights into the role of alternative splicing in influencing transcriptional and chromatin regulatory programs in neuronal development.

KEYWORDS

post-transcriptional regulation, alternative splicing, RNA-binding protein, neuronal development, transcription, epigenetic regulation, transcription factor, histone-modifying enzyme

Introduction

Alternative splicing (AS) plays a major role in expanding proteomic diversity by allowing a limited number of eukaryotic genes to generate multiple protein variants, and thereby significantly enhancing the functional complexity of the genome. Current knowledge indicates that roughly 95% of the pre-mRNA transcripts of human multiexon genes undergo AS (Pan et al., 2008; Wang et al., 2008). AS ensures the appropriate removal of introns and the inclusion or skipping of specific exons through the selective use of splice sites in pre-mRNA transcripts. This process often occurs in a tissue-specific or developmental-stage-specific manner, orchestrated by the binding of specific *trans*-acting splicing regulatory proteins to their cognate *cis*-regulatory elements dispersed in the alternatively spliced exons and/or their flanking introns (Black, 2003; Nazim et al., 2016, 2018; Ohno et al., 2017; Vuong C. K. et al., 2016). As in many other tissues, AS is common for genes involved in the development of the nervous system, where alternatively spliced protein isoforms determine the cell fate decisions and properties of different cell types within the neuronal lineage. Changes in the expression of specific splicing regulatory proteins during neuronal development induce alterations in splicing of a large set of exons (Boutz et al., 2007; Gueroussov et al., 2015; Li et al., 2014; Vuong J. K. et al., 2016). The resulting alternative protein isoforms regulate diverse functions of neuronal development, including transcription, chromatin remodeling, apoptosis, synaptogenesis, and axonogenesis (Lin et al., 2020; Linares et al., 2015; Nazim et al., 2024; Zhang M. et al., 2019; Zheng et al., 2012). The critical role of specific splicing decisions and splicing regulatory factors in the nervous system development and function is becoming increasingly evident. Below, we discuss the molecular mechanisms that govern post-transcriptional regulation of transcriptional and chromatin regulators during neuronal development and highlight several cellular processes where splicing regulation plays a critical role.

Neuronal alternative splicing programs

Alternative splicing is highly prevalent in complex organisms such as vertebrates, where the brain displays a considerably greater number of alternative splicing events than other tissues (Pan et al., 2008; Xu, 2002; Yeo et al., 2004). Brain-specific alternative splicing programs are highly conserved throughout vertebrate evolution, indicating the functional importance of the alternatively spliced variants (Barbosa-Morais et al., 2012; Merkin et al., 2012). Notably, multiple studies from several groups have highlighted the neocortex as a major site for alternative splicing and demonstrated its effect on cortical development (Belgard et al., 2011; McKee et al., 2005; Zhang et al., 2016, 2014).

The primary machinery for splicing, the spliceosome, determines which pre-mRNA segments will be included or excluded in the mature mRNA. The spliceosome is a dynamic macromolecular RNA-protein complex composed of five RNA subunits (U1, U2, U4, U5, and U6), associated small ribonucleoproteins (RNPs), and a large number of auxiliary factors that assist the spliceosome to recognize splice sites (Black, 2003; Matera and Wang, 2014; Nazim et al., 2018; Vuong C. K. et al., 2016; Wahl et al., 2009; Wilkinson et al., 2020). The spliceosomal assembly process begins when U1 snRNP binds to the 5′ splice site (SS), SF1 protein binds to the branch point (BP), and U2 auxiliary factor heterodimer (U2AF65 and U2AF35) binds to the polypyrimidine tract and the 3′ splice site, respectively. This initial complex formation, known as the E-complex, is ATP-independent. In the next step, SF1 is replaced by U2 snRNP at the BP in an ATP-dependent manner, forming the A-complex. Subsequent recruitment of the U4/U6.U5 tri-snRNPs leads to the formation of the B-complex. At this stage, the spliceosome undergoes extensive remodeling and conformational changes, releasing U1 and U4 snRNPs to form the catalytically active C-complex. Subsequently, the intron forms a lariat structure and is excised, followed by the ligation of the two neighboring exons to complete the splicing reaction.

Although most spliceosome components discussed above are ubiquitously expressed, many alternative splicing events are regulated in a developmental-stage-specific or tissue-specific manner. This is achieved by tissue-specific expression of specific splicing regulatory proteins that direct the spliceosome to particular splice sites. Neuron-specific splicing, for example, is controlled by various brain-specific splicing regulatory programs (Figures 1A,B and Table 1). The splicing regulation by neuronal splicing factors is often context-dependent, and multiple RNA-binding proteins can regulate splicing events synergistically or antagonistically (Figure 1C). Recent reviews have extensively discussed the mechanisms and roles of these splicing regulators in brain development (Lara-Pezzi et al., 2017; Lee et al., 2023; Porter et al., 2018; Raj and Blencowe, 2015; Vuong C. K. et al., 2016). Below, we summarize how tissue-specific splicing regulatory RNA binding proteins influence splicing programs during neuronal development and how their dysregulation leads to neurological diseases.

Splicing regulation by PTB proteins

The polypyrimidine tract-binding protein (PTBP) family of splicing regulators, including PTBP1, PTBP2, and PTBP3, share structural and RNA-binding similarities but differ in cell type expression (Keppetipola et al., 2012; Spellman et al., 2007). PTBP1, also known as PTB, is widely expressed in most cell/tissue types except in neurons, muscle cells, and specific mature cells. The paralog PTBP2 (nPTB or brPTB) is found in neurons, myoblasts, and spermatocytes, while PTBP3 (ROD1) is expressed in hematopoietic and liver cells and does not affect neuronal splicing. Each PTB protein has four RNA recognition motif (RRM) domains that bind to extended CU-rich elements (Keppetipola et al., 2012). The two PTB proteins, PTBP1 and PTBP2, significantly influence post-transcriptional regulation during neuronal development (Boutz et al., 2007; Keppetipola et al., 2012; Nazim et al., 2024; Vuong C. K. et al., 2016; Vuong J. K. et al., 2016). By binding to CU-rich elements in pre-mRNAs, these proteins mainly repress a large number of exons but also stimulate splicing of some exons or cause retention of some introns (Hamid and Makeyev, 2017; Llorian et al., 2010; Yap et al., 2012; Yeom et al., 2021). Some exons maintain their repression through the switch from PTBP1 to PTBP2, while others, more sensitive to PTBP1, shift their splicing earlier when its expression level changes (Boutz et al., 2007; Li et al., 2014; Licatalosi et al., 2012; Linares et al., 2015; Zheng et al., 2012). Moreover, PTBP1 can dimerize and bridge RNA segments, causing looping out of exons or intronic segments to modulate exon splicing (Ye et al., 2023). The differential sensitivity of the two PTB paralogs may be due to exons requiring PTBP1 dimerization for repression, a property not seen in PTBP2.

PTBP1 is highly expressed in neural stem cells and progenitors but is sharply reduced upon mitotic exit by the induction of microRNA miR-124 (Makeyev et al., 2007). This reduction in PTBP1 level enhances miR-124 mediated repression of the REST complex (discussed below), a transcriptional suppressor of neuronal genes (Xue et al., 2013). Exons repressed by PTBP1 early in development affect functions such as axonogenesis, cell polarity, reduced apoptotic potential, and transcriptional programs of early neurons (Lin et al., 2020; Linares et al., 2015; Zhang M. et al., 2019). PTBP1 also represses exon 10 of the PTBP2 gene (Figure 1D), whose skipping leads to nonsense-mediated mRNA decay (NMD) of the PTBP2 transcript, preventing its expression in PTBP1-expressing cells (Boutz et al., 2007; Makeyev et al., 2007; Spellman et al., 2007). Reduced expression of PTBP1 during neuronal development derepresses exon 10 and allows PTBP2 expression, which is required for proper neuronal maturation. In contrast, PTBP2 exon 10 inclusion is promoted by the neural-specific SR-related protein SRRM4 in later stages of neuronal development, when PTBP1 expression is downregulated (Calarco et al., 2009). Additionally, PTBP1 represses the inclusion of many neural exons that are positively regulated by SRRM4, showing opposing regulation by these two RBPs during neuronal development (Raj et al., 2014).

Knockout of *Ptbp1* in mouse germline results in early embryonic lethality, implicating that many PTBP1 splicing targets are involved in maintaining pluripotency and inhibiting

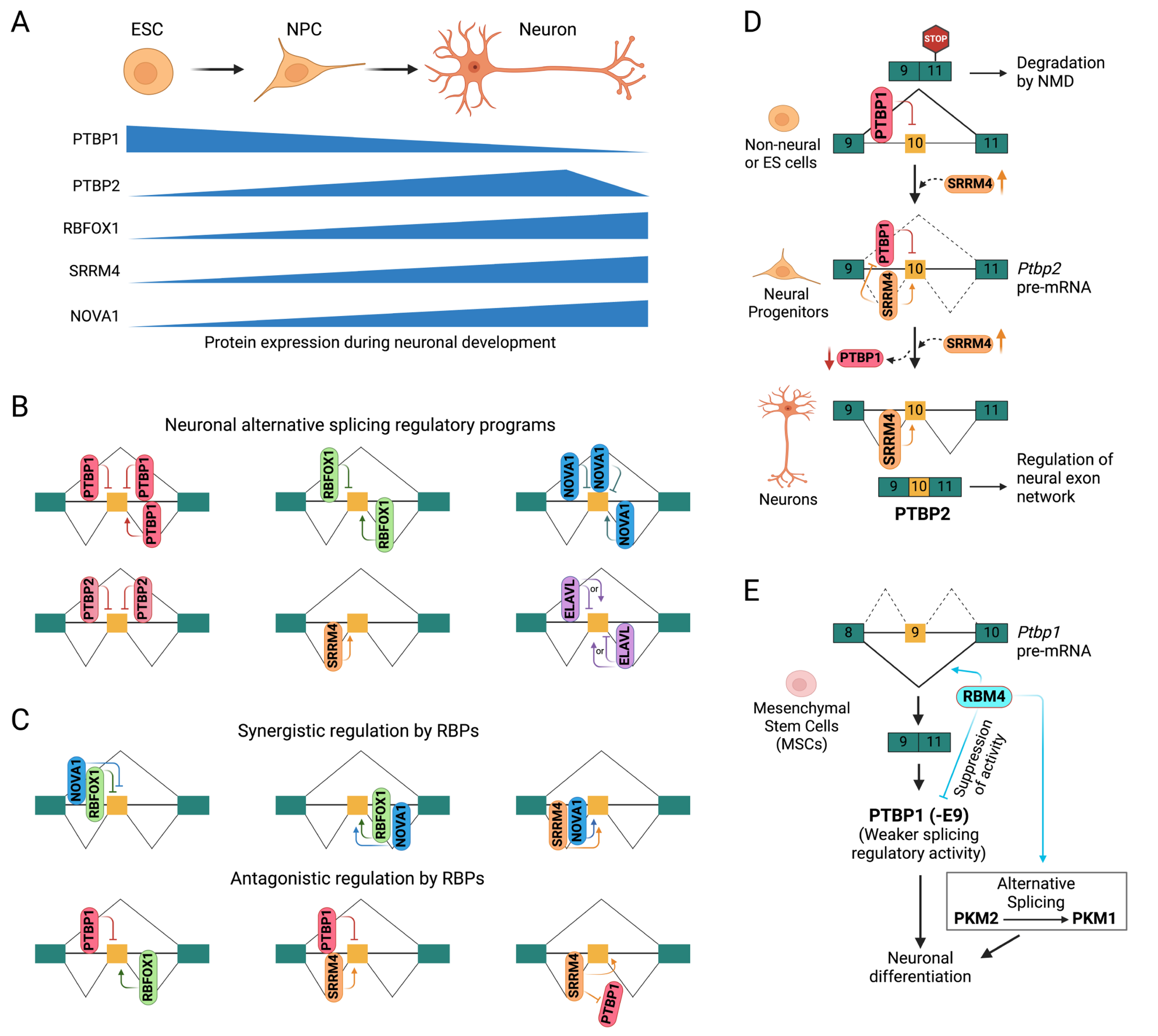


FIGURE 1
Neuronal splicing regulatory programs. **(A)** Schematic showing developmental stage-specific expression of splicing regulatory RNA binding proteins in embryonic stem cells (ESCs), neural progenitor cells (NPCs), and mature neurons. **(B)** Position/context-dependent alternative splicing regulation by neuronal RNA-binding proteins. Constitutive and alternatively spliced exons are shown as green and orange boxes, respectively. **(C)** Coordinated regulation (synergistic and antagonistic) of alternative splicing by multiple RNA binding proteins. **(D)** Functional antagonism between PTBP1 and SRRM4 in regulating the alternative splicing of PTBP2 exon 10 during neuronal development. **(E)** Functional antagonism between PTBP1 and RBM4 in alternative splicing regulation during neuronal differentiation.

differentiation (Shibayama et al., 2009; Suckale et al., 2011). Pan-neuronal loss of Ptbp1 initially shows normal brain morphology but later shows progressive loss of ependymal cells in the lateral ventricles, leading to severe hydrocephaly and death by ~10 weeks of age (Shibasaki et al., 2013). One possibility is that the loss of PTBP1 may cause premature differentiation of radial glial cells into neurons, depleting the pool of radial glial cells necessary for generating ependymal cells (Spassky et al., 2005). In contrast, mice carrying germline null alleles or pan-neuronal conditional alleles of *Ptbp2* show perinatal lethality with respiratory failure and unresponsive to touch at birth (Li et al., 2014; Licatalosi et al., 2012). Depletion of PTBP2 in excitatory neurons of the dorsal telencephalon using an *Emx1-Cre* line showed similar brain morphology in *Emx1–Ptbp2*$^{-/-}$ brains compared to wild-type mice at birth, followed by cortical atrophy as early as P5, and extensive cell death and neuronal degeneration by P15 (Li et al., 2014). Moreover, *Ptbp2*$^{-/-}$ embryonic cortical neurons initially display similar plating efficiency and neurite outgrowth but show progressive cell death starting in the following weeks, possibly due to failed synapse formation or other maturation defects, contributing to perinatal lethality.

Interestingly, recent studies reported that depletion of PTBP1 or co-depletion of PTBP1 and PTBP2 were sufficient to induce the transdifferentiation of cells such as fibroblasts or astrocytes into fully mature neurons (Maimon et al., 2021; Qian et al., 2020; Xue et al., 2013; Zhou et al., 2020), although other groups have not replicated these findings (Chen et al., 2022; Hoang et al., 2022; Wang L. L. et al., 2021).

TABLE 1 Neuronal alternative splicing regulatory RNA binding proteins (RBPs) and their target transcription and chromatin regulators.

RBP	RNA binding domain (RBD)	Number of RBDs	Binding elements in RNA	General mechanism of splicing	Target transcription and chromatin regulators
PTB proteins	RNA recognition motif (RRM)	4	CU-rich motifs	Promotes exon skipping	DPF2, PBX1
RBFOX proteins	RNA recognition motif (RRM)	1	(U)GCAUG	Promotes exon inclusion when binds downstream of alternative exon Promotes exon skipping when binds upstream of alternative exon	–
NOVA proteins	(KH)-type RNA-binding domain	3	YCAY clusters	Promotes exon inclusion when binds downstream of alternative exon Promotes exon skipping when binds upstream of alternative exon	LSD1
SRRM4	–	–	UGC containing motifs	Promotes exon inclusion when binds upstream of alternative exon	REST, MEF2C, MEF2D, TAF1, LSD1
Hu/ELAVL	RNA recognition motif (RRM)	3	U-rich and AU-rich motifs	Exon inclusion and exon skipping	–

"Y" represents a pyrimidine (C or U).

Splicing regulation by RBFOX proteins

The highly conserved RBFOX family of RNA-binding proteins includes three paralogs: RBFOX1 (A2BP1), RBFOX2 (RBM9), and RBFOX3 (NeuN) with varying expression in different cell/tissue types (Conboy, 2017; Kuroyanagi, 2009). RBFOX1 and RBFOX2 are mainly expressed in neurons, skeletal muscle, and cardiac muscle, with RBFOX2 exhibiting a broader expression pattern across other tissues. In contrast, RBFOX3 is predominantly expressed in post-mitotic neurons. Upregulation of these splicing factors during neuronal development generally promotes the inclusion of many neuronal exons. RBFOX proteins contain a single high-affinity RRM domain that specifically recognizes and binds (U)GCAUG elements in pre-mRNA transcripts (Auweter et al., 2006; Jin et al., 2003; Lambert et al., 2014). Their splicing regulatory functions are context-dependent: binding to the downstream intron of an alternative exon typically promotes splicing, while binding to the upstream intron or within the alternative exon generally inhibits exon inclusion (Farshadyeganeh et al., 2023; Jangi et al., 2014; Lovci et al., 2013; Tang et al., 2009; Weyn-Vanhentenryck et al., 2014; Yeo et al., 2009; Zhang et al., 2008). Rbfox proteins are also part of a larger complex known as Large Assembly of Splicing Regulators (LASR) (Damianov et al., 2016; Ying et al., 2017). Within this complex, RBFOX can be indirectly recruited to RNA via interactions with other components like the hnRNP M and hnRNP H proteins, which partially explains why some of the RBFOX binding motifs identified in genome-wide assays do not contain a (U)GCAUG element (Peyda et al., 2024). This recruitment allows RBFOX to crosslink to RNA and function as a splicing regulator even in the absence of its typical (U)GCAUG binding motifs.

A large number of studies underscore the significant roles of RBFOX proteins in neuronal development and function from Drosophila to humans. In Drosophila, RBFOX-related genes were shown to regulate diverse developmental processes including germ cell differentiation and enhancing memory (Carreira-Rosario et al., 2016; Guven-Ozkan et al., 2016). Central Nervous System (CNS)-specific knockouts of *Rbfox1* or *Rbfox2* in mice exhibit distinct neurological phenotypes corresponding to their differential expression patterns in the cerebellum. *Rbfox1*$^{-/-}$ mice experience spontaneous seizures and heightened sensitivity to the neuroexcitatory agent kainic acid (Gehman et al., 2011). On the other hand, *Rbfox2*$^{-/-}$ mice have smaller cerebellums, abnormal Purkinje cell function, progressive motor difficulties, and often develop hydrocephalus early in life (Gehman et al., 2012). Exon-junction microarrays comparing the brains of *Rbfox1* and *Rbfox2* knockout mice to those of normal mice revealed significant splicing differences in alternative exons, many of which have adjacent (U)GCAUG motifs, suggesting they are direct targets of RBFOX proteins. Despite the complexity of correlating particular splicing changes to distinct phenotypes, some changes in ion channels and neurotransmitter genes in *Rbfox1* knockout mice were linked to the seizure phenotype. Notably, previous research indicated that splicing disruptions in genes such as *Gabrg2a* and *Grin1* have been associated with epilepsy in humans and altered seizure susceptibility in mice (Chapman et al., 1996; Gehman et al., 2011; Mulley et al., 2003; Zapata et al., 1997). Moreover, RBFOX1 expression is reduced in the post-mortem brains of individuals with autism, correlating with splicing irregularities in genes critical for synaptogenesis (Voineagu et al., 2011). Genome-wide mapping has shown that RBFOX1, RBFOX2, and RBFOX3 directly control the splicing of genes that are upregulated during brain development and whose dysregulation is linked to autism (Weyn-Vanhentenryck et al., 2014). Additionally, RBFOX1 regulates alternative splicing of an exon of the CaV1.2 voltage-gated calcium channel, affecting the channel's electrophysiological properties in neurons (Tang et al., 2009). These observations collectively highlight the crucial role of RBFOX proteins in regulating splicing in neuronal development and function.

Splicing regulation by NOVA proteins

The NOVA (neuro-oncologic ventral antigen) protein was first identified as an autoantigen in a neurological disease called paraneoplastic opsoclonus-myoclonus ataxia, characterized by motor and cognitive impairments (Buckanovich et al., 1993; Luque et al., 1991), and was the first RNA-binding protein described as a splicing regulator of neuron-specific exons (Jensen et al., 2000). NOVA1 and NOVA2, its two paralogs, each possess three K homology (KH)-type RNA-binding domains and bind to clusters of YCAY elements (Ule et al., 2006). The expression of NOVA proteins is upregulated during neuronal development. NOVA1 is mainly expressed in the ventral spinal cord and the hindbrain. In contrast, NOVA2 is predominantly expressed in the forebrain and dorsal spinal cord, with some overlapping expression in the midbrain and hindbrain regions (Yang et al., 1998). NOVA plays diverse roles in mRNA regulation, controlling alternative splicing and polyadenylation site selection to create brain-specific 3′ UTRs (Licatalosi et al., 2008; Ule et al., 2005). The binding of NOVA to an exonic YCAY cluster blocks U1 snRNP recruitment at the 5′ splice site (SS) and subsequently inhibits exon inclusion (Ule et al., 2006). Conversely, NOVA binding to a YCAY cluster in the downstream intron promotes spliceosome assembly and facilitates exon inclusion, whereas binding in the upstream intron generally inhibits exon splicing (Ule et al., 2006). These observations demonstrate a position-dependent regulation of splicing by NOVA (Ule et al., 2006). High-throughput sequencing data suggests that the regulatory network of NOVA encompasses a large number of alternative splicing events, including transcripts encoding synaptic proteins crucial for synaptic plasticity (Licatalosi et al., 2008; Zhang C. et al., 2010).

Genetic knockouts of *Nova1*, *Nova2*, or both have revealed their crucial roles in various aspects of brain development. *Nova1*$^{-/-}$ mice appear normal at birth but die within weeks of birth, exhibiting motor dysfunction, neuronal apoptosis, and action-induced tremors (Jensen et al., 2000). *Nova2*$^{-/-}$ mice shows mislocalization of neurons in different cortical layers and perturbed long-term potentiation of inhibitory postsynaptic current in hippocampal neurons (Yano et al., 2010). *Nova1/Nova2*-double knockout mice are paralyzed and die shortly after birth from respiratory failure (Ruggiu et al., 2009). The double knockout mice exhibit reduced acetylcholine receptor (AChR) clusters and a lack of alignment between AChR clusters and phrenic nerve terminals, which are not observed in single-knockout mice, suggesting that the NOVA proteins have redundant roles in regulating neuromuscular junction (NMJ) development and function. Altogether, these findings highlight the essential role of the NOVA proteins in the development and plasticity of the nervous system.

Splicing regulation by SRRM4/nSR100

The neural-specific SR-related protein SRRM4, also known as nSR100, is a vertebrate-specific splicing factor containing a Serine/Arginine-repeat region uniquely expressed in neurons across multiple brain regions and sensory organs (Calarco et al., 2009; Irimia et al., 2014; Quesnel-Vallières et al., 2015; Raj et al., 2014). Expression of SRRM4 increases during neuronal maturation (Irimia et al., 2014) and is essential for neurogenesis and neuronal differentiation, as demonstrated in mammalian cell cultures and zebrafish models (Calarco et al., 2009; Raj et al., 2014). SRRM4 is highly conserved among vertebrates but absent in invertebrates, suggesting that it likely emerged as an alternative strategy that evolved to support the enhanced regulatory complexities of the vertebrate nervous system (Torres-Méndez et al., 2022). SRRM4 promotes the inclusion of specific neuronal exons by recognizing UGC-containing motifs near the 3′ splice site and interacting with U2-RNP components to facilitate early spliceosome assembly (Raj et al., 2014). It regulates a network of brain-enriched alternative splicing events in genes crucial for neural functions, such as GTPase signaling, cytoskeletal organization, and synaptic membrane dynamics. Of particular interest is exon 10 of PTBP2 gene, which is repressed by its paralog PTBP1 in non-neuronal cells, causing the transcript to be targeted by NMD (Figure 1D). SRRM4 promotes the inclusion of *Ptbp2* exon 10, preventing its transcripts from undergoing NMD and promoting PTBP2 (nPTB) expression (Calarco et al., 2009). It also promotes the inclusion of a neural-specific exon in the transcription factor REST/NRSF, relieving its repressive effect and enhancing the expression of a subset of neural genes (Figure 2C) (Raj et al., 2011). Similarly, SRRM4 promotes the inclusion of neural microexons in several other transcription and chromatin regulators, including MEF2C, MEF2D, TAF1, and LSD1, which are discussed below (Figures 2E, 3B,C).

Loss of SRRM4 exhibits severe neuronal phenotypes in cultured cells, zebrafish, and mice. Depletion of SRRM4 in Neuro2a cells impairs neurite outgrowth, and affect neurosphere formation from differentiating ESCs or adult neural stem cells (Calarco et al., 2009). SRRM4 also promotes the inclusion of a microexon (exon L) in the protrudin pre-mRNA, resulting in a longer protrudin-L protein isoform that promotes neurite outgrowth (Ohnishi et al., 2017). In contrast, depletion of SRRM4 in Neuro2a cells suppresses the inclusion of exon L, resulting in the expression of a shorter protrudin-S isoform, which is less efficient in promoting neurite extension. One report showed that mutation in the *Srrm4* gene causes splicing defects and deafness in the sensory hair cells essential for hearing and balance in a Bronx Waltzer mouse model (Nakano et al., 2012). Knockdown of SRRM4 in zebrafish embryos shows severe neural degeneration and impaired axonal extension and branching (Calarco et al., 2009). In contrast, mice with SRRM4 haploinsufficiency exhibit severe neuronal phenotypes including altered neuronal excitability and synaptic transmission, and behavioral anomalies resembling autism spectrum disorder (Quesnel-Vallières et al., 2015, 2016). These observations highlight the essential functions of SRRM4 in the development of the nervous system.

Splicing regulation by Hu/ELAVL

The Hu (also known as ELAVL) family of splicing regulators was first identified as autoantigens in a paraneoplastic neurological syndrome (Szabo et al., 1991). This family consists of four highly homologous members: HuA or HuR (ELAVL1), HuB (ELAVL2), HuC (ELAVL3), and HuD (ELAVL4) (Wei and Lai, 2022). While HuA is widely expressed in non-neural tissues, HuB, HuC, and HuD are predominantly found in neurons (Okano and Darnell, 1997) and are collectively known as neural ELAVLs (nELAVLs). Initially, Hu proteins have been shown to bind to U- and AU-rich elements in the 3′ UTR of mRNAs, enhancing their cytoplasmic stability and translation (Jain et al., 1997; Wang and Tanaka Hall, 2001). Further studies uncovered Hu proteins' roles in alternative splicing of neuronal pre-mRNAs (Zhou et al., 2011; Zhu et al., 2006). Several studies have confirmed its roles in the alternative splicing and polyadenylation of genes related to neuronal function and diseases, such as *Bdnf* (Brain-derived neurotrophic factor) and *Nf1* (Neurofibromatosis type 1) (Allen et al., 2013; Zhu et al., 2008). Hu proteins interfere with U1 and U6 snRNP binding at the 5' SS of an alternative exon (exon 23a) in the *Nf1* gene, whereas it causes decreased U2AF binding at the 3′ SS, thus influencing the alternative splicing outcome of the NF1 gene (Zhu et al., 2008). Moreover, Hu-mediated alternative polyadenylation generates differential 3′-UTRs that stabilize mRNAs in dendrites, facilitating local protein synthesis and contributing to synaptic plasticity (Allen et al., 2013; Bronicki and Jasmin, 2013; Zhou et al., 2011).

Regulation of RNA metabolism by Hu/ELAVL proteins is critically linked to neuronal differentiation and plasticity, as loss of nELAVLs in the brain results in various neurological abnormalities (Akamatsu et al., 2005; DeBoer et al., 2014; Ince-Dunn et al., 2012). HuC-null mice appear normal at birth and are fertile, but most adults exhibit impaired motor coordination, likely due to HuC being the sole nELAVL protein present in Purkinje cells (Ince-Dunn et al., 2012). These mice also experience non-convulsive electrographic seizures and spontaneous cortical hypersynchrony, possibly because of disrupted glutamate levels as nELAVLs bind to the 3′ UTRs of genes involved in glutamate synthesis. The prevalence of seizure phenotypes in other neuronal splicing regulator mutants, including HuC, suggests that many membrane and synaptic proteins are regulated through splicing. In contrast, HuD-null mice display motor and sensory neuron defects, particularly hind limb clasping, and a reduced number of cortical neurons despite an average count of neural stem cells (Akamatsu et al., 2005). Genome-wide profiling of nELAVL binding in HuC/HuD double-knockout brains has revealed hundreds of splicing changes regulated by nELAVL binding to specific intronic sites. Most of these splicing targets are associated with proteins that regulate microtubule dynamics at synapses and axons, suggesting crucial roles of Hu/ELAVLs in nervous system development and function (Ince-Dunn et al., 2012).

Additional splicing regulatory proteins implicated in the nervous system

Several other RNA-binding proteins are also implicated in regulating alternative splicing in the nervous system. For instance, the RNA-binding protein RBM4 suppresses exon 9 of the *PTBP1* gene during neuronal differentiation of mesenchymal stem cells, resulting in a shorter PTBP1 isoform, PTBP1 (−E9), with significantly reduced splicing regulatory activity (Figure 1E), alleviating the repressive effect of PTBP1 on neuronal exons (Su et al., 2017). Interestingly, both RBM4 and PTBP1 prefer to bind CU-rich elements in pre-mRNA transcripts and antagonize each other's function during differentiation. This functional antagonism is implicated in the alternative splicing regulation of pyruvate kinase M (PKM), where RBM4 antagonizes PTBP1 to promote a switch from the embryonic PKM2 isoform to the adult PKM1 isoform (Su et al., 2017). Additionally, RMB4 was shown to modulate alternative splicing of *Numb* exons 3 and 9 and promote neuronal differentiation and neurite outgrowth in mouse P19 cells (Tarn et al., 2016).

The KH-domain containing KHDRBS family of RNA-binding proteins, including SAM68 (KHDRBS1), SLM1 (KHDRBS2), and SLM2 (KHDRBS3), have been shown to control the splicing of neurexins, influencing synaptic functions (Vuong C. K. et al., 2016). The muscleblind-like 2 (MBNL2) splicing regulator, a member of the MBNL family of RNA-binding proteins, has been implicated in the neurological symptoms of myotonic dystrophy (Vuong C. K. et al., 2016). Another report showed that the RNA-binding proteins hnRNP H1 and H2 regulate the use of an alternative splice site of the telomere repeat-binding factor 2 (TRF2) pre-mRNA, encoding a shorter protein isoform (TRF2-S), a factor implicated in neuronal differentiation (Grammatikakis et al., 2016). On the other hand, mutations or dysfunction of TDP43 and FUS are associated with widespread splicing misregulation, which leads to neurodegenerative disorders such as amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD) (Vuong C. K. et al., 2016). These studies highlight the diverse functional roles of different RNA-binding proteins in controlling the splicing regulatory programs in the nervous system.

Alternative splicing of transcription factors in neuronal development

Among other tissues, the brain is particularly susceptible to splicing and transcriptional dysregulation, highlighting the necessity of studying neuron-specific splicing events in transcription regulators. One recent study developed a comprehensive transcriptome database for eight different cell types from the mouse cerebral cortex (neurons, astrocytes, microglia, oligodendrocyte precursors, newly formed oligodendrocytes, myelinating oligodendrocytes, endothelial cells, and pericytes) by RNA sequencing, identifying a large number of alternative splicing events that are cell type-specific, including genes encoding transcriptional regulators (Zhang et al., 2014). Another group created a manually curated database called "EpiFactors," which includes expression data for various epigenetic regulators, their complexes, and targets (Medvedeva et al., 2015). By intersecting these two databases, Porter et al. (2018) identified 115 chromatin regulators exhibiting neuron-specific alternative splicing patterns. Additionally, comparing the EpiFactors dataset with a list of neuronally regulated microexons revealed 76 transcriptional regulators containing alternatively spliced microexons (Porter et al., 2018). The substantial number of transcriptional regulators undergoing neuron-specific alternative splicing events underscores their crucial role in the transcriptional regulation of neuronal development. Despite this, only a few studies have delved into the functional consequences of these alternative splicing switches. Below, we explore the role of alternative splicing in regulating transcription factor genes and its overall impact on neuronal development.

Alternative splicing of the chromatin modifier DPF2

The mammalian chromatin-remodeling SWI/SNF complex (also known as BRG1/BRM-associated factor (BAF) complex) subunit DPF2 is a member of the BAF45 family of paralogous genes.

The four BAF45 paralogs, including PH10 (BAF45a), DPF1 (BAF45b), DPF2 (BAF45d), and DPF3 (BAF45c), each contain two plant homeodomain (PHD) finger domains at the C-terminus, which facilitate the targeting of BAF complex to specific genomic loci bearing distinct histone marks and regulate gene transcription (Chestkov et al., 1996; Kadoch and Crabtree, 2015; Lessard et al., 2007). DPF2 is broadly expressed in different cell and tissue types and has been implicated in programmed cell death (apoptosis) in myeloid cells (Gabig et al., 1994), maintenance of pluripotency by interaction with pluripotency transcription factors in embryonic stem cells (Pardo et al., 2010; van den Berg et al., 2010), and in mesendodermal differentiation (Zhang W. et al., 2019). In a recent study, we reported that during neuronal differentiation, the DPF2 subunit switches from the canonical DPF2-Short (S) isoform to a longer DPF2-Long (L) isoform containing a new exon 7 (Figure 2A). In embryonic stem cells (ESCs), the splicing regulator PTBP1

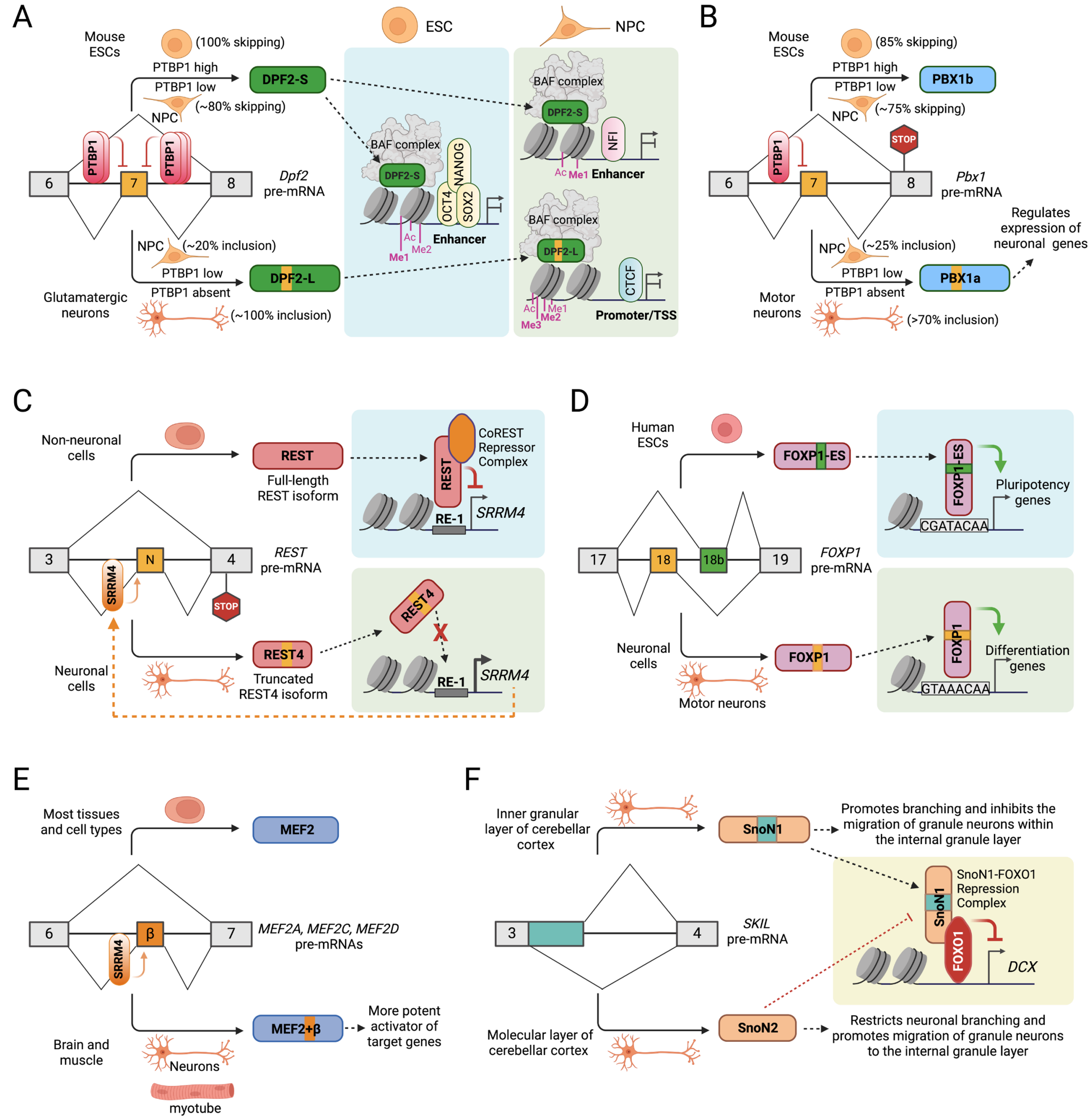


FIGURE 2
Alternative splicing of transcription factors in neuronal development. **(A)** PTBP1 regulated alternative splicing of *Dpf2* exon 7 alters the transcriptional and chromatin regulatory programs of stem cell maintenance and neuronal differentiation. **(B)** PTBP1 regulated alternative splicing of *Pbx1* exon 7 controls the expression of neuronal genes in motor neurons. **(C)** Cross-regulation between the neuronal alternative splicing activator SRRM4 and the transcription repressor REST controls the expression of neuronal genes. **(D)** Alternative splicing of mutually exclusive exons 18 and 18b in *FOXP1* gene controls the expression of pluripotency and differentiation genes in ESCs and motor neurons, respectively. **(E)** Brain- and muscle-specific inclusion of a microexon (β) in *MEF2A*, *MEF2C*, and *MEF2D* genes create a more potent activator of their target genes. **(F)** Alternative 5′ splice site selection in exon 3 of the *SKIL* gene to generate two SnoN isoforms that modulate neuronal branching and migration of granule neurons.

suppresses *Dpf2* exon 7 to produce the DPF2-S isoform. Loss of PTBP1 during neuronal differentiation allows exon 7 inclusion, leading to the expression of the DPF2-L isoform (Nazim et al., 2024).

The two DPF2 isoforms differentially affect cellular phenotypes and transcriptional regulatory programs of ESCs, neural progenitor cells (NPCs), and glutamatergic neurons (GNs) (Nazim et al., 2024). Transcriptomic profiling in genome-edited mouse ESC lines that force expression of only DPF2-S or DPF2-L revealed that DPF2-S upregulates stem cell identity and pluripotency-associated genes such as *Lefty1*, *Lefty2*, *Myc*, *Zic2*, *Zic3*, *Wt1*, *Bmp4*, *Otx2*, *Lef1*, *Nodal*, and *Tcf15*, indicating its function in pluripotency maintenance. In contrast, DPF2-L upregulates neuron-specific genes in ESC-derived glutamatergic neurons, including *Vamp1*, *Syt2*, *Sncg*, *Nefh*, *Rph3a*, *Lynx1*, *Glra3*, *Hapln4*, and *Chrm2*, suggesting that DPF2-L modulates a subset of neuronal genes. Interestingly, forced expression of DPF2-L in ESCs exhibited flat-shaped colonies instead of the characteristic dome-shaped colonies, and a subpopulation of these cells showed reduced immunofluorescence of the stem cell pluripotency marker OCT4, indicating that DPF2-S is required for proper pluripotency maintenance in ES cells. In contrast, loss of DPF2-L in developing neurons that cannot switch to this isoform promotes the proliferation of an unidentified population of non-neuronal cells that do not stain for neuronal markers Map2 and GluR1, indicating that DPF2-L is required for proper glutamatergic differentiation (Nazim et al., 2024).

The two DPF2 isoforms exhibit overlapping but distinct binding preferences in chromatin (Nazim et al., 2024). DPF2-S preferentially targets chromatin regions bound by several stem cell pluripotency factors in ESCs, such as OCT4, SOX2, NANOG, ZIC2, and ZIC3. In NPCs, DPF2-S preferentially targets chromatin sites bound by NFI and several SOX proteins, while DPF2-L preferentially targets sites bound by CTCF and BORIS (CTCFL), suggesting that alternative DPF2 isoforms differentially target regulatory regions in NPCs. Moreover, the DPF2-S and -L preferential binding sites are marked by distinct chromatin modifications (Nazim et al., 2024). DPF2-S binds to chromatin sites with enhancer-specific modifications, including H3K4me1, H3K4me2, and H3K27ac, while DPF2-L binds to sites enriched for promoter modifications, including H3K4me3, H3K9ac, H3K4me2, and H3K27ac. These findings show that the timely alternative splicing switch of the highly conserved *Dpf2* exon 7 is critical in regulating BAF function and epigenetic programs during neuronal development.

Alternative splicing of the transcription factor PBX1

The pre-B-cell leukemia homeobox transcription factor 1 (PBX1) belongs to the PBX1-4 family, which regulates diverse developmental programs, including cell proliferation and differentiation, malignant cell transformation, and apoptosis (Bourette et al., 2007; Dedera et al., 1993; Smith et al., 1997; Sykes and Kamps, 2004). PBX1 forms heterodimers with Hox homeodomain proteins to bind DNA/chromatin to promote gene transcription (Charboneau et al., 2006; LaRonde-LeBlanc and Wolberger, 2003; Piper et al., 1999). A conserved exon 7 in *Pbx1* is alternatively spliced during neuronal development (Linares et al., 2015). In early embryonic tissues, high expression of splicing regulatory protein PTBP1 represses exon 7 to generate the PBX1b isoform, where the translational reading frame is shifted to introduce a premature termination codon (PTC) in exon 8 (Figure 2B). The PTC does not result in Nonsense-mediated mRNA decay (NMD) but instead generates the shorter protein isoform, which lacks 83 amino acids at the C-terminus but retains the DNA binding homeodomain. In neural tissues, PTBP1 expression is downregulated, which allows the inclusion of exon 7 to generate the PBX1a isoform. PBX1 is thus a target of the larger PTBP1 regulatory program in neuronal development (Linares et al., 2015).

Interestingly, deletion of intronic regions to eliminate PTBP1 binding sites upstream to exon 7 upregulates PBX1a expression in ESCs. Differentiation of these mutant ESCs into motor neuron lineage induces a subset of neuronal genes involved in axonogenesis, regulation of transcription, pattern specification, cell fate commitment, cell adhesion, cell motion, and heart development as early as 2 days in culture, indicating that early expression of PBX1a activates the neuronal transcriptional program (Linares et al., 2015). Roughly a quarter of the PBX1a-induced genes also exhibited nearby PBX1 binding. Interestingly, several transcription factors with neuronal functions, including the homeobox C5 transcription factor (Hoxc5), were among the upregulated genes. The upregulation of Hoxc5 in motor neurons is potentially regulated by increased binding of PBX1 and its cofactor Meis1 at the Hoxc5 locus. These findings suggest that the alternative splicing of *Pbx1* exon 7 is critical in determining neuronal fate during differentiation.

Alternative splicing of the transcription factor REST/NRSF

The Neuron-Restrictive Silencer Factor (NRSF), commonly referred to as RE-1 Silencing Transcription factor (REST), was first identified in non-neuronal tissues where it represses neuronal genes (Chong et al., 1995). REST binds to RE-1 elements located in the promoter regions of specific neuronal genes and recruits a co-repressor complex, facilitating suppression of neuronal genes (Bruce et al., 2004; Chen et al., 1998; Schoenherr and Anderson, 1995). The splicing regulatory protein SRRM4 (nSR100) promotes the inclusion of a 16-nucleotide microexon between exons 3 and 4 in the REST gene, producing the neuron-specific "REST4" isoform (Figure 2C) (Palm et al., 1999; Raj et al., 2011). In non-neuronal cells, skipping of this microexon ensures full-length REST protein expression. However, in neuronal cells, the inclusion of this microexon changes the reading frame and generates a premature termination codon in exon 4, ultimately resulting in a truncated REST4 protein isoform lacking four zinc finger domains and a C-terminal repressor domain, which are required for DNA binding and gene repressive activities, respectively. The shorter REST4 protein may also act in a dominant-negative manner by sequestering full-length REST into nonfunctional hetero-oligomers, relieving the suppressive effect of REST on neuronal genes (Shimojo et al., 1999).

In non-neuronal cells, REST directly represses nSR100 expression, creating a regulatory loop that maintains the downregulation of neuronal genes. In neurons, expression of nSR100 is upregulated as neuronal differentiation progresses, leading to the microexon inclusion that produces the REST4 isoform with significantly reduced repressive activity and, therefore, activating the expression of REST targets in neural cells (Raj et al., 2011). Although overall REST expression is decreased in neurons, nSR100-mediated alternative splicing ensures complete loss of REST function and expression of neuronal genes. Intriguingly, the loss of nSR100 expression in the developing mouse brain disrupts neurogenesis, consistent with the crucial role of nSR100 in inhibiting REST activity (Raj et al., 2011). These findings emphasize the antagonistic molecular relationship between the transcriptional repressor REST and the neuronal splicing activator nSR100, which is crucial for maintaining the identity of neuronal and non-neuronal cells.

Alternative splicing of the transcription factor FOXP1

Forkhead Box P1 (FOXP1) is one of four members of the FOXP subfamily of transcription factors that regulate numerous genes involved in cell proliferation, differentiation, and development (Wijchers et al., 2006). The forkhead domain of FOXP proteins is known to bind a canonical consensus motif GTAAACA on its target genes as either a monomer or homo- and/or heterodimers. Previous studies have shown that knockout of *Foxp1* in mice disrupts the establishment of specific cell types and results in early embryonic lethality (Dasen et al., 2008; Wang et al., 2004; Zhang Y. et al., 2010). In human pluripotent ESCs, a highly conserved exon 18b in the *FOXP1* transcript becomes included instead of exon 18, whereas exon 18 is included in other differentiated cell lines (Figure 2D). Similarly, mouse *Foxp1* exon 16 but not exon 16b (orthologous exons 18 and 18b in humans) is included during ESC differentiation into embryoid bodies or motor neurons (Gabut et al., 2011). The inclusion of exon 18b (FOXP1-ES) in ESC maintains the reading frame but alters critical amino acid residues within the forkhead domain. Interestingly, protein-binding microarray analysis showed that FOXP1 and FOXP1-ES forkhead domains prefer distinct DNA-binding motifs. While FOXP1 predominantly recognizes and binds the canonical binding motif GTAAACAA, FOXP1-ES prefers CGATACAA or closely related sequences (Gabut et al., 2011). These findings suggest that the specific inclusion of exon 18b in human ESCs modifies the DNA-binding specificity of FOXP1.

Alternatively spliced FOXP1 isoforms regulate distinct programs of gene expression in human ESCs. In undifferentiated human ESCs, the two FOXP1 isoforms regulate distinct and overlapping sets of target genes, although FOXP1-ES regulates a larger set of genes than FOXP1. The altered DNA-binding specificity switches the transcriptional output of FOXP1-ES such that the pluripotency genes *OCT4*, *NANOG*, *GDF3*, *NR5A2*, and *TDGF1* are stimulated while genes involved in cell-lineage specification and differentiation are repressed. Moreover, induced expression of the FOXP1-ES isoform inhibits neural cell differentiation and promotes ESC self-renewal and pluripotency maintenance. In contrast, the mouse Foxp1-ES is required for efficient reprogramming of mouse embryonic fibroblasts (MEFs) into iPSCs. Thus, alternative splicing of an evolutionarily conserved exon reconfigures transcriptional regulatory programs required for ESC self-renewal, pluripotency maintenance, and neuronal differentiation.

Alternative splicing of the transcription factor MEF2

Myocyte Enhancer-binding Factor 2 (MEF2), also known as MADS box transcription enhancer factor 2, is a family of four paralogous transcription factors, including MEF2A, MEF2B, MEF2C, and MEF2D, which are involved in the development of both the muscle and nervous system. Notably, MEF2 factors have previously been shown to regulate genes associated with synapse development (Flavell et al., 2008; Flavell et al., 2006). Previous studies have also identified that the MEF2C gene exhibits alternative pre-mRNA splicing at multiple sites, resulting in various isoforms, including some that are brain-specific (Janson et al., 2001; Leifer et al., 1993). Interestingly, three MEF2 family members, MEF2A, MEF2C, and MEF2D, have a highly conserved 24-nucleotide exon encoding a short domain designated as β (Figure 2E), that is only expressed in striated muscle and neurons (Leifer et al., 1993; Zhu et al., 2005). Multiple reports showed that SRRM4 directly regulates the inclusion of this microexon in *MEF2C* and *MEF2D* by binding to UGC motifs adjacent to the polypyrimidine tract upstream of the alternative exon (Raj et al., 2014; Torres-Méndez et al., 2022). Reporter assays show that the inclusion of the β domain, which is adjacent to the MEF2 transactivating domains, creates a more potent activator of MEF2 target genes (Zhu et al., 2005). The authors showed that the observed activity is not attributable to *cis* effects on MEF2 DNA binding or dimerization, nor does it involve interactions with established transcription factors or coactivators, but instead generates an acidic activation domain selectively in muscle and neurons.

Alternative splicing of the transcription factor SKIL/SnoN

The transcription factor SKI-like proto-oncogene (SKIL), also known as SnoN, plays a vital role in axon morphogenesis in the cerebellar cortex (Ikeuchi et al., 2009; Stegmüller et al., 2006). The SnoN gene undergoes alternative splicing, where activation of the canonical 5’ SS produces the full-length SnoN1 isoform, while activation of an alternative 5’ SS within exon 3 results in a 46 amino acid deletion, generating the shorter SnoN2 isoform (Figure 2F) (Pelzer et al., 1996). Both SnoN isoforms function in neurons, but their roles are confined to specific cerebellar layers (Huynh et al., 2011). SnoN1 is predominantly found in the inner granular layer, while SnoN2 is primarily expressed in the molecular layer.

Interestingly, SnoN1 and SnoN2 exhibit opposing functional roles in coordinating neuronal branching and positioning (Huynh et al., 2011). Knockdown of SnoN1 results in the suppression of neural branching but promotes the migration of granule neurons in the cerebellar cortex, while knockdown of SnoN2 produces the opposite effect. Intriguingly, SnoN1, but not SnoN2, can form a complex with the transcription factor FOXO1 and repress the expression of doublecortin (DCX) in cerebellar granule neurons (Figure 2F), thereby controlling neuronal branching and positioning (Huynh et al., 2011). These observations highlight an isoform-specific SnoN1-FOXO1 complex that orchestrates the transcriptional regulation of neuronal branching and positioning in the brain.

Alternative splicing of chromatin-modifying enzymes in neuronal development

Chromatin-modifying enzymes are pivotal in maintaining the chromatin architecture, influencing the accessibility of the transcriptional machinery, and thereby regulating gene expression. A significant number of these enzymes (histone Readers, Writers, Erasers) undergo neuron-specific alternative splicing, producing isoforms essential for the epigenetic regulatory programs involved in neurodevelopment (Porter et al., 2018). The resulting isoforms from these splicing events play crucial roles in shaping the chromatin landscape and transcriptional regulatory programs during neuronal development. Below, we delve into how these neuron-specific alternative splicing events impact the regulation of chromatin and transcriptional processes in neuronal development.

Alternative splicing of histone methyltransferase EHMT2/G9a

The histone methyltransferase (HMTase) EHMT2, also known as G9a, belongs to a family of six members, including GLP (EHMT1), SETDB1, SETDB2, SUV39H1, and SUV39H2. These HMTases control the mono-, di-, or tri-methylation of histone H3 at lysine 9 (H3K9me1/2/3) (Fritsch et al., 2010), histone marks generally associated with transcriptional repression (Kouzarides, 2007). G9a plays a critical role in the differentiation of various cell and tissue types, including tenocyte growth and differentiation (Wada et al., 2015), skeletal muscle differentiation (Ling et al., 2012), differentiation of monocyte and T helper cells (Lehnertz et al., 2010; Wierda et al., 2015), cardiac development (Inagawa et al., 2013), and maturation of gametes (Tachibana et al., 2002). G9a has also been implicated as a critical regulator in pluripotent stem cells and the nervous system. G9a promotes specific gene silencing by local heterochromatinization through a pronounced increase in histone H3K9 methylation (H3K9me1/2), which causes irreversible epigenetic inactivation of pluripotency transcription factors Oct-3/4 and prevents reprogramming of ESCs during differentiation (Epsztejn-Litman et al., 2008; Feldman et al., 2006). In the nervous system, G9a is crucial for controlling cognition and adaptive behavior in mice, indicating that G9a-mediated histone H3K9 di-methylation is essential for regulating brain function by maintaining transcriptional homeostasis in adult neurons (Schaefer et al., 2009). In Drosophila, G9a regulates peripheral dendrite growth, classical learning, and expression of memory-related genes (Kramer et al., 2011). G9a has also been shown to affect the specification of different neuronal subtypes in the striatum (Maze et al., 2014) and the regulation of ethanol-induced neurodegeneration in neonatal mice brains (Subbanna et al., 2013).

Accumulating evidence has shed light on the role of alternative splicing of G9a in neuronal development and function. The existence of two alternatively spliced transcripts of G9a with the presence or absence of exon 10 was first described in 2001 (Brown et al., 2001). More recent reports show that G9a exon 10 is alternatively spliced in a tissue-specific and developmental-stage-specific manner (Fiszbein et al., 2016; Mauger et al., 2015). SiRNA-mediated depletion experiments suggest that Sam68 represses, but RBM39 promotes G9a exon 10 inclusion in HeLa, MCF7, and SKOV3-ip cells (Mauger et al., 2015). The inclusion of G9a exon 10 generates a longer protein isoform without altering the organization of G9A protein domains (Figure 3A). The methyltransferase activity of G9a is required for proper neuronal differentiation of N2a cells in culture, and exon 10 inclusion increases during neuronal differentiation (Fiszbein et al., 2016; Fiszbein and Kornblihtt, 2016). Exon 10 inclusion does not affect the intrinsic catalytic activity of G9a but results in increased global levels of H3K9me2. This is in part due to the higher nuclear localization of G9a containing exon 10, although the mechanism of its nuclear localization is unclear. Interestingly, G9a methylates its own intragenic histone marks, leading to a more compact chromatin structure, which subsequently promotes the inclusion of exon 10 (Figure 3A). The data imply a positive feedback loop highlighting the crucial roles of alternatively spliced isoforms of G9a in cellular commitment to differentiation.

Alternative splicing of histone acetyltransferase TAF1

The histone acetyltransferase TAF1 is a TFIID transcription initiation complex component that recruits RNA Polymerase II to transcription start sites (TSS) (Jacobson et al., 2000; Mizzen et al., 1996). A six-nucleotide microexon (34′) close to the two bromodomains of TAF1 near the C-terminus is alternatively spliced during neuronal maturation to create the neuronal TAF1 isoform, also known as N-TAF1 (Figure 3B) (Ito et al., 2016; Jambaldorj et al., 2012). A recent report showed that the *TAF1* neural microexon inclusion is directly regulated by SRRM4 through the recognition of UGC elements upstream of the regulated microexon (Capponi et al., 2020). Interestingly, depletion of N-TAF1 in neuroblastoma cells downregulates genes involved with synaptic function, vesicular transport, and dopamine metabolism, suggesting its essential roles in the nervous system (Herzfeld et al., 2013). The N-TAF1 isoform has been implicated in X-linked Dystonia-Parkinsonism (XDP), an adult-onset neurodegenerative disorder presenting features of dystonia and parkinsonism. XDP is caused by a ~ 2.6 kb SINE-VNTR-Alu (SVA)-type retrotransposon insertion into intron 32 of the *TAF1* gene (Domingo et al., 2015; Makino et al., 2007; Nolte et al., 2003). XDP patient-derived neural cells show significantly reduced expression of the N-TAF1 protein, suggesting that the SVA retrotransposon may disrupt the expression of N-TAF1 in neurons (Makino et al., 2007). Recent studies also identified that the SVA insertion into intron 32 of the *TAF1* gene generates a partially intron-retained (IR) aberrant RNA transcript that reduces exon usage in proximity to the SVA and overall TAF1 expression in patient-derived neural cells (Aneichyk et al., 2018). However, the molecular mechanisms leading to partial intron 32 retention, whether the SVA insertion has additional effects on RNA metabolism, and the ultimate fate of the mutant *TAF1* mRNA remain unclear. Moreover, multiple point mutations and duplications in the *TAF1* gene were implicated in X-linked intellectual disability in males, presenting various neurological features, although the molecular mechanism of pathogenesis remains poorly understood. Altogether, the data suggests a vital role of the alternatively spliced N-TAF1 isoform in neurons and warrants further functional studies in both *in vitro* and *in vivo* settings to address its specific function in normal physiology and genetic diseases.

Alternative splicing of histone demethylase KDM1A/LSD1

Lysine (K)-Specific Demethylase 1A (KDM1A), commonly known as LSD1, is a histone-modifying enzyme that demethylates

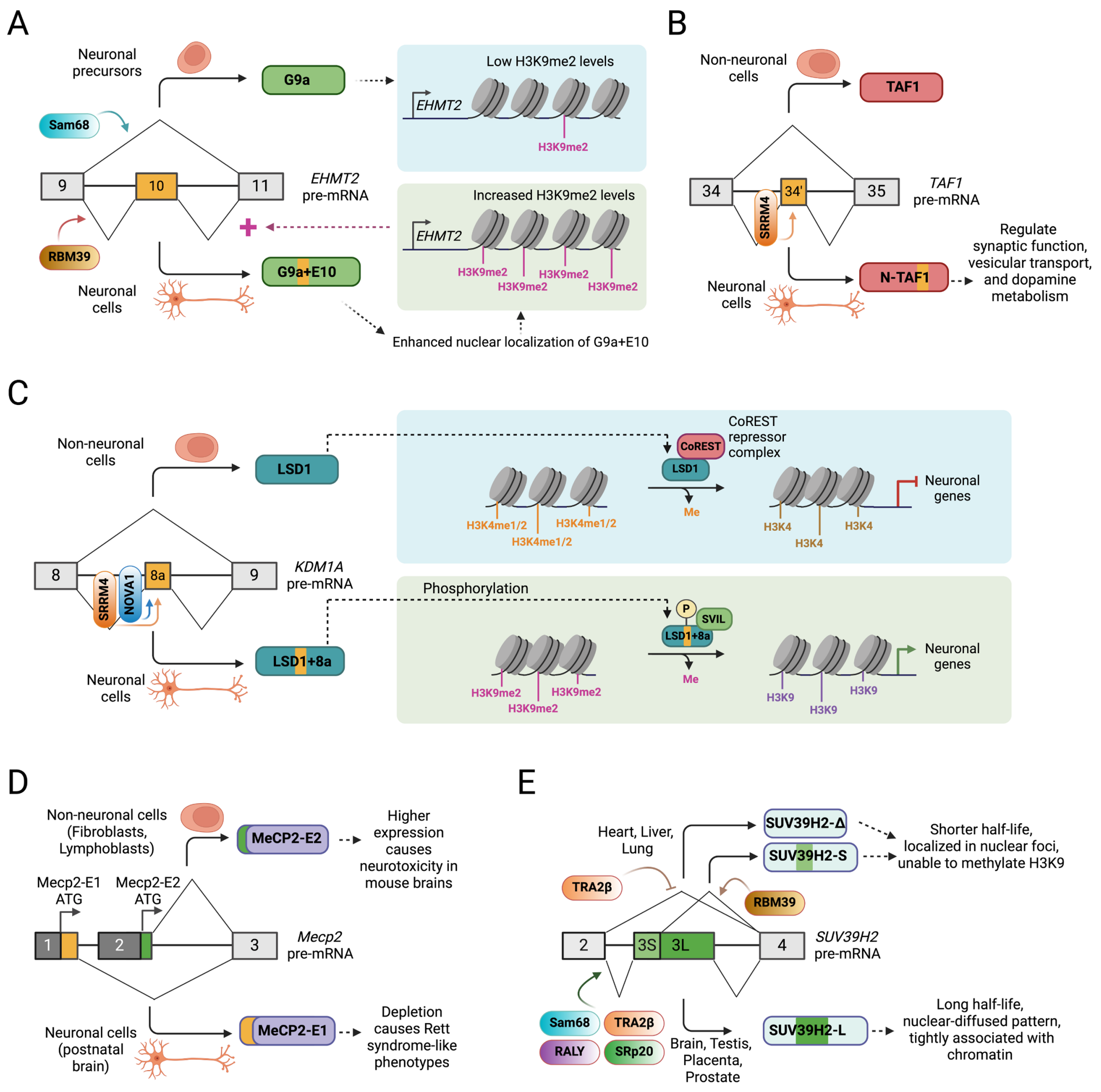


FIGURE 3
Alternative splicing of chromatin-modifying enzymes in neuronal development. **(A)** A positive feedback regulatory loop between histone methylation by histone methyltransferase G9a (EHMT2) and its alternative splicing regulation during neuronal development. **(B)** Alternative splicing of a 6 nucleotide microexon (exon 34') in the *TAF1* creates a neuronal TAF1 isoform that regulates synaptic function, vesicular transport, and dopamine metabolism. **(C)** Alternative splicing of a 12 nucleotide microexon (8a) in histone demethylase LSD1 gene (*KDM1A*) allows its detachment from the CoREST repressor complex and the expression of neuronal genes. **(D)** Selection of alternative promoter exons create alternative MeCP2 isoforms with distinct N-terminus and biological functions. **(E)** Tissue-specific alternative splicing of exon 3 in *SUV39H2* gene generates multiple protein isoforms with distinct function and cellular localization.

mono- and di-methylated lysine 4 residues on histone H3 (H3K4me1 and H3K4me2), leading to repression of target genes (Shi et al., 2004). The canonical KDM1A was found to be an essential component of the CoREST repressor complex that represses neuronal genes in non-neuronal cells (discussed above) (Ballas et al., 2001; Shi et al., 2004).

The LSD1 gene has two alternatively spliced exons, namely, E2a (60 bp long) and E8a (12 bp long), whose inclusion does not alter the reading frame of the LSD1 protein. In neurons, the 12 nucleotide E8a microexon is included to produce a neuronal isoform (Figure 3C) (Zibetti et al., 2010). The newly encoded four amino acids by E8a (with sequence Asp-Thr-Val-Lys) immediately precede the CoREST-binding domain of LSD1. Multiple lines of evidence suggest that the expression of the neuronal LSD1 isoform (LSD1 + 8a) is upregulated during neuronal maturation, which plays essential roles in synaptogenesis and neurite morphogenesis and ensures proper transcriptional response to neuronal depolarization (Laurent et al., 2015; Toffolo et al., 2014; Wang et al., 2015; Zibetti et al., 2010). It was also shown that in neuronal cells, the splicing regulatory proteins NOVA1 and SRRM4 binds to LSD1 pre-mRNA and promote the inclusion of exon 8a (Rusconi et al., 2015). Knockdown of LSD1 + 8a isoform in mouse cortical neurons inhibits, whereas its overexpression promotes neurite morphogenesis (Toffolo et al., 2014; Zibetti et al., 2010). In contrast, LSD1 exon 8a limited-knockout mice display reduced neuronal excitability and are less susceptible to seizures (Rusconi et al., 2015). One report showed that the LSD1 + 8a interacts with the nuclear factor supervillin (SVIL) and demethylates the repressive H3K9me2 mark but loses its intrinsic capability to demethylate H3K4me2 and, therefore, function as an activator of its target genes (Figure 3C) (Laurent et al., 2015). Interestingly, phosphorylation of the threonine residue at position 369 encoded by the neuronal exon causes a conformational change that leads to its detachment from the CoREST complex (Toffolo et al., 2014). Another report suggests that the LSD1 + 8a acquires a unique substrate specificity to demethylate H4K20me1/2, a histone mark associated with transcriptionally repressed chromatin regions, and regulates the expression of genes related to learning and memory formation (Wang et al., 2015). Collectively, these findings highlight the critical functional roles of the neuronal splice variant of LSD1 in the nervous system.

Alternative splicing of methyl DNA reader MeCP2

The methyl-CpG binding protein 2 (MeCP2) is highly expressed in neurons and functions as an epigenetic silencer by binding to methylated CpG sites and interacting with the corepressor SIN3A (Amir et al., 1999; Jones et al., 1998; Nan et al., 1998). Loss-of-function mutations in the *MECP2* gene typically result in a pediatric neurodevelopmental disorder called Rett syndrome, which affects young females exhibiting clinical features such as intellectual impairment, reduced language and motor skills, and hand stereotypies (Amir et al., 1999; Pohodich and Zoghbi, 2015). Maintenance of appropriate levels of MeCP2 is crucial for normal brain function.

The splicing of alternative first exons in the *MECP2* gene generates two distinct isoforms: one that encodes a 21 amino acid peptide (MeCP2-E1) and another encoding a nine amino acid peptide (MeCP2-E2) at the N-terminus of the protein (Figure 3D) (Kriaucionis and Bird, 2004; Mnatzakanian et al., 2004). The MeCP2-E1 isoform is expressed at higher levels than the MeCP2-E2 isoform in postnatal brains (Dragich et al., 2007; Zachariah et al., 2012). The alternative N-terminal peptides are positioned close to the Methyl-Cytosine Binding Domain (MBD), potentially affecting its ability to bind to methyl-CpG sites. In parallel work, two groups showed that knockout of *Mecp2* in mice results in Rett syndrome-like phenotypes (Chen et al., 2001; Guy et al., 2001). Interestingly, the deletion of MeCP2-E1 in mice recapitulated the neurological features associated with Rett syndrome (Yasui et al., 2014), but the deletion of MeCP2-E2 did not show these neurological features (Itoh et al., 2012). These data suggest that the haploinsufficiency of the MeCP2-E1 variant is specifically associated with Rett syndrome. In contrast, higher levels of the MeCP2-E2 isoform, but not the MeCP2-E1 isoform, show neurotoxicity in mouse brains (Dastidar et al., 2012). Interestingly, MeCP2-E2 can directly interact with the transcription factor FoxG1, which inhibits the MeCP2-E2 mediated neurotoxicity. These observations suggest that the two alternatively spliced MeCP2 isoforms play different functional roles in the nervous system.

Alternative splicing of histone methyltransferase SUV39H2

SUV39H2 and its paralog SUV39H1 are histone methyltransferases that catalyze the H3K9me3 mark. SUV39H2 was initially described as an early embryonic (embryonic stem cells, embryoid bodies, and early mouse embryos) and adult testis-specific protein (O'Carroll et al., 2000). However, the study of *Suv39h1* knockout and *Suv39h1*/*Suv39h2* double-knockout mice indicates that it may have functions in other tissues (Peters et al., 2001). SUV39H2 is a ubiquitously expressed protein, but in adult tissues, the expression is enriched in the cerebellum and testis (Weirich et al., 2021). SUV39H2 promotes the maintenance of trophoblast stem cells, restrains trophoblast cell differentiation, and contributes to the epigenetic landscape of placental development (Wang L. et al., 2021). Another study has shown that the knockdown of SUV39H2 inhibits stemness and cell proliferation of glioma cells and promotes their chemosensitivity (Wang et al., 2019). Recent studies have also shown that SUV39H1 and SUV39H2 control the differentiation of NPCs in the adult hippocampus (Guerra et al., 2022). Another study identified a loss-of-function variant of SUV39H2 in autism-spectrum disorder that causes altered H3K9 trimethylation and dysregulation of protocadherin β-cluster (Pcdhb cluster) genes in the developing brain (Balan et al., 2021). These observations delineate a critical role of SUV39H2 in the nervous system.

A study by Mauger et al. (2015) showed a broad expression pattern of SUV39H2 in different human tissues, including the brain. The authors showed that SUV39H2 exon 3 is alternatively spliced in a tissue-specific manner, where exon 3 can be skipped (SUV39H2-Δ), partially included (SUV39H2-S) using a cryptic 5′ splice site, or fully included (SUV39H2-L) (Figure 3E). Multiple RNA-binding proteins, including Sam68, RALY, TRA2β, SRp20, RBM9, and RBM39, modulate the alternative splicing of exon 3. Like the G9a protein, SUV39H2 protein also contains an evolutionarily conserved SET domain required for their HMTase activities. Total or partial skipping of *SUV39H2* exon 3 causes a large deletion in the SET domain (in SUV39H2-S and SUV39H2-Δ isoforms) and in the chromodomain (in SUV39H2-Δ isoform) that binds methylated H3K9. The shorter SUV39H2 isoforms (SUV39H2-S and SUV39H2-Δ) show a shorter half-life in protein stability assays, suggesting that exon 3 inclusion determines SUV39H2 protein stability. The inclusion of exon 3 also regulates SUV39H2 sub-nuclear localization, where the full-length SUV39H2-L shows a nuclear-diffused pattern, but SUV39H2-S and SUV39H2-Δ isoforms are concentrated in the nuclear foci (Mauger et al., 2015). Biochemical fractionation of HeLa cells showed that the longer SUV39H2-L isoform does not co-fractionate with the shorter isoforms. SUV39H2-L is codistributed with H3 and heterochromatin protein 1α (HP1α), suggesting it is more tightly associated with chromatin than the shorter isoforms. The differential distribution of alternative SUV39H2 isoforms in the chromatin may indicate that they are involved in different

10.3389/fnmol.2024.1483901

complexes (Mauger et al., 2015). *In vitro* methylation assay indicates that the SUV39H2-S and SUV39H2-Δ isoforms, lacking a full-length SET domain, are unable to methylate H3K9, suggesting that the skipping of exon 3 affects its H3K9 methyltransferase activity. Moreover, alternative splicing of *SUV39H2* exon 3 was also shown to regulate various target genes. Transcriptomic profiling of HeLa cells expressing exogenous SUV39H2-L and -S isoforms showed that a subset of target genes was differentially regulated by the two isoforms, suggesting that the ratio between the alternatively spliced SUV39H2 isoforms is crucial for the normal regulation of their target genes. Further ChIP assays revealed that the promoter regions of some of the target genes were occupied by SUV39H2-L, indicating that the full-length isoform acts directly on the promoters of its target genes. Altogether, the data suggests that the alternative splicing of SUV39H2 generates protein isoforms with different tissue-specific functions.

Perspectives

Alternative splicing of pre-mRNA transcripts is highly prevalent in vertebrates. The brain, in particular, exhibits the most intricate patterns of alternative splicing, producing a wide array of protein isoforms not typically found in other tissues. Recent high-throughput transcriptomic profiling has identified numerous neuronal alternatively spliced exons regulated by specialized neuron-specific splicing regulatory proteins/programs, resulting in isoforms with distinct functions. Among the many hundreds of RNA-binding proteins, only a few have been implicated in controlling neuronal splicing programs so far. It is likely that other RNA-binding proteins, yet to be analyzed in detail, also contribute to the neuronal splicing programs, adding further layers of complexity to gene regulation in the brain. While the functions of some alternatively spliced variants of transcription and chromatin regulators have been studied in greater detail, many alternative splicing events still need to be examined. As highlighted in this review, understanding the functional consequences of these events is crucial for fully grasping the various aspects of neuronal development and function, as well as comprehending the pathomechanisms of related neurodevelopmental disorders.

Emerging genetic tools and advanced next-generation sequencing technologies will aid future researchers in providing a more detailed understanding of the dynamic role of splicing programs in determining cell fate and differentiation of stem/progenitor cells into various neuronal lineages and the development of neural circuits. The study of splicing factors in knockout models is complicated due to their highly pleiotropic effects, as these modulations are often lethal or result in developmental defects that mask functions that would appear later in development. To circumvent this, prior studies have used Cre recombinase-expressing conditional knockout mouse lines. This strategy allows the depletion of specific genes in specific tissue or cell types and at specific time points, which is particularly advantageous for studying the function of regulatory proteins in different tissues and developmental stages. However, knocking out specific regulators can affect many target genes involved in common biological pathways, making it difficult to link specific phenotypes with specific splicing events or variants. One approach to circumvent this limitation is to modulate genes by techniques such as CRISPR/Cas9-mediated gene-editing so that cells can generate one particular splice variant and not the other. This methodology has been used to study the function of specific splice variants in genes such as *Dpf2* and *Mecp2* (discussed earlier), where researchers modulated target genes to allow the expression of specific isoforms of these proteins.

Recent advancements in single-cell/nuclei RNA sequencing (sc/snRNA-seq) technologies provided unparalleled advantages for examining the individual cell-level transcriptome, revealing cellular heterogeneity that bulk RNA-seq often obscures. This is especially valuable in complex tissues like the brain, where diverse cell types and states coexist. Additionally, sc/snRNA-seq can trace cell lineage and differentiation pathways, offering unique insights into the development of various cell types, which is crucial for understanding cellular and tissue development. When combined with spatial transcriptomics or time-course studies, scRNA-seq can demonstrate how gene expression varies across tissue regions or changes over time, offering a dynamic perspective on cellular processes. However, analysis of isoform-specific expression driven by alternative splicing is particularly challenging due to factors such as uneven or low capturing of the transcript coverage from single cells, variability in the number of RNA molecules in cells, number of cells sequenced, low cDNA conversion efficiency, and sequencing errors and artifacts, which often result in low coverage and high technical noise. However, recent advances in single-cell long-read sequencing enabled researchers to distinguish isolated and coordinated alternative splicing events and assign the events to the cell of origin. The utilization of genetically engineered fluorescent proteins and cell-surface markers, combined with fluorescence-activated cell sorting (FACS), has made it possible to isolate different cell types of the neuronal lineage, including neural progenitor cells and specific neuronal subtypes. Another method that can be used to capture cell-type specific splicing signatures is the utilization of Ribo-Tag/TRAP, where a tag is added to a protein of the large ribosomal subunit. This method is particularly useful for analyzing ribosome-bound/translating mRNAs in particular cells expressing the tagged ribosomal protein. Improved single-cell fluorescence *in situ* hybridization (FISH) and immunofluorescence (IF) methods also have the potential to uncover topological alterations in alternative splicing within the brain network. Additionally, spatial transcriptomic techniques, such as multiplexed error-robust fluorescence *in situ* hybridization (MERFISH), could be highly effective for characterizing the expression and spatial distribution of alternative spliced transcripts in a high-throughput manner. These cutting-edge molecular genetic tools will enable future researchers to explore gene regulation in the nervous system with unprecedented precision and depth, providing new insights into the complexities of neural gene expression and function.

Author contributions

MN: Writing – original draft, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This work was supported by NIH grant R35 GM136426, a SEED grant from the Jonsson Comprehensive Cancer Center (UCLA), and an Innovation Award from the Broad Stem Cell Research Center (UCLA) to Douglas L. Black at UCLA.

Acknowledgments

The author thanks Douglas L. Black for helpful discussions and guidance. The illustrations used in the figures in this manuscript were created using the scientific image and illustration software BioRender.

Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

Akamatsu, W., Fujihara, H., Mitsuhashi, T., Yano, M., Shibata, S., Hayakawa, Y., et al. (2005). The RNA-binding protein HuD regulates neuronal cell identity and maturation. *Proc. Natl. Acad. Sci. USA* 102, 4625–4630. doi: 10.1073/pnas.0407523102

Allen, M., Bird, C., Feng, W., Liu, G., Li, W., Perrone-Bizzozero, N. I., et al. (2013). HuD promotes BDNF expression in brain neurons via selective stabilization of the BDNF long 3'UTR mRNA. *PLoS One* 8:e55718. doi: 10.1371/journal.pone.0055718

Amir, R. E., Van den Veyver, I. B., Wan, M., Tran, C. Q., Francke, U., and Zoghbi, H. Y. (1999). Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. *Nat. Genet.* 23, 185–188. doi: 10.1038/13810

Aneichyk, T., Hendriks, W. T., Yadav, R., Shin, D., Gao, D., Vaine, C. A., et al. (2018). Dissecting the causal mechanism of X-linked dystonia-parkinsonism by integrating genome and transcriptome assembly. *Cell* 172, 897–909.e21. doi: 10.1016/j.cell.2018.02.011

Auweter, S. D., Fasan, R., Reymond, L., Underwood, J. G., Black, D. L., Pitsch, S., et al. (2006). Molecular basis of RNA recognition by the human alternative splicing factor Fox-1. *EMBO J.* 25, 163–173. doi: 10.1038/sj.emboj.7600918

Balan, S., Iwayama, Y., Ohnishi, T., Fukuda, M., Shirai, A., Yamada, A., et al. (2021). A loss-of-function variant in SUV39H2 identified in autism-spectrum disorder causes altered H3K9 trimethylation and dysregulation of protocadherin β-cluster genes in the developing brain. *Mol. Psychiatry* 26, 7550–7559. doi: 10.1038/s41380-021-01199-7

Ballas, N., Battaglioli, E., Atouf, F., Andres, M. E., Chenoweth, J., Anderson, M. E., et al. (2001). Regulation of neuronal traits by a novel transcriptional complex. *Neuron* 31, 353–365. doi: 10.1016/s0896-6273(01)00371-3

Barbosa-Morais, N. L., Irimia, M., Pan, Q., Xiong, H. Y., Gueroussov, S., Lee, L. J., et al. (2012). The evolutionary landscape of alternative splicing in vertebrate species. *Science* 338, 1587–1593. doi: 10.1126/science.1230612

Belgard, T. G., Marques, A. C., Oliver, P. L., Abaan, H. O., Sirey, T. M., Hoerder-Suabedissen, A., et al. (2011). A transcriptomic atlas of mouse neocortical layers. *Neuron* 71, 605–616. doi: 10.1016/j.neuron.2011.06.039

Black, D. L. (2003). Mechanisms of alternative pre-messenger RNA splicing. *Annu. Rev. Biochem.* 72, 291–336. doi: 10.1146/annurev.biochem.72.121801.161720

Bourette, R. P., Grasset, M.-F., and Mouchiroud, G. (2007). E2a/Pbx1 oncogene inhibits terminal differentiation but not myeloid potential of pro-T cells. *Oncogene* 26, 234–247. doi: 10.1038/sj.onc.1209777

Boutz, P. L., Stoilov, P., Li, Q., Lin, C.-H., Chawla, G., Ostrow, K., et al. (2007). A post-transcriptional regulatory switch in polypyrimidine tract-binding proteins reprograms alternative splicing in developing neurons. *Genes Dev.* 21, 1636–1652. doi: 10.1101/gad.1558107

Bronicki, L. M., and Jasmin, B. J. (2013). Emerging complexity of the HuD/ELAVl4 gene; implications for neuronal development, function, and dysfunction. *RNA* 19, 1019–1037. doi: 10.1261/rna.039164.113

Brown, S. E., Campbell, R. D., and Sanderson, C. M. (2001). Novel NG36/G9a gene products encoded within the human and mouse MHC class III regions. *Mamm. Genome* 12, 916–924. doi: 10.1007/s00335-001-3029-3

Bruce, A. W., Donaldson, I. J., Wood, I. C., Yerbury, S. A., Sadowski, M. I., Chapman, M., et al. (2004). Genome-wide analysis of repressor element 1 silencing transcription factor/neuron-restrictive silencing factor (REST/NRSF) target genes. *Proc. Natl. Acad. Sci. USA* 101, 10458–10463. doi: 10.1073/pnas.0401827101

Buckanovich, R. J., Posner, J. B., and Darnell, R. B. (1993). Nova, the paraneoplastic Ri antigen, is homologous to an RNA-binding protein and is specifically expressed in the developing motor system. *Neuron* 11, 657–672. doi: 10.1016/0896-6273(93)90077-5

Calarco, J. A., Superina, S., O'Hanlon, D., Gabut, M., Raj, B., Pan, Q., et al. (2009). Regulation of vertebrate nervous system alternative splicing and development by an SR-related protein. *Cell* 138, 898–910. doi: 10.1016/j.cell.2009.06.012

Capponi, S., Stöffler, N., Irimia, M., Van Schaik, F. M. A., Ondik, M. M., Biniossek, M. L., et al. (2020). Neuronal-specific microexon splicing of *TAF1* mRNA is directly regulated by SRRM4/nSR100. *RNA Biol.* 17, 62–74. doi: 10.1080/15476286.2019.1667214

Carreira-Rosario, A., Bhargava, V., Hillebrand, J., Kollipara, R. K., Ramaswami, M., and Buszczak, M. (2016). Repression of Pumilio protein expression by Rbfox1 promotes germ cell differentiation. *Dev. Cell* 36, 562–571. doi: 10.1016/j.devcel.2016.02.010

Chapman, A. G., Woodburn, V. L., Woodruff, G. N., and Meldrum, B. S. (1996). Anticonvulsant effect of reduced NMDA receptor expression in audiogenic DBA/2 mice. *Epilepsy Res.* 26, 25–35. doi: 10.1016/s0920-1211(96)00036-8

Charboneau, A., East, L., Mulholland, N., Rohde, M., and Boudreau, N. (2006). Pbx1 is required for Hox D3-mediated angiogenesis. *Angiogenesis* 8, 289–296. doi: 10.1007/s10456-005-9016-7

Chen, R. Z., Akbarian, S., Tudor, M., and Jaenisch, R. (2001). Deficiency of methyl-CpG binding protein-2 in CNS neurons results in a Rett-like phenotype in mice. *Nat. Genet.* 27, 327–331. doi: 10.1038/85906

Chen, Z. F., Paquette, A. J., and Anderson, D. J. (1998). NRSF/REST is required in vivo for repression of multiple neuronal target genes during embryogenesis. *Nat. Genet.* 20, 136–142. doi: 10.1038/2431

Chen, W., Zheng, Q., Huang, Q., Ma, S., and Li, M. (2022). Repressing PTBP1 fails to convert reactive astrocytes to dopaminergic neurons in a 6-hydroxydopamine mouse model of Parkinson's disease. *eLife* 11:e75636. doi: 10.7554/eLife.75636

Chestkov, A. V., Baka, I. D., Kost, M. V., Georgiev, G. P., and Buchman, V. L. (1996). The d4 gene family in the human genome. *Genomics* 36, 174–177. doi: 10.1006/geno.1996.0440

Chong, J. A., Tapia-Ramírez, J., Kim, S., Toledo-Aral, J. J., Zheng, Y., Boutros, M. C., et al. (1995). REST: a mammalian silencer protein that restricts sodium channel gene expression to neurons. *Cell* 80, 949–957. doi: 10.1016/0092-8674(95)90298-8

Conboy, J. G. (2017). Developmental regulation of RNA processing by Rbfox proteins. *WIREs RNA* 8:e1398. doi: 10.1002/wrna.1398

Damianov, A., Ying, Y., Lin, C.-H., Lee, J.-A., Tran, D., Vashisht, A. A., et al. (2016). Rbfox proteins regulate splicing as part of a large multiprotein complex LASR. *Cell* 165, 606–619. doi: 10.1016/j.cell.2016.03.040

Dasen, J. S., De Camilli, A., Wang, B., Tucker, P. W., and Jessell, T. M. (2008). Hox repertoires for motor neuron diversity and connectivity gated by a single accessory factor, FoxP1. *Cell* 134, 304–316. doi: 10.1016/j.cell.2008.06.019

Dastidar, S. G., Bardai, F. H., Ma, C., Price, V., Rawat, V., Verma, P., et al. (2012). Isoform-specific toxicity of Mecp2 in postmitotic neurons: suppression of neurotoxicity by FoxG1. *J. Neurosci.* 32, 2846–2855. doi: 10.1523/JNEUROSCI.5841-11.2012

DeBoer, E. M., Azevedo, R., Vega, T. A., Brodkin, J., Akamatsu, W., Okano, H., et al. (2014). Prenatal deletion of the RNA-binding protein HuD disrupts postnatal cortical circuit maturation and behavior. *J. Neurosci.* 34, 3674–3686. doi: 10.1523/JNEUROSCI.3703-13.2014

Dedera, D. A., Waller, E. K., LeBrun, D. P., Sen-Majumdar, A., Stevens, M. E., Barsh, G. S., et al. (1993). Chimeric homeobox gene E2A-PBX1 induces proliferation, apoptosis, and malignant lymphomas in transgenic mice. *Cell* 74, 833–843. doi: 10.1016/0092-8674(93)90463-Z

Domingo, A., Westenberger, A., Lee, L. V., Brænne, I., Liu, T., Vater, I., et al. (2015). New insights into the genetics of X-linked dystonia-parkinsonism (XDP, DYT3). *Eur. J. Hum. Genet.* 23, 1334–1340. doi: 10.1038/ejhg.2014.292

Dragich, J. M., Kim, Y.-H., Arnold, A. P., and Schanen, N. C. (2007). Differential distribution of the MeCP2 splice variants in the postnatal mouse brain. *J. Comp. Neurol.* 501, 526–542. doi: 10.1002/cne.21264

Epsztejn-Litman, S., Feldman, N., Abu-Remaileh, M., Shufaro, Y., Gerson, A., Ueda, J., et al. (2008). De novo DNA methylation promoted by G9a prevents reprogramming of embryonically silenced genes. *Nat. Struct. Mol. Biol.* 15, 1176–1183. doi: 10.1038/nsmb.1476

Farshadyeganeh, P., Nazim, M., Zhang, R., Ohkawara, B., Nakajima, K., Rahman, M. A., et al. (2023). Splicing regulation of GFPT1 muscle-specific isoform and its roles in glucose metabolisms and neuromuscular junction. *iScience* 26:107746. doi: 10.1016/j.isci.2023.107746

Feldman, N., Gerson, A., Fang, J., Li, E., Zhang, Y., Shinkai, Y., et al. (2006). G9a-mediated irreversible epigenetic inactivation of Oct-3/4 during early embryogenesis. *Nat. Cell Biol.* 8, 188–194. doi: 10.1038/ncb1353

Fiszbein, A., Giono, L. E., Quaglino, A., Berardino, B. G., Sigaut, L., von Bilderling, C., et al. (2016). Alternative splicing of G9a regulates neuronal differentiation. *Cell Rep.* 14, 2797–2808. doi: 10.1016/j.celrep.2016.02.063

Fiszbein, A., and Kornblihtt, A. R. (2016). Histone methylation, alternative splicing and neuronal differentiation. *Neurogenesis* 3:e1204844. doi: 10.1080/23262133.2016.1204844

Flavell, S. W., Cowan, C. W., Kim, T.-K., Greer, P. L., Lin, Y., Paradis, S., et al. (2006). Activity-dependent regulation of MEF2 transcription factors suppresses excitatory synapse number. *Science* 311, 1008–1012. doi: 10.1126/science.1122511

Flavell, S. W., Kim, T.-K., Gray, J. M., Harmin, D. A., Hemberg, M., Hong, E. J., et al. (2008). Genome-wide analysis of MEF2 transcriptional program reveals synaptic target genes and neuronal activity-dependent polyadenylation site selection. *Neuron* 60, 1022–1038. doi: 10.1016/j.neuron.2008.11.029

Fritsch, L., Robin, P., Mathieu, J. R. R., Souidi, M., Hinaux, H., Rougeulle, C., et al. (2010). A subset of the histone H3 lysine 9 methyltransferases Suv39h1, G9a, GLP, and SETDB1 participate in a multimeric complex. *Mol. Cell* 37, 46–56. doi: 10.1016/j.molcel.2009.12.017

Gabig, T. G., Mantel, P. L., Rosli, R., and Crean, C. D. (1994). Requiem: a novel zinc finger gene essential for apoptosis in myeloid cells. *J. Biol. Chem.* 269, 29515–29519. doi: 10.1016/S0021-9258(18)43910-5

Gabut, M., Samavarchi-Tehrani, P., Wang, X., Slobodeniuc, V., O'Hanlon, D., Sung, H.-K., et al. (2011). An alternative splicing switch regulates embryonic stem cell pluripotency and reprogramming. *Cell* 147, 132–146. doi: 10.1016/j.cell.2011.08.023

Gehman, L. T., Meera, P., Stoilov, P., Shiue, L., O'Brien, J. E., Meisler, M. H., et al. (2012). The splicing regulator Rbfox2 is required for both cerebellar development and mature motor function. *Genes Dev.* 26, 445–460. doi: 10.1101/gad.182477.111

Gehman, L. T., Stoilov, P., Maguire, J., Damianov, A., Lin, C.-H., Shiue, L., et al. (2011). The splicing regulator Rbfox1 (A2BP1) controls neuronal excitation in the mammalian brain. *Nat. Genet.* 43, 706–711. doi: 10.1038/ng.841

Grammatikakis, I., Zhang, P., Panda, A. C., Kim, J., Maudsley, S., Abdelmohsen, K., et al. (2016). Alternative splicing of neuronal differentiation factor TRF2 regulated by HNRNPH1/H2. *Cell Rep.* 15, 926–934. doi: 10.1016/j.celrep.2016.03.080

Gueroussov, S., Gonatopoulos-Pournatzis, T., Irimia, M., Raj, B., Lin, Z.-Y., Gingras, A.-C., et al. (2015). An alternative splicing event amplifies evolutionary differences between vertebrates. *Science* 349, 868–873. doi: 10.1126/science.aaa8381

Guerra, M. V., Cáceres, M. I., Herrera-Soto, A., Arredondo, S. B., Varas-Godoy, M., Van Zundert, B., et al. (2022). H3K9 methyltransferases Suv39h1 and Suv39h2 control the differentiation of neural progenitor cells in the adult Hippocampus. *Front. Cell Dev. Biol.* 9:778345. doi: 10.3389/fcell.2021.778345

Guven-Ozkan, T., Busto, G. U., Schutte, S. S., Cervantes-Sandoval, I., O'Dowd, D. K., and Davis, R. L. (2016). MiR-980 is a memory suppressor MicroRNA that regulates the autism-susceptibility gene A2bp1. *Cell Rep.* 14, 1698–1709. doi: 10.1016/j.celrep.2016.01.040

Guy, J., Hendrich, B., Holmes, M., Martin, J. E., and Bird, A. (2001). A mouse Mecp2-null mutation causes neurological symptoms that mimic Rett syndrome. *Nat. Genet.* 27, 322–326. doi: 10.1038/85899

Hamid, F. M., and Makeyev, E. V. (2017). A mechanism underlying position-specific regulation of alternative splicing. *Nucleic Acids Res.* 45, 12455–12468. doi: 10.1093/nar/gkx901

Herzfeld, T., Nolte, D., Grznarova, M., Hofmann, A., Schultze, J. L., and Müller, U. (2013). X-linked dystonia parkinsonism syndrome (XDP, lubag): disease-specific sequence change DSC3 in TAF1/DYT3 affects genes in vesicular transport and dopamine metabolism. *Hum. Mol. Genet.* 22, 941–951. doi: 10.1093/hmg/dds499

Hoang, T., Kim, D. W., Appel, H., Pannullo, N. A., Leavey, P., Ozawa, M., et al. (2022). Genetic loss of function of Ptbp1 does not induce glia-to-neuron conversion in retina. *Cell Rep.* 39:110849. doi: 10.1016/j.celrep.2022.110849

Huynh, M. A., Ikeuchi, Y., Netherton, S., de la Torre-Ubieta, L., Kanadia, R., Stegmüller, J., et al. (2011). An isoform-specific SnoN1-FOXO1 repressor complex controls neuronal morphogenesis and positioning in the mammalian brain. *Neuron* 69, 930–944. doi: 10.1016/j.neuron.2011.02.008

Ikeuchi, Y., Stegmüller, J., Netherton, S., Huynh, M. A., Masu, M., Frank, D., et al. (2009). A SnoN-Ccd1 pathway promotes axonal morphogenesis in the mammalian brain. *J. Neurosci.* 29, 4312–4321. doi: 10.1523/JNEUROSCI.0126-09.2009

Inagawa, M., Nakajima, K., Makino, T., Ogawa, S., Kojima, M., Ito, S., et al. (2013). Histone H3 lysine 9 methyltransferases, G9a and GLP are essential for cardiac morphogenesis. *Mech. Dev.* 130, 519–531. doi: 10.1016/j.mod.2013.07.002

Ince-Dunn, G., Okano, H. J., Jensen, K. B., Park, W.-Y., Zhong, R., Ule, J., et al. (2012). Neuronal Elav-like (Hu) proteins regulate RNA splicing and abundance to control glutamate levels and neuronal excitability. *Neuron* 75, 1067–1080. doi: 10.1016/j.neuron.2012.07.009

Irimia, M., Weatheritt, R. J., Ellis, J. D., Parikshak, N. N., Gonatopoulos-Pournatzis, T., Babor, M., et al. (2014). A highly conserved program of neuronal microexons is misregulated in autistic brains. *Cell* 159, 1511–1523. doi: 10.1016/j.cell.2014.11.035

Ito, N., Hendriks, W. T., Dhakal, J., Vaine, C. A., Liu, C., Shin, D., et al. (2016). Decreased N-TAF1 expression in X-linked dystonia-parkinsonism patient-specific neural stem cells. *Dis. Model. Mech.* 9, 451–462. doi: 10.1242/dmm.022590

Itoh, M., Tahimic, C. G. T., Ide, S., Otsuki, A., Sasaoka, T., Noguchi, S., et al. (2012). Methyl CpG-binding protein isoform MeCP2_e2 is dispensable for Rett syndrome phenotypes but essential for embryo viability and placenta development. *J. Biol. Chem.* 287, 13859–13867. doi: 10.1074/jbc.M111.309864

Jacobson, R. H., Ladurner, A. G., King, D. S., and Tjian, R. (2000). Structure and function of a human TAF $_{II}$ 250 double Bromodomain module. *Science* 288, 1422–1425. doi: 10.1126/science.288.5470.1422

Jain, R. G., Andrews, L. G., McGowan, K. M., Pekala, P. H., and Keene, J. D. (1997). Ectopic expression of Hel-N1, an RNA-binding protein, increases glucose transporter (GLUT1) expression in 3T3-L1 adipocytes. *Mol. Cell. Biol.* 17, 954–962. doi: 10.1128/MCB.17.2.954

Jambaldorj, J., Makino, S., Munkhbat, B., and Tamiya, G. (2012). Sustained expression of a neuron-specific isoform of the Taf1 gene in development stages and aging in mice. *Biochem. Biophys. Res. Commun.* 425, 273–277. doi: 10.1016/j.bbrc.2012.07.081

Jangi, M., Boutz, P. L., Paul, P., and Sharp, P. A. (2014). Rbfox2 controls autoregulation in RNA-binding protein networks. *Genes Dev.* 28, 637–651. doi: 10.1101/gad.235770.113

Janson, C. G., Chen, Y., Li, Y., and Leifer, D. (2001). Functional regulatory regions of human transcription factor MEF2C. *Brain Res. Mol. Brain Res.* 97, 70–82. doi: 10.1016/s0169-328x(01)00187-5

Jensen, K. B., Dredge, B. K., Stefani, G., Zhong, R., Buckanovich, R. J., Okano, H. J., et al. (2000). Nova-1 regulates neuron-specific alternative splicing and is essential for neuronal viability. *Neuron* 25, 359–371. doi: 10.1016/s0896-6273(00)80900-9

Jin, Y., Suzuki, H., Maegawa, S., Endo, H., Sugano, S., Hashimoto, K., et al. (2003). A vertebrate RNA-binding protein Fox-1 regulates tissue-specific splicing via the pentanucleotide GCAUG. *EMBO J.* 22, 905–912. doi: 10.1093/emboj/cdg089

Jones, P. L., Veenstra, G. J., Wade, P. A., Vermaak, D., Kass, S. U., Landsberger, N., et al. (1998). Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nat. Genet.* 19, 187–191. doi: 10.1038/561

Kadoch, C., and Crabtree, G. R. (2015). Mammalian SWI/SNF chromatin remodeling complexes and cancer: mechanistic insights gained from human genomics. *Sci. Adv.* 1:e1500447. doi: 10.1126/sciadv.1500447

Keppetipola, N., Sharma, S., Li, Q., and Black, D. L. (2012). Neuronal regulation of pre-mRNA splicing by polypyrimidine tract binding proteins, PTBP1 and PTBP2. *Crit. Rev. Biochem. Mol. Biol.* 47, 360–378. doi: 10.3109/10409238.2012.691456

Kouzarides, T. (2007). Chromatin modifications and their function. *Cell* 128, 693–705. doi: 10.1016/j.cell.2007.02.005

Kramer, J. M., Kochinke, K., Oortveld, M. A. W., Marks, H., Kramer, D., de Jong, E. K., et al. (2011). Epigenetic regulation of learning and memory by Drosophila EHMT/G9a. *PLoS Biol.* 9:e1000569. doi: 10.1371/journal.pbio.1000569

Kriaucionis, S., and Bird, A. (2004). The major form of MeCP2 has a novel N-terminus generated by alternative splicing. *Nucleic Acids Res.* 32, 1818–1823. doi: 10.1093/nar/gkh349

Kuroyanagi, H. (2009). Fox-1 family of RNA-binding proteins. *Cell. Mol. Life Sci.* 66, 3895–3907. doi: 10.1007/s00018-009-0120-5

Lambert, N., Robertson, A., Jangi, M., McGeary, S., Sharp, P. A., and Burge, C. B. (2014). RNA bind-n-Seq: quantitative assessment of the sequence and structural binding specificity of RNA binding proteins. *Mol. Cell* 54, 887–900. doi: 10.1016/j.molcel.2014.04.016

Lara-Pezzi, E., Desco, M., Gatto, A., and Gómez-Gaviro, M. V. (2017). Neurogenesis: regulation by alternative splicing and related posttranscriptional processes. *Neuroscientist* 23, 466–477. doi: 10.1177/1073858416678604

LaRonde-LeBlanc, N. A., and Wolberger, C. (2003). Structure of HoxA9 and Pbx1 bound to DNA: Hox hexapeptide and DNA recognition anterior to posterior. *Genes Dev.* 17, 2060–2072. doi: 10.1101/gad.1103303

Laurent, B., Ruitu, L., Murn, J., Hempel, K., Ferrao, R., Xiang, Y., et al. (2015). A specific LSD1/KDM1A isoform regulates neuronal differentiation through H3K9 demethylation. *Mol. Cell* 57, 957–970. doi: 10.1016/j.molcel.2015.01.010

Lee, S., Aubee, J. I., and Lai, E. C. (2023). Regulation of alternative splicing and polyadenylation in neurons. *Life Sci. Alliance* 6:e202302000. doi: 10.26508/lsa.202302000

Lehnertz, B., Northrop, J. P., Antignano, F., Burrows, K., Hadidi, S., Mullaly, S. C., et al. (2010). Activating and inhibitory functions for the histone lysine methyltransferase G9a in T helper cell differentiation and function. *J. Exp. Med.* 207, 915–922. doi: 10.1084/jem.20100363

Leifer, D., Krainc, D., Yu, Y. T., McDermott, J., Breitbart, R. E., Heng, J., et al. (1993). MEF2C, a MADS/MEF2-family transcription factor expressed in a laminar distribution in cerebral cortex. *Proc. Natl. Acad. Sci. USA* 90, 1546–1550. doi: 10.1073/pnas.90.4.1546

Lessard, J., Wu, J. I., Ranish, J. A., Wan, M., Winslow, M. M., Staahl, B. T., et al. (2007). An essential switch in subunit composition of a chromatin remodeling complex during neural development. *Neuron* 55, 201–215. doi: 10.1016/j.neuron.2007.06.019

Li, Q., Zheng, S., Han, A., Lin, C.-H., Stoilov, P., Fu, X.-D., et al. (2014). The splicing regulator PTBP2 controls a program of embryonic splicing required for neuronal maturation. *eLife* 3:e01201. doi: 10.7554/eLife.01201

Licatalosi, D. D., Mele, A., Fak, J. J., Ule, J., Kayikci, M., Chi, S. W., et al. (2008). HITS-CLIP yields genome-wide insights into brain alternative RNA processing. *Nature* 456, 464–469. doi: 10.1038/nature07488

Licatalosi, D. D., Yano, M., Fak, J. J., Mele, A., Grabinski, S. E., Zhang, C., et al. (2012). Ptbp2 represses adult-specific splicing to regulate the generation of neuronal precursors in the embryonic brain. *Genes Dev.* 26, 1626–1642. doi: 10.1101/gad.191338.112

Lin, L., Zhang, M., Stoilov, P., Chen, L., and Zheng, S. (2020). Developmental attenuation of neuronal apoptosis by neural-specific splicing of Bak1 microexon. *Neuron* 107, 1180–1196.e8. doi: 10.1016/j.neuron.2020.06.036

Linares, A. J., Lin, C.-H., Damianov, A., Adams, K. L., Novitch, B. G., and Black, D. L. (2015). The splicing regulator PTBP1 controls the activity of the transcription factor Pbx1 during neuronal differentiation. *eLife* 4:e09268. doi: 10.7554/eLife.09268

Ling, B. M. T., Gopinadhan, S., Kok, W. K., Shankar, S. R., Gopal, P., Bharathy, N., et al. (2012). G9a mediates Sharp-1–dependent inhibition of skeletal muscle differentiation. *MBoC* 23, 4778–4785. doi: 10.1091/mbc.e12-04-0311

Llorian, M., Schwartz, S., Clark, T. A., Hollander, D., Tan, L.-Y., Spellman, R., et al. (2010). Position-dependent alternative splicing activity revealed by global profiling of alternative splicing events regulated by PTB. *Nat. Struct. Mol. Biol.* 17, 1114–1123. doi: 10.1038/nsmb.1881

Lovci, M. T., Ghanem, D., Marr, H., Arnold, J., Gee, S., Parra, M., et al. (2013). Rbfox proteins regulate alternative mRNA splicing through evolutionarily conserved RNA bridges. *Nat. Struct. Mol. Biol.* 20, 1434–1442. doi: 10.1038/nsmb.2699

Luque, F. A., Furneaux, H. M., Ferziger, R., Rosenblum, M. K., Wray, S. H., Schold, S. C., et al. (1991). Anti-Ri: an antibody associated with paraneoplastic opsoclonus and breast cancer. *Ann. Neurol.* 29, 241–251. doi: 10.1002/ana.410290303

Maimon, R., Chillon-Marinas, C., Snethlage, C. E., Singhal, S. M., McAlonis-Downes, M., Ling, K., et al. (2021). Therapeutically viable generation of neurons with antisense oligonucleotide suppression of PTB. *Nat. Neurosci.* 24, 1089–1099. doi: 10.1038/s41593-021-00864-y

Makeyev, E. V., Zhang, J., Carrasco, M. A., and Maniatis, T. (2007). The MicroRNA miR-124 promotes neuronal differentiation by triggering brain-specific alternative pre-mRNA splicing. *Mol. Cell* 27, 435–448. doi: 10.1016/j.molcel.2007.07.015

Makino, S., Kaji, R., Ando, S., Tomizawa, M., Yasuno, K., Goto, S., et al. (2007). Reduced neuron-specific expression of the TAF1 gene is associated with X-linked dystonia-parkinsonism. *Am. J. Hum. Genet.* 80, 393–406. doi: 10.1086/512129

Matera, A. G., and Wang, Z. (2014). A day in the life of the spliceosome. *Nat. Rev. Mol. Cell Biol.* 15, 108–121. doi: 10.1038/nrm3742

Mauger, O., Klinck, R., Chabot, B., Muchardt, C., Allemand, E., and Batsché, E. (2015). Alternative splicing regulates the expression of G9A and SUV39H2 methyltransferases, and dramatically changes SUV39H2 functions. *Nucleic Acids Res.* 43, 1869–1882. doi: 10.1093/nar/gkv013

Maze, I., Chaudhury, D., Dietz, D. M., Von Schimmelmann, M., Kennedy, P. J., Lobo, M. K., et al. (2014). G9a influences neuronal subtype specification in striatum. *Nat. Neurosci.* 17, 533–539. doi: 10.1038/nn.3670

McKee, A. E., Minet, E., Stern, C., Riahi, S., Stiles, C. D., and Silver, P. A. (2005). A genome-wide in situ hybridization map of RNA-binding proteins reveals anatomically restricted expression in the developing mouse brain. *BMC Dev. Biol.* 5:14. doi: 10.1186/1471-213X-5-14

Medvedeva, Y. A., Lennartsson, A., Ehsani, R., Kulakovskiy, I. V., Vorontsov, I. E., Panahandeh, P., et al. (2015). EpiFactors: a comprehensive database of human epigenetic factors and complexes. *Database* 2015:bav067. doi: 10.1093/database/bav067

Merkin, J., Russell, C., Chen, P., and Burge, C. B. (2012). Evolutionary dynamics of gene and isoform regulation in mammalian tissues. *Science* 338, 1593–1599. doi: 10.1126/science.1228186

Mizzen, C. A., Yang, X.-J., Kokubo, T., Brownell, J. E., Bannister, A. J., Owen-Hughes, T., et al. (1996). The TAFII250 subunit of TFIID has histone acetyltransferase activity. *Cell* 87, 1261–1270. doi: 10.1016/S0092-8674(00)81821-8

Mnatzakanian, G. N., Lohi, H., Munteanu, I., Alfred, S. E., Yamada, T., MacLeod, P. J. M., et al. (2004). A previously unidentified MECP2 open reading frame defines a new protein isoform relevant to Rett syndrome. *Nat. Genet.* 36, 339–341. doi: 10.1038/ng1327

Mulley, J. C., Scheffer, I. E., Petrou, S., and Berkovic, S. F. (2003). Channelopathies as a genetic cause of epilepsy. *Curr. Opin. Neurol.* 16, 171–176. doi: 10.1097/01.wco.0000063767.15877.c7

Nakano, Y., Jahan, I., Bonde, G., Sun, X., Hildebrand, M. S., Engelhardt, J. F., et al. (2012). A mutation in the Srrm4 gene causes alternative splicing defects and deafness in the Bronx Waltzer mouse. *PLoS Genet.* 8:e1002966. doi: 10.1371/journal.pgen.1002966

Nan, X., Ng, H. H., Johnson, C. A., Laherty, C. D., Turner, B. M., Eisenman, R. N., et al. (1998). Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature* 393, 386–389. doi: 10.1038/30764

Nazim, M., Lin, C.-H., Feng, A.-C., Xiao, W., Yeom, K.-H., Li, M., et al. (2024). Alternative splicing of a chromatin modifier alters the transcriptional regulatory programs of stem cell maintenance and neuronal differentiation. *Cell Stem Cell* 31, 754–771.e6. doi: 10.1016/j.stem.2024.04.001

Nazim, M., Masuda, A., Rahman, M. A., Nasrin, F., Takeda, J., Ohe, K., et al. (2016). Competitive regulation of alternative splicing and alternative polyadenylation by hnRNP H and CstF64 determines acetylcholinesterase isoforms. *Nucleic Acids Res.* 22:gkw823. doi: 10.1093/nar/gkw823

Nazim, M., Nasrin, F., and Rahman, M. A. (2018). Coordinated regulation of alternative splicing and alternative polyadenylation. *J. Genet. Genetic Eng.* 2, 26–34. doi: 10.22259/2637-5370.0203005

Nolte, D., Niemann, S., and Müller, U. (2003). Specific sequence changes in multiple transcript system DYT3 are associated with X-linked dystonia parkinsonism. *Proc. Natl. Acad. Sci. USA* 100, 10347–10352. doi: 10.1073/pnas.1831949100

O'Carroll, D., Scherthan, H., Peters, A. H., Opravil, S., Haynes, A. R., Laible, G., et al. (2000). Isolation and characterization of Suv39h2, a second histone H3 methyltransferase gene that displays testis-specific expression. *Mol. Cell. Biol.* 20, 9423–9433. doi: 10.1128/MCB.20.24.9423-9433.2000

Ohnishi, T., Shirane, M., and Nakayama, K. I. (2017). SRRM4-dependent neuron-specific alternative splicing of protrudin transcripts regulates neurite outgrowth. *Sci. Rep.* 7:41130. doi: 10.1038/srep41130

Ohno, K., Rahman, M. A., Nazim, M., Nasrin, F., Lin, Y., Takeda, J., et al. (2017). Splicing regulation and dysregulation of cholinergic genes expressed at the neuromuscular junction. *J. Neurochem.* 142, 64–72. doi: 10.1111/jnc.13954

Okano, H. J., and Darnell, R. B. (1997). A hierarchy of Hu RNA binding proteins in developing and adult neurons. *J. Neurosci.* 17, 3024–3037. doi: 10.1523/JNEUROSCI.17-09-03024.1997

Palm, K., Metsis, M., and Timmusk, T. (1999). Neuron-specific splicing of zinc finger transcription factor REST/NRSF/XBR is frequent in neuroblastomas and conserved in human, mouse and rat. *Brain Res. Mol. Brain Res.* 72, 30–39. doi: 10.1016/s0169-328x(99)00196-5

Pan, Q., Shai, O., Lee, L. J., Frey, B. J., and Blencowe, B. J. (2008). Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. *Nat. Genet.* 40, 1413–1415. doi: 10.1038/ng.259

Pardo, M., Lang, B., Yu, L., Prosser, H., Bradley, A., Babu, M. M., et al. (2010). An expanded Oct4 interaction network: implications for stem cell biology, development, and disease. *Cell Stem Cell* 6, 382–395. doi: 10.1016/j.stem.2010.03.004

Pelzer, T., Lyons, G. E., Kim, S., and Moreadith, R. W. (1996). Cloning and characterization of the murine homolog of the sno proto-oncogene reveals a novel splice variant. *Dev. Dyn.* 205, 114–125. doi: 10.1002/(SICI)1097-0177(199602)205:2<114::AID-AJA3>3.0.CO;2-L

Peters, A. H., O'Carroll, D., Scherthan, H., Mechtler, K., Sauer, S., Schöfer, C., et al. (2001). Loss of the Suv39h histone methyltransferases impairs mammalian heterochromatin and genome stability. *Cell* 107, 323–337. doi: 10.1016/s0092-8674(01)00542-6

Peyda, P., Lin, C.-H., Onwuzurike, K., and Black, D. L. (2024). The Rbfox1/LASR complex controls alternative pre-mRNA splicing by recognition of multi-part RNA regulatory modules. *Biorxiv*. doi: 10.1101/2024.07.12.603345

Piper, D. E., Batchelor, A. H., Chang, C.-P., Cleary, M. L., and Wolberger, C. (1999). Structure of a HoxB1–Pbx1 heterodimer bound to DNA. *Cell* 96, 587–597. doi: 10.1016/S0092-8674(00)80662-5

Pohodich, A. E., and Zoghbi, H. Y. (2015). Rett syndrome: disruption of epigenetic control of postnatal neurological functions. *Hum. Mol. Genet.* 24, R10–R16. doi: 10.1093/hmg/ddv217

Porter, R. S., Jaamour, F., and Iwase, S. (2018). Neuron-specific alternative splicing of transcriptional machineries: implications for neurodevelopmental disorders. *Mol. Cell. Neurosci.* 87, 35–45. doi: 10.1016/j.mcn.2017.10.006

Qian, H., Kang, X., Hu, J., Zhang, D., Liang, Z., Meng, F., et al. (2020). Reversing a model of Parkinson's disease with in situ converted nigral neurons. *Nature* 582, 550–556. doi: 10.1038/s41586-020-2388-4

Quesnel-Vallières, M., Dargaei, Z., Irimia, M., Gonatopoulos-Pournatzis, T., Ip, J. Y., Wu, M., et al. (2016). Misregulation of an activity-dependent splicing network as a common mechanism underlying autism Spectrum disorders. *Mol. Cell* 64, 1023–1034. doi: 10.1016/j.molcel.2016.11.033

Quesnel-Vallières, M., Irimia, M., Cordes, S. P., and Blencowe, B. J. (2015). Essential roles for the splicing regulator nSR100/SRRM4 during nervous system development. *Genes Dev.* 29, 746–759. doi: 10.1101/gad.256115.114

Raj, B., and Blencowe, B. J. (2015). Alternative splicing in the mammalian nervous system: recent insights into mechanisms and functional roles. *Neuron* 87, 14–27. doi: 10.1016/j.neuron.2015.05.004

Raj, B., Irimia, M., Braunschweig, U., Sterne-Weiler, T., O'Hanlon, D., Lin, Z.-Y., et al. (2014). A global regulatory mechanism for activating an exon network required for neurogenesis. *Mol. Cell* 56, 90–103. doi: 10.1016/j.molcel.2014.08.011

Raj, B., O'Hanlon, D., Vessey, J. P., Pan, Q., Ray, D., Buckley, N. J., et al. (2011). Cross-regulation between an alternative splicing activator and a transcription repressor controls neurogenesis. *Mol. Cell* 43, 843–850. doi: 10.1016/j.molcel.2011.08.014

Ruggiu, M., Herbst, R., Kim, N., Jevsek, M., Fak, J. J., Mann, M. A., et al. (2009). Rescuing Z$^+$ agrin splicing in *Nova* null mice restores synapse formation and unmasks a physiologic defect in motor neuron firing. *Proc. Natl. Acad. Sci. USA* 106, 3513–3518. doi: 10.1073/pnas.0813112106

Rusconi, F., Paganini, L., Braida, D., Ponzoni, L., Toffolo, E., Maroli, A., et al. (2015). LSD1 Neurospecific alternative splicing controls neuronal excitability in mouse models of epilepsy. *Cereb. Cortex* 25, 2729–2740. doi: 10.1093/cercor/bhu070

Schaefer, A., Sampath, S. C., Intrator, A., Min, A., Gertler, T. S., Surmeier, D. J., et al. (2009). Control of cognition and adaptive behavior by the GLP/G9a epigenetic suppressor complex. *Neuron* 64, 678–691. doi: 10.1016/j.neuron.2009.11.019

Schoenherr, C. J., and Anderson, D. J. (1995). The neuron-restrictive silencer factor (NRSF): a coordinate repressor of multiple neuron-specific genes. *Science* 267, 1360–1363. doi: 10.1126/science.7871435

Shi, Y., Lan, F., Matson, C., Mulligan, P., Whetstine, J. R., Cole, P. A., et al. (2004). Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell* 119, 941–953. doi: 10.1016/j.cell.2004.12.012

Shibasaki, T., Tokunaga, A., Sakamoto, R., Sagara, H., Noguchi, S., Sasaoka, T., et al. (2013). PTB deficiency causes the loss of adherens junctions in the dorsal telencephalon and leads to lethal hydrocephalus. *Cereb. Cortex* 23, 1824–1835. doi: 10.1093/cercor/bhs161

Shibayama, M., Ohno, S., Osaka, T., Sakamoto, R., Tokunaga, A., Nakatake, Y., et al. (2009). Polypyrimidine tract-binding protein is essential for early mouse development and embryonic stem cell proliferation. *FEBS J.* 276, 6658–6668. doi: 10.1111/j.1742-4658.2009.07380.x

Shimojo, M., Paquette, A. J., Anderson, D. J., and Hersh, L. B. (1999). Protein kinase a regulates cholinergic gene expression in PC12 cells: REST4 silences the silencing activity of neuron-restrictive silencer factor/REST. *Mol. Cell. Biol.* 19, 6788–6795. doi: 10.1128/MCB.19.10.6788

Smith, K. S., Jacobs, Y., Chang, C.-P., and Cleary, M. L. (1997). Chimeric oncoprotein E2a-Pbx1 induces apoptosis of hematopoietic cells by a p53-independent mechanism that is suppressed by Bcl-2. *Oncogene* 14, 2917–2926. doi: 10.1038/sj.onc.1201249

Spassky, N., Merkle, F. T., Flames, N., Tramontin, A. D., García-Verdugo, J. M., and Alvarez-Buylla, A. (2005). Adult ependymal cells are postmitotic and are derived from radial glial cells during embryogenesis. *J. Neurosci.* 25, 10–18. doi: 10.1523/JNEUROSCI.1108-04.2005

Spellman, R., Llorian, M., and Smith, C. W. J. (2007). Crossregulation and functional redundancy between the splicing regulator PTB and its paralogs nPTB and ROD1. *Mol. Cell* 27, 420–434. doi: 10.1016/j.molcel.2007.06.016

Stegmüller, J., Konishi, Y., Huynh, M. A., Yuan, Z., DiBacco, S., and Bonni, A. (2006). Cell-intrinsic regulation of axonal morphogenesis by the Cdh1-APC target SnoN. *Neuron* 50, 389–400. doi: 10.1016/j.neuron.2006.03.034

Su, C.-H., Hung, K.-Y., Hung, S.-C., and Tarn, W.-Y. (2017). RBM4 regulates neuronal differentiation of mesenchymal stem cells by modulating alternative splicing of pyruvate kinase M. *Mol. Cell. Biol.* 37:e00466-16. doi: 10.1128/MCB.00466-16

Subbanna, S., Shivakumar, M., Umapathy, N. S., Saito, M., Mohan, P. S., Kumar, A., et al. (2013). G9a-mediated histone methylation regulates ethanol-induced neurodegeneration in the neonatal mouse brain. *Neurobiol. Dis.* 54, 475–485. doi: 10.1016/j.nbd.2013.01.022

Suckale, J., Wendling, O., Masjkur, J., Jäger, M., Münster, C., Anastassiadis, K., et al. (2011). PTBP1 is required for embryonic development before gastrulation. *PLoS One* 6:e16992. doi: 10.1371/journal.pone.0016992

Sykes, D. B., and Kamps, M. P. (2004). E2a/Pbx1 induces the rapid proliferation of stem cell factor-dependent murine pro-T cells that cause acute T-lymphoid or myeloid Leukemias in mice. *Mol. Cell. Biol.* 24, 1256–1269. doi: 10.1128/MCB.24.3.1256-1269.2004

Szabo, A., Dalmau, J., Manley, G., Rosenfeld, M., Wong, E., Henson, J., et al. (1991). HuD, a paraneoplastic encephalomyelitis antigen, contains RNA-binding domains and is homologous to Elav and sex-lethal. *Cell* 67, 325–333. doi: 10.1016/0092-8674(91)90184-z

Tachibana, M., Sugimoto, K., Nozaki, M., Ueda, J., Ohta, T., Ohki, M., et al. (2002). G9a histone methyltransferase plays a dominant role in euchromatic histone H3 lysine 9 methylation and is essential for early embryogenesis. *Genes Dev.* 16, 1779–1791. doi: 10.1101/gad.989402

Tang, Z. Z., Zheng, S., Nikolic, J., and Black, D. L. (2009). Developmental control of CaV1.2 L-type calcium channel splicing by fox proteins. *Mol. Cell. Biol.* 29, 4757–4765. doi: 10.1128/MCB.00608-09

Tarn, W.-Y., Kuo, H.-C., Yu, H.-I., Liu, S.-W., Tseng, C.-T., Dhananjaya, D., et al. (2016). RBM4 promotes neuronal differentiation and neurite outgrowth by modulating numb isoform expression. *Mol. Biol. Cell* 27, 1676–1683. doi: 10.1091/mbc.E15-11-0798

Toffolo, E., Rusconi, F., Paganini, L., Tortorici, M., Pilotto, S., Heise, C., et al. (2014). Phosphorylation of neuronal lysine-specific demethylase 1LSD1/KDM1A impairs transcriptional repression by regulating interaction with CoREST and histone deacetylases HDAC1/2. *J. Neurochem.* 128, 603–616. doi: 10.1111/jnc.12457

Torres-Méndez, A., Pop, S., Bonnal, S., Almudi, I., Avola, A., Roberts, R. J. V., et al. (2022). Parallel evolution of a splicing program controlling neuronal excitability in flies and mammals. *Sci. Adv.* 8:eabk0445. doi: 10.1126/sciadv.abk0445

Ule, J., Stefani, G., Mele, A., Ruggiu, M., Wang, X., Taneri, B., et al. (2006). An RNA map predicting Nova-dependent splicing regulation. *Nature* 444, 580–586. doi: 10.1038/nature05304

Ule, J., Ule, A., Spencer, J., Williams, A., Hu, J.-S., Cline, M., et al. (2005). Nova regulates brain-specific splicing to shape the synapse. *Nat. Genet.* 37, 844–852. doi: 10.1038/ng1610

van den Berg, D. L. C., Snoek, T., Mullin, N. P., Yates, A., Bezstarosti, K., Demmers, J., et al. (2010). An Oct4-centered protein interaction network in embryonic stem cells. *Cell Stem Cell* 6, 369–381. doi: 10.1016/j.stem.2010.02.014

Voineagu, I., Wang, X., Johnston, P., Lowe, J. K., Tian, Y., Horvath, S., et al. (2011). Transcriptomic analysis of autistic brain reveals convergent molecular pathology. *Nature* 474, 380–384. doi: 10.1038/nature10110

Vuong, C. K., Black, D. L., and Zheng, S. (2016). The neurogenetics of alternative splicing. *Nat. Rev. Neurosci.* 17, 265–281. doi: 10.1038/nrn.2016.27

Vuong, J. K., Lin, C.-H., Zhang, M., Chen, L., Black, D. L., and Zheng, S. (2016). PTBP1 and PTBP2 serve both specific and redundant functions in neuronal pre-mRNA splicing. *Cell Rep.* 17, 2766–2775. doi: 10.1016/j.celrep.2016.11.034

Wada, S., Ideno, H., Shimada, A., Kamiunten, T., Nakamura, Y., Nakashima, K., et al. (2015). H3K9MTase G9a is essential for the differentiation and growth of tenocytes in vitro. *Histochem. Cell Biol.* 144, 13–20. doi: 10.1007/s00418-015-1318-2

Wahl, M. C., Will, C. L., and Lührmann, R. (2009). The spliceosome: design principles of a dynamic RNP machine. *Cell* 136, 701–718. doi: 10.1016/j.cell.2009.02.009

Wang, L., Chakraborty, D., Iqbal, K., and Soares, M. J. (2021). SUV39H2 controls trophoblast stem cell fate. *Biochim. Biophys. Acta Gen. Subj.* 1865:129867. doi: 10.1016/j.bbagen.2021.129867

Wang, R., Cheng, L., Yang, X., Chen, X., Miao, Y., Qiu, Y., et al. (2019). Histone methyltransferase SUV39H2 regulates cell growth and chemosensitivity in glioma via regulation of hedgehog signaling. *Cancer Cell Int.* 19:269. doi: 10.1186/s12935-019-0982-z

Wang, E. T., Sandberg, R., Luo, S., Khrebtukova, I., Zhang, L., Mayr, C., et al. (2008). Alternative isoform regulation in human tissue transcriptomes. *Nature* 456, 470–476. doi: 10.1038/nature07509

Wang, L.-L., Serrano, C., Zhong, X., Ma, S., Zou, Y., and Zhang, C.-L. (2021). Revisiting astrocyte to neuron conversion with lineage tracing in vivo. *Cell* 184, 5465–5481. doi: 10.1016/j.cell.2021.09.005

Wang, X., and Tanaka Hall, T. M. (2001). Structural basis for recognition of AU-rich element RNA by the HuD protein. *Nat. Struct. Biol.* 8, 141–145. doi: 10.1038/84131

Wang, J., Telese, F., Tan, Y., Li, W., Jin, C., He, X., et al. (2015). LSD1n is an H4K20 demethylase regulating memory formation via transcriptional elongation control. *Nat. Neurosci.* 18, 1256–1264. doi: 10.1038/nn.4069

Wang, B., Weidenfeld, J., Lu, M. M., Maika, S., Kuziel, W. A., Morrisey, E. E., et al. (2004). *Foxp1* regulates cardiac outflow tract, endocardial cushion morphogenesis and myocyte proliferation and maturation. *Development* 131, 4477–4487. doi: 10.1242/dev.01287

Wei, L., and Lai, E. C. (2022). Regulation of the alternative neural transcriptome by ELAV/Hu RNA binding proteins. *Front. Genet.* 13:848626. doi: 10.3389/fgene.2022.848626

Weirich, S., Khella, M. S., and Jeltsch, A. (2021). Structure, activity and function of the Suv39h1 and Suv39h2 protein lysine methyltransferases. *Life* 11:703. doi: 10.3390/life11070703

Weyn-Vanhentenryck, S. M., Mele, A., Yan, Q., Sun, S., Farny, N., Zhang, Z., et al. (2014). HITS-CLIP and integrative modeling define the Rbfox splicing-regulatory network linked to brain development and autism. *Cell Rep.* 6, 1139–1152. doi: 10.1016/j.celrep.2014.02.005

Wierda, R. J., Goedhart, M., van Eggermond, M. C. J. A., Muggen, A. F., Miggelbrink, X. M., Geutskens, S. B., et al. (2015). A role for KMT1c in monocyte to dendritic cell differentiation: epigenetic regulation of monocyte differentiation. *Hum. Immunol.* 76, 431–437. doi: 10.1016/j.humimm.2015.03.017

Wijchers, P. J. E. C., Burbach, J. P. H., and Smidt, M. P. (2006). In control of biology: of mice, men and foxes. *Biochem. J.* 397, 233–246. doi: 10.1042/BJ20060387

Wilkinson, M. E., Charenton, C., and Nagai, K. (2020). RNA splicing by the spliceosome. *Annu. Rev. Biochem.* 89, 359–388. doi: 10.1146/annurev-biochem-091719-064225

Xu, Q. (2002). Genome-wide detection of tissue-specific alternative splicing in the human transcriptome. *Nucleic Acids Res.* 30, 3754–3766. doi: 10.1093/nar/gkf492

Xue, Y., Ouyang, K., Huang, J., Zhou, Y., Ouyang, H., Li, H., et al. (2013). Direct conversion of fibroblasts to neurons by reprogramming PTB-regulated MicroRNA circuits. *Cell* 152, 82–96. doi: 10.1016/j.cell.2012.11.045

Yang, Y. Y., Yin, G. L., and Darnell, R. B. (1998). The neuronal RNA-binding protein Nova-2 is implicated as the autoantigen targeted in POMA patients with dementia. *Proc. Natl. Acad. Sci. USA* 95, 13254–13259. doi: 10.1073/pnas.95.22.13254

Yano, M., Hayakawa-Yano, Y., Mele, A., and Darnell, R. B. (2010). Nova2 regulates neuronal migration through an RNA switch in disabled-1 signaling. *Neuron* 66, 848–858. doi: 10.1016/j.neuron.2010.05.007

Yap, K., Lim, Z. Q., Khandelia, P., Friedman, B., and Makeyev, E. V. (2012). Coordinated regulation of neuronal mRNA steady-state levels through developmentally controlled intron retention. *Genes Dev.* 26, 1209–1223. doi: 10.1101/gad.188037.112

Yasui, D. H., Gonzales, M. L., Aflatooni, J. O., Crary, F. K., Hu, D. J., Gavino, B. J., et al. (2014). Mice with an isoform-ablating Mecp2 exon 1 mutation recapitulate the neurologic deficits of Rett syndrome. *Hum. Mol. Genet.* 23, 2447–2458. doi: 10.1093/hmg/ddt640

Ye, R., Hu, N., Cao, C., Su, R., Xu, S., Yang, C., et al. (2023). Capture RIC-seq reveals positional rules of PTBP1-associated RNA loops in splicing regulation. *Mol. Cell* 83, 1311–1327. doi: 10.1016/j.molcel.2023.03.001

Yeo, G. W., Coufal, N. G., Liang, T. Y., Peng, G. E., Fu, X.-D., and Gage, F. H. (2009). An RNA code for the FOX2 splicing regulator revealed by mapping RNA-protein interactions in stem cells. *Nat. Struct. Mol. Biol.* 16, 130–137. doi: 10.1038/nsmb.1545

Yeo, G., Holste, D., Kreiman, G., and Burge, C. B. (2004). Variation in alternative splicing across human tissues. *Genome Biol.* 5:R74. doi: 10.1186/gb-2004-5-10-r74

Yeom, K.-H., Pan, Z., Lin, C.-H., Lim, H. Y., Xiao, W., Xing, Y., et al. (2021). Tracking pre-mRNA maturation across subcellular compartments identifies developmental gene regulation through intron retention and nuclear anchoring. *Genome Res.* 31, 1106–1119. doi: 10.1101/gr.273904.120

Ying, Y., Wang, X.-J., Vuong, C. K., Lin, C.-H., Damianov, A., and Black, D. L. (2017). Splicing activation by Rbfox requires self-aggregation through its tyrosine-rich domain. *Cell* 170, 312–323. doi: 10.1016/j.cell.2017.06.022

Zachariah, R. M., Olson, C. O., Ezeonwuka, C., and Rastegar, M. (2012). Novel MeCP2 isoform-specific antibody reveals the endogenous MeCP2E1 expression in murine brain, primary neurons and astrocytes. *PLoS One* 7:e49763. doi: 10.1371/journal.pone.0049763

Zapata, A., Capdevila, J. L., Tarrason, G., Adan, J., Martínez, J. M., Piulats, J., et al. (1997). Effects of NMDA-R1 antisense oligodeoxynucleotide administration: behavioral and radioligand binding studies. *Brain Res.* 745, 114–120. doi: 10.1016/s0006-8993(96)01134-1

Zhang, Y., Chen, K., Sloan, S. A., Bennett, M. L., Scholze, A. R., O'Keeffe, S., et al. (2014). An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. *J. Neurosci.* 34, 11929–11947. doi: 10.1523/JNEUROSCI.1860-14.2014

Zhang, X., Chen, M. H., Wu, X., Kodani, A., Fan, J., Doan, R., et al. (2016). Cell-type-specific alternative splicing governs cell fate in the developing cerebral cortex. *Cell* 166, 1147–1162. doi: 10.1016/j.cell.2016.07.025

Zhang, W., Chronis, C., Chen, X., Zhang, H., Spalinskas, R., Pardo, M., et al. (2019). The BAF and PRC2 complex subunits Dpf2 and Eed antagonistically converge on Tbx3 to control ESC differentiation. *Cell Stem Cell* 24, 138–152. doi: 10.1016/j.stem.2018.12.001

Zhang, M., Ergin, V., Lin, L., Stork, C., Chen, L., and Zheng, S. (2019). Axonogenesis is coordinated by neuron-specific alternative splicing programming and splicing regulator PTBP2. *Neuron* 101, 690–706. doi: 10.1016/j.neuron.2019.01.022

Zhang, C., Frias, M. A., Mele, A., Ruggiu, M., Eom, T., Marney, C. B., et al. (2010). Integrative modeling defines the Nova splicing-regulatory network and its combinatorial controls. *Science* 329, 439–443. doi: 10.1126/science.1191150

Zhang, Y., Li, S., Yuan, L., Tian, Y., Weidenfeld, J., Yang, J., et al. (2010). Foxp1 coordinates cardiomyocyte proliferation through both cell-autonomous and nonautonomous mechanisms. *Genes Dev.* 24, 1746–1757. doi: 10.1101/gad.1929210

Zhang, C., Zhang, Z., Castle, J., Sun, S., Johnson, J., Krainer, A. R., et al. (2008). Defining the regulatory network of the tissue-specific splicing factors Fox-1 and Fox-2. *Genes Dev.* 22, 2550–2563. doi: 10.1101/gad.1703108

Zheng, S., Gray, E. E., Chawla, G., Porse, B. T., O'Dell, T. J., and Black, D. L. (2012). PSD-95 is post-transcriptionally repressed during early neural development by PTBP1 and PTBP2. *Nat. Neurosci.* 15, 381–388. doi: 10.1038/nn.3026

Zhou, H.-L., Hinman, M. N., Barron, V. A., Geng, C., Zhou, G., Luo, G., et al. (2011). Hu proteins regulate alternative splicing by inducing localized histone hyperacetylation in an RNA-dependent manner. *Proc. Natl. Acad. Sci. USA* 108, E627–E635. doi: 10.1073/pnas.1103344108

Zhou, H., Su, J., Hu, X., Zhou, C., Li, H., Chen, Z., et al. (2020). Glia-to-neuron conversion by CRISPR-CasRx alleviates symptoms of neurological disease in mice. *Cell* 181, 590–603. doi: 10.1016/j.cell.2020.03.024

Zhu, H., Hasman, R. A., Barron, V. A., Luo, G., and Lou, H. (2006). A nuclear function of Hu proteins as neuron-specific alternative RNA processing regulators. *Mol. Biol. Cell* 17, 5105–5114. doi: 10.1091/mbc.e06-02-0099

Zhu, H., Hinman, M. N., Hasman, R. A., Mehta, P., and Lou, H. (2008). Regulation of neuron-specific alternative splicing of neurofibromatosis type 1 pre-mRNA. *Mol. Cell. Biol.* 28, 1240–1251. doi: 10.1128/MCB.01509-07

Zhu, B., Ramachandran, B., and Gulick, T. (2005). Alternative pre-mRNA splicing governs expression of a conserved acidic transactivation domain in myocyte enhancer factor 2 factors of striated muscle and brain. *J. Biol. Chem.* 280, 28749–28760. doi: 10.1074/jbc.M502491200

Zibetti, C., Adamo, A., Binda, C., Forneris, F., Toffolo, E., Verpelli, C., et al. (2010). Alternative splicing of the histone demethylase LSD1/KDM1 contributes to the modulation of neurite morphogenesis in the mammalian nervous system. *J. Neurosci.* 30, 2521–2532. doi: 10.1523/JNEUROSCI.5500-09.2010

frontiers | Frontiers in Molecular Neuroscience

TYPE Original Research
PUBLISHED 20 February 2025
DOI 10.3389/fnmol.2025.1427036

OPEN ACCESS

EDITED BY
Clémence Bernard,
University of Exeter, United Kingdom

REVIEWED BY
Dolores Irala,
Duke University, United States
Anne-Cécile Boulay,
Institut National de la Santé et de la Recherche Médicale (INSERM), France

*CORRESPONDENCE
Jimena Baleriola
✉ jimena.baleriola@achucarro.org

RECEIVED 02 May 2024
ACCEPTED 24 January 2025
PUBLISHED 20 February 2025

CITATION
de la Cruz-Gambra A and Baleriola J (2025) Astrocyte-secreted factors modulate synaptic protein synthesis as revealed by puromycin labeling of isolated synaptosomes.
Front. Mol. Neurosci. 18:1427036.
doi: 10.3389/fnmol.2025.1427036

Astrocyte-secreted factors modulate synaptic protein synthesis as revealed by puromycin labeling of isolated synaptosomes

Aida de la Cruz-Gambra[1,2] and Jimena Baleriola[1,3]*

[1]Laboratory of Local Translation in Neurons and Glia, Achucarro Basque Center for Neuroscience, Leioa, Spain, [2]Neuroscience Department, University of the Basque Country (UPV/EHU), Leioa, Spain, [3]IKERBASQUE, Basque Foundation for Science, Bilbao, Spain

The synaptic proteome can be shaped by proteins transported from the neuronal soma and/or by mRNAs that are delivered to synapses where proteins are locally synthesized. This last mechanism is known as local translation. Local translation has been extensively studied in neurons in physiological conditions and, more recently, in neurological disorders, in which local transcriptomes and translatomes become dysregulated. It is widely believed that in neurons, the main source of localized transcripts is the neuronal soma and that localized translation is primarily regulated by the neuron itself. However, we wondered whether glial cells, especially astrocytes, could contribute to the modulation of synaptic local protein synthesis. To address this question, we compared levels of proteins produced in synaptic compartments in neuronal and neuron–astrocyte co-cultures using modified Boyden chambers or astrocyte-conditioned medium. We developed a methodology to measure local protein synthesis by puromycin labeling of isolated synaptosomes devoid of somatic input. Our results show that synaptic local translation is enhanced or retained when neurons are cultured in the presence of astrocytes and in response to astrocyte-conditioned medium. Puromycin labeling coupled with proximity ligation identified Rpl26 as one of the proteins whose local synthesis is regulated by astrocyte-secreted factors. Our results thus unravel the contribution of glia to synaptic protein synthesis and point to a previously unexplored extra layer of complexity in the regulation of local translation in neurons.

KEYWORDS

local translation, proteins, synaptosomes, astrocyte-secreted factors, puromycilation assays and astrocyte-neuron communication

1 Introduction

In the central nervous system (CNS), neuronal connectivity in the brain is accomplished by synaptic connections among neurons (Lynn et al., 2024), which are simultaneously in constant interaction with non-neuronal cells known as glia. Astrocytes are the most abundant glial cells of the CNS, which support neurons with energy through the lactate shuttle, and they regulate processes such as blood flow, axon myelination, long-term memory, and neurotransmitters clearance (Farizatto and Baldwin, 2023; Sun et al., 2024). Astrocytes also contribute to the maintenance and formation of synaptic connections, and they regulate synaptic plasticity through the so-called tripartite synapse (Murai and Pasquale, 2011; Perea et al., 2009). Thus, the constant communication between neurons and astrocytes leads to a proper synaptic function. Neuron–astrocyte communication can occur via direct contact or through secreted factors (Farizatto and Baldwin, 2023; Garrett and Weiner, 2009; Murai et al., 2003; Pyka et al., 2011). The first evidence of astrocytes enhancing synapse formation through secreted factors was observed in primary cultures of purified retinal ganglion cells, where neurons treated with astrocyte-conditioned medium presented more synapses (Ullian et al., 2001). Interestingly, synapse formation, function, and maintenance are partially regulated by local protein synthesis (Leal et al., 2014; Martin, 2004; Yoon et al., 2012; Zhang and Poo, 2002). However, whether astrocytes contribute to local translation in synaptic compartments through secreted molecules is largely unexplored, and it is the focus of this report.

Local translation enables the shaping of local proteomes in neurons, which were originally thought to be maintained by proteins synthesized in the soma and then transported to subneuronal compartments. Local translation requires the delivery of mRNAs rather than proteins to distal subcellular domains (e.g., dendrites, axons, and synapses), where they are locally translated into proteins (Bernard et al., 2022; Hafner et al., 2019; Leal et al., 2014; Liu-Yesucevitz et al., 2011; Wong et al., 2024). This mechanism enables neurons to respond to their environment in an acute manner as proteins are newly produced only where and when they are needed.

Local translation in neurons has been deeply studied in the nervous system under physiological conditions and, more recently in neurodegenerative diseases, in which this mechanism becomes dysregulated (Baleriola et al., 2014; Gamarra et al., 2021). However, there is one question that remains greatly unanswered in this field: Is local protein synthesis in neurons fully regulated by the neuron itself or could non-neuronal cells contribute to this phenomenon to regulate neuronal functions? In this study, we demonstrate that neuron–astrocyte crosstalk through secreted factors regulates local protein synthesis in synapses, which could contribute to synaptic function. Additionally, in this article, we provide a new method to measure local translation by performing puromycin labeling and puromycin-based proximity ligation assays (Puro-PLA) in isolated synaptosomes by immunocytochemical approaches.

2 Materials and methods

2.1 Animals

All animal protocols followed the European directive 2010/63/EU and were approved by the University of the Basque Country (UPV/EHU) Ethics Committee. Sprague–Dawley rats were bred in local facilities, and embryonic brains (E18) were obtained from CO_2 euthanized pregnant rats for neuronal and fibroblast cultures, whereas postnatal rats P0-P2 were used for primary astrocytic culture.

2.2 Primary neuronal culture

Cultured hippocampal neurons were prepared from embryonic day 18 Sprague–Dawley rat embryos (E18). In brief, hippocampi of rat embryos were dissected in ice-cold 1X Hank's balanced salt solution (HBSS, Gibco, Thermo Fisher Scientific, Waltham MA, United States). Then, an enzymatic dissociation was performed in 1X TrypLE Express (Gibco) for 10 min at 37°C in a 5% CO_2 humidified incubator followed by a mechanical homogenization. Cells were centrifuged for 5 min at 200 g, and the resulting pellet was resuspended in plating medium containing filtered Neurobasal medium (Gibco) supplemented with 10% fetal bovine serum (Sigma-Aldrich Aldrich, Merck, Darmstadt, Germany) and 10 U/μL penicillin (Gibco), 10 μg/μL streptomycin (Gibco), and 29.2 μg/μL L-glutamine (Gibco). Hippocampal neurons were plated on poly-D-lysine-coated (Sigma-Aldrich, #P1149) 12-well plates at a density of 100,000 cells/cm^2 for synaptosomal isolation or 20,000 cells/cm^2 for puromycilation assay in neurites. Neurons were maintained at 37°C in a 5% CO_2 humidified incubator. To avoid glial growth, at 1 day *in vitro* (DIV), plating medium was replaced with growth medium containing filtered Neurobasal medium supplemented with 1X B27 (Gibco) and 10 U/μL penicillin, 10 μg/μL streptomycin, and 29.2 μg/μL L-glutamine containing 20 μM of 5-fluorodeoxyuridine (Fdu, Sigma-Aldrich) and 20 μM uridine (Sigma-Aldrich). At 3 DIV, half of the medium was replaced with fresh growth medium supplemented with 20 μM Fdu and 20 μM uridine. At 7 DIV, half of the medium was replaced with growth medium, and neurons were maintained for >21 DIV to ensure mature synapses (Hafner et al., 2019).

2.3 Primary astrocytic culture

Primary astrocytes were cultured from mixed glial culture. In brief, brain hemispheres of two postnatal Sprague–Dawley rats (P0-P2) were dissected in 1X HBSS (Gibco). The four hemispheres were placed in a tube containing 4 mL of 1X HBSS (Gibco) and enzymatically dissociated with 0.25% trypsin (Sigma-Aldrich) and 0.004% DNAse (Sigma-Aldrich) during 15 min at 37°C in a 5% CO_2 humidified incubator. Afterward, the enzymatic dissociation was stopped by adding the same amount of glial plating medium containing IMDM (Gibco), 10% fetal bovine serum Hyclone (Cytiva, Thermo Fisher Scientific), and 10% of a mixture of antibiotics and antimycotics (Gibco). Cells were centrifuged for 6 min at 300 g at room temperature. The pellet was resuspended in 1 mL of glia plating medium and mechanically dissociated using syringes of 21G and 23G needles, respectively. Cells were centrifuged again for 6 min at 300 g at room temperature, and cells were resuspended in 1 mL glia plating medium and seeded onto 75 cm^2 flasks (BioLite, Thermo Fisher Scientific) and incubated at 37°C in a 5% CO_2 humidified incubator. Medium was changed to glia medium containing glucose Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 10 U/μL penicillin, 10 μg/μL streptomycin, and 29.2 μg/μL L-glutamine after 1 DIV and every 3 days.

Astrocytes were isolated by agitating the 11 DIV mixed glial culture flasks at 180 rpm for 4 h at 37°C. The medium containing microglial cells was discarded, and the astrocytes attached to the surface of the flask were enzymatically dissociated by adding 7 mL of 1X TrypLE Express (Gibco) for 15 min at 37°C in a 5% CO_2 humidified incubator. Trypsin reaction was stopped by adding 7 mL of glia medium. Cells were centrifuged for 5 min at 300 g at room temperature. The pellet containing astrocytes was washed ones with 1 mL of growth medium and resuspended in 1 mL of growth medium. For the co-cultures, astrocytes were seeded in a ratio of 1:10 (1 astrocyte: 10 neurons) at the bottom of 1 μm pore Modified Boyden

Chambers (Corning, Sigma-Aldrich) previously coated with Poly-D-lysine-coated and co-cultured with 21 DIV hippocampal neurons for 3 days.

2.4 Primary fibroblast culture

Primary fibroblast culture was performed from E18 Sprague–Dawley rat embryos ears. Ears of 10 embryos were dissected and then cut into smaller pieces to facilitate their dissociation. The ears were placed in a tube containing 4 mL of 1X HBSS and enzymatically dissociated with 0.25% trypsin and 0.004% DNAse during 30 min at 37°C in a 5% CO_2 humidified incubator. After 30 min, 4 mL of filtered IMDM supplemented with 10% fetal bovine serum Hyclone and 10% of a mixture of antibiotics and antimycotics was added to stop the enzymatic dissociation. Cells were centrifuged for 6 min at 580 g at room temperature (Khan and Gasser, 2016; Pyka et al., 2011). The pellet was resuspended in 1 mL of the previous supplemented IMDM medium and mechanically dissociated using syringes of 21G and 23G needles, respectively. Fibroblasts were centrifuged again for 6 min at 580 g at room temperature, and cells were resuspended in 1 mL supplemented IMDM medium and seeded onto 75 cm^2 flasks and incubated at 37°C in a 5% CO_2 humidified incubator. Medium was changed to DMEM with glucose supplemented with 10% fetal bovine serum, 10 U/μL penicillin, 10 μg/μL streptomycin, and 29.2 μg/μL L-glutamine after 1 DIV and every 3 days.

At 11 DIV, fibroblasts were trypsinized with 7 mL of 1X TrypLE Express for 15 min at 37°C. Subsequently, 7 mL of supplemented DMEM with glucose medium was added to stop trypsin reaction. Fibroblasts were centrifuged for 5 min at 300 g at room temperature, and the pellet containing fibroblasts was washed once with 1 mL of growth medium and resuspended in 1 mL of growth medium. For the co-cultures, fibroblasts were plated in a ratio of 1:10 (1 fibroblast: 10 neurons) at the bottom of 1 μm pore modified Boyden chambers previously coated with Poly-D-lysine-coated and co-cultured with 21 DIV hippocampal neuron for 3 days.

2.5 Astrocyte- or fibroblast-conditioned meidum treatment

Astrocytes or fibroblasts were seeded 3 days prior to the treatment day, in 12-well plates as previously described. On the day of the treatment, the conditioned medium (CM) of astrocytes or fibroblasts was collected and immediately placed into 21 DIV hippocampal neurons. Neurons were kept with astrocytic or fibroblast CM for 3 days at 37°C in a 5% CO_2 humidified incubator. At 24 DIV, a pool of 3 wells of hippocampal neurons were used for each condition to isolate synaptosomes.

2.6 *In vitro* synaptosome isolation

Synaptosome isolation was performed using Syn-PER buffer (Thermo Scientific). In brief, neurons were washed twice with cold 1X PBS and of 200 μL/well of Syn-PER reagent supplemented with 1X EDTA-free protease and phosphatase inhibitor (#A32961, Thermo Fisher Scientific) and 0.04 U/μL ribonuclease inhibitor (Fisher BioReagents, Thermo Fisher) was added to each culture well. Neurons were gently detached from the culture substrate with a cell lifter and transferred to a tube at 4°C (a pool from 3 to 5 wells were used). Cell debris was removed by centrifugation at 1,200 g for 10 min at 4°C. The pellet containing nuclear components was discarded, and the supernatant was collected and centrifuged at 20,000 g for 30 min at 4°C. Finally, the supernatant (cytosolic fraction) was reserved for immunoblotting, and the pellet (crude synaptosomal fraction) was resuspended in 40 μL of Syn-PER buffer or PBS for immunoblotting or immunofluorescence studies, respectively.

2.7 Cryo-electron microscopy

For the vitrification of the sample, freshly glow-discharged 200-mesh grids (R 3.5/1; QUANTIFOIL) were placed inside the chamber of an EM GP2 Automatic Plunge Freezing (Leica, Wetzlar, Germany), which was maintained at 8°C and relative humidity close to saturation (90% rH). Then, 4 μL of the sample were dropped onto the grid for 30 s. After incubation, most of the liquid on the grid was removed by blotting with absorbent standard filter paper (Ø55mm, Grade 595, Hahnemühle). After the blotting step, the grid was abruptly plunged into a liquid ethane bath, automatically set to −184°C. Once the specimen was frozen, the vitrified grid was removed from the plunger and stored under liquid nitrogen inside a cryo-grid storage box.

Cryo-TEM analysis of the samples was performed on a JEM-2200FS/CR (JEOL Europe) transmission electron microscope. This microscope is equipped with a field emission gun (FEG) operated at 200 kV and an in-column Ω energy filter. During imaging, no-tilted zero-loss two-dimensional (2D) images were recorded under low-dose conditions, utilizing the 'Minimum Dose System (MDS)' of Jeol software, with a total dose on the order of 30–40 electrons/$Å^2$ per exposure, at defocus values ranging from 1.5 to 4.0 μm. The in-column Omega energy filter of the microscope helped us to record images with improved signal-to-noise ratio (SNR) by zero-loss filtering, using an energy selecting slit width of 30 eV centered at the zero-loss peak of the energy spectra. Digital images were recorded in linear mode on a 3,840 × 3,712 (5 μm pixels) Gatan K2 Summit direct detection camera (Gatan Inc.) using DigitalMicrograph™ (Gatan Inc.) software, at nominal magnifications of 1,500X and 8,000X with a pixel size of 2.7 nm and 0.49 nm, respectively.

2.8 Immunoblotting

Protein quantification of whole lysates, synaptosomal fraction, and cytosolic fraction was carried out with the Pierce BCA Protein Assay Kit (Thermo Fisher), following manufacturer's instructions. Proteins (3–5 μg) were fractioned by SDS-PAGE electrophoresis under reducing conditions (5% β-mercaptoethanol, Gibco) at a 135 V voltage for 90 min in 1.0 mm 4–12% Tris-glycine gels (Invitrogen, Thermo Fisher Scientific). Samples were then transferred at 30 V for 1 h in a 0.2 μm PVDF blotting membrane (Amersham, Sigma-Aldrich), previously activated with methanol (Fisher BioReagents, Thermo Fisher) for 15 min at room temperature. Proteins were visualized with ponceau (Thermo Fisher), washed in TBS-0.1% Tween20 (TBS-T), and blocked in 5% BSA (Sigma-Aldrich) in TBS-T for 1 h at room temperature (RT). The following primary antibodies, namely, mouse anti-PSD95 (1:1000, 95 kDa, Merck #MAB1596), rabbit anti-NR2A (1:1000, 180 kDa, Merck #AB1555P), rabbit anti-Homer1 (1:500, 45 kDa, Synaptic Systems #160003), mouse anti-synaptophysin1 (1:2000, 38 kDa, BioLegend #837102), and rabbit anti-actin (1:10000, 42 kDa, Sigma-Aldrich #SAB4301137), were incubated in 3% BSA in TBS-T overnight in agitation at 4°C. Membranes were washed three times in TBS-T and incubated with the appropriate secondary HRP-conjugated antibodies for 1 h at RT. Membranes were washed three times in TBS-T, and the signal was detected by West Pico Plus chemiluminescent substrate (Protein biology, Thermo Fisher) using the ChemiDoc imaging system (Bio-Rad, Hercules, CA, United States). Total amount of protein was quantified with amido black staining solution (Sigma-Aldrich).

2.9 Pharmacological treatments

For puromycilation assays, cells were treated with 2 µM puromycin dihydrochloride from *Streptomyces alboniger* (Sigma-Aldrich) for 2, 10, or 30 min at 37°C prior to the end of the treatment. Neurites were exposed to 2 min of puromycin, whereas isolated synaptosomes were exposed to 10 min of puromycin, unless otherwise stated. DMSO was used as a vehicle. To block protein synthesis, cells were treated with 40 µM anisomycin dissolved in DMSO (Sigma-Aldrich) for 30 min at 37°C.

Soluble oligomeric Aβ was prepared as previously described (Dahlgren et al., 2002; Gamarra et al., 2020). In brief, synthetic $A\beta_{1-42}$ peptides (Bachem, Bubendorf, Switzerland) were resuspended in dry dimethylsulfoxide (DMSO; 5 mM, Sigma-Aldrich) and Hams F-12 pH 7.4 (PromoCell Labclinics, Barcelona, Spain) to 100 µM final concentration. Peptides were incubated overnight at 4°C. Oligomerized Aβ was added to neurons culture or neuron–astrocyte co-cultures at 23 DIV at a 3 µM concentration and incubated for 24 h at 37°C in a 5% CO_2 humidified incubator. DMSO was used as vehicle control.

2.10 Puromycilation assay

Puromycin is an aminoacyl-tRNA analog that incorporates into nascent polypeptide chains during elongation in a ribosome-catalyzed reaction (Schmidt et al., 2009), and specific anti-puromycin antibodies can be used to detect *de novo* protein synthesis. A total of 24 DIV neurons or neuron–astrocyte co-cultures were exposed to 2 µM puromycin diluted in culture medium for 2 min. To remove puromycin excess, cells were washed once with 1X PBS supplemented with 3 µg/mL of digitonin (Sigma-Aldrich) followed by another wash with 1X PBS. Finally, neurons were fixed with 4% paraformaldehyde (PFA) (Sigma-Aldrich) with 4% sucrose in PBS for 20 min at RT.

In the case of isolated synaptosomes, these were resuspended in 1 mL 1X PBS and divided into 167 µL aliquots that were transferred to a 24-well plate containing poly-D-lysine-coated 12 mm coverslips. Synaptosomes were exposed to puromycin diluted in PBS for 10 min at 37°C, except for the experiments on Aβ-treated cells in which the exposure was extended to 30 min. For the short exposure to conditioned medium (Figure 1B), puromycilation assays were performed with 2 µM puromycin diluted in astroglia-conditioned medium. In any case, after treatments, the plates were centrifuged at 1,400 g for 30 min at room temperature. Synaptosomes were fixed with 4% PFA and 4% sucrose in PBS for 20 min at room temperature.

To block protein synthesis and whenever stated, both neurons and isolated synaptosomes were pretreated with 40 µM anisomycin for 20–28 (depending on the puromycin pulse duration) min at 37°C prior to the puromycin exposure. Samples treated with neither puromycin nor anisomycin were used as negative controls and subjected to the same procedures as experimental samples.

2.11 Immunofluorescence

After fixation, cells or isolated synaptosomes were washed three times with 1X PBS (5 min each wash) and blocked for 30 min in agitation in 3% BSA, 100 mM glycine, and 0.25% Triton X-100 (Thermo Fisher Scientific). Samples were incubated overnight at 4°C with primary antibodies including mouse anti-puromycin (1:500, Merck Millipore #MABE343), chicken anti-synaptophysin 1 (1:500, Synaptic Systems #101006), mouse anti-PSD95 (1:500, Merck #MAB1596), rabbit anti-homer 1 (1:500, Synaptic Systems #160003), guinea pig anti-Homer1 (1:500, Synaptic Systems #160005), rabbit anti-SNAP25 (1:250, Abcam #S9684), and rabbit anti-Rpl26 (1:120, Abcam #ab59567). The following day, after three washes with 1X PBS, cells were incubated with fluorophore-conjugated secondary antibodies Alexa Fluor 594 goat anti-mouse IgG (H + L) (1:200, Invitrogen #A11005), Alexa Fluor 488 goat anti-chicken Ig Y (H + L) (1:200, Abcam #ab150169), Alexa Fluor 647 donkey anti-rabbit IgG (H + L) (1:200, Invitrogen #A31573), and Alexa Fluor 647 goat anti-guinea pig IgG (H + L) (1:200, Invitrogen #A21450) for 1 h at room temperature. Samples were washed three times with 1X PBS and mounted with ProLong Gold Antifade Reagent with DAPI (Invitrogen). Of each secondary antibody, a no-primary-antibody negative control was used.

2.12 Proximity ligation assay

Proximity ligation assays (PLA) were performed using Duolink® *In Situ* Red Started Kit Mouse/Rabbit (Sigma-Aldrich #DU092008). In brief, fixed cells or synaptosomes were washed three times with 1X PBS for 5 min and permeabilized with 3% BSA, 100 mM glycine, and 0.25% Triton X-100 (Thermo Fisher Scientific) for 30 min at room temperature in agitation. The blocking was performed by adding a drop of Duolink® Blocking solution (Sigma-Aldrich) into coverslips at 37°C for 1 h. The mix of primary antibodies was diluted in Duolink® antibody diluent (Sigma-Aldrich) and incubated overnight at 4°C. In brief, the presynaptic antibody chicken anti-synaptophysin (1:500, Synaptic Systems #101006) and the postsynaptic antibody guinea pig anti-Homer1 (1:500, Synaptic Systems #160005) were co-incubated with the primary antibodies required for the proximity ligation assay (PLA). On the one hand, newly synthesized Rpl26 proteins (Puro-Rpl26 PLA) were detected by adding mouse anti-puromycin (1:500, Merck Millipore #MABE343) and rabbit anti-Rpl26 (1:120, Abcam #ab59567) antibodies to the mix of synaptic antibodies, while mouse anti-puromycin (1:500, Merck Millipore #MABE343) and rabbit anti-SNAP25 (1:250, Abcam #S9684) were co-incubated with pre- and postsynaptic antibodies to detect the PLA between puromycin and SNAP25 (Puro-SNAP25 PLA).

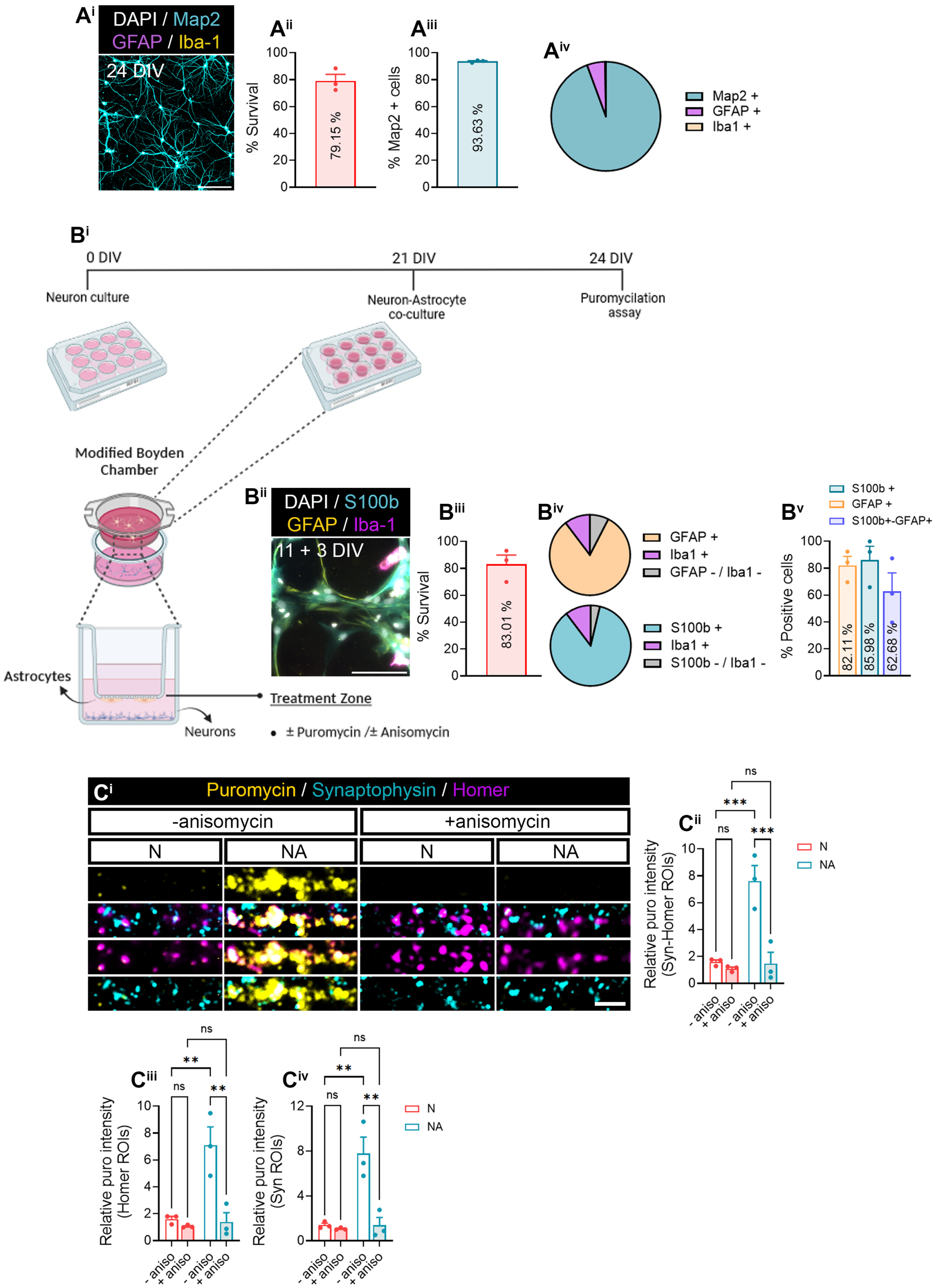


FIGURE 1
Neuron–astrocyte-secreted factors induce local translation in synaptic compartments. **(A[i])** Primary hippocampal neuron cultures were characterized by staining with MAP2 dendritic marker (cyan), GFAP astrocytic marker (magenta), Iba1 microglial marker (yellow), and DAPI (gray). Scale bar 50 μm. Survival of cells is shown in **(A[ii])**. The bar graph represents the mean ± SEM of three independent experiments. The percentage of MAP2+ neurons is

(Continued)

FIGURE 1 (Continued)
quantified in (**A**^{iii}). The bar graph represents the mean ± SEM of three independent experiments. The pie chart (**A**^{iv}) depicts the percentage of neurons (MAP2+) as well as other cell types such as astrocytes (GFAP+) or microglia (Iba1+) observed in neuronal cultures. **(B)** Neurons were cultured in modified Boyden chambers in the presence or absence of astrocytes. (**B**^{i}) Depiction of the culture approach used to culture neurons or to perform neuron–astrocyte co-cultures in modified Boyden chambers. Image created with BioRender. (**B**^{ii}) Astroglial cultures were characterized by staining with S100b astrocytic markers S100b (cyan) and GFAP (yellow), Iba1 microglial marker (magenta), and DAPI (gray). Scale bar 50 µm. Survival of cells is shown in (**B**^{iii}). The bar graph represents the mean ± SEM of three independent experiments. The pie charts (**B**^{iv}) depict the percentage of astrocytes (S100b or GFAP-positive) as well as microglia (Iba1+) and other undetermined cells (negative for S100b–, GFAP, and Iba–1). The percentage of S100b and/or GFAP-positive cells is quantified in (**B**^{v}). The bar graph represents the mean ± SEM of three independent experiments. **(C)** Local translation in synaptic compartments is enhanced by the presence of astrocytes in culture. Twenty four DIV neuron cultures (N) or neuron–astrocyte co-cultures (NA) in modified Boyden chambers in were exposed 2-min with 2 µM puromycin. Translation was blocked with 40 µM anisomycin. Representative micrographs are shown in (**C**^{i}). Bar graphs show changes in puromycin labeling in distinct synaptic compartments in the presence of astrocytes (**C**^{ii}–**C**^{iv}). Data were analyzed in three independent cultures and analyzed by two-way ANOVA followed by Holm-Sidak's *post-hoc* analysis for selected pairs of columns. $^{**}p < 0.01$; $^{***}p < 0.001$; n.s: not significant.

The following day, coverslips were washed twice with wash buffer A (Sigma-Aldrich) for 5 min, and subsequently, PLA signal was developed following manufacturer's instructions. In brief, plus PLA (rabbit probe, 1:5, Sigma-Aldrich) and minus PLA (mouse probe, 1:5, Sigma-Aldrich) probes were diluted in Duolink® antibody diluent (Sigma-Aldrich) at 37°C for 60 min. Samples were washed twice with wash buffer A for 5 min. The ligation of both probes was carried out by incubating coverslips in Duolink® ligation buffer 5X (1:5, Sigma-Aldrich) and 1 U/µL ligase (1:40, Sigma-Aldrich) in ddH_2O at 37°C for 30 min. Before the amplification step, cells were washed twice with wash buffer A and incubated with Duolink® amplification buffer 5X (1:5, Sigma-Aldrich) and 10 U/µL polymerase (1:80, Sigma-Aldrich) in ddH_2O at 37°C for 100 min. Coverslips were washed twice with wash buffer B (10 min each wash), followed by 10 min wash with 0.01% wash buffer B and a final wash with 1X PBS for 5 min. Samples were incubated at room temperature for 1 h with secondary antibodies for synaptic makers Alexa Fluor 488 goat anti-chicken Ig Y (H + L) (1:200, Abcam #ab150169) and Alexa Fluor 647 goat anti-guinea pig IgG (H + L) (1:200, Invitrogen # A21450). Finally, coverslips were washed three times with 1X PBS and mounted with ProLong Gold Antifade Reagent with DAPI (Invitrogen).

2.13 Image acquisition

Images were acquired using an EC Plan-Neofluar 63×/1.4 Oil DIC M27 objective on an Axio Observer Z1 microscope equipped with AxioCam MRm Rev. 3 (Zeiss, Oberkochen, Germany) digital camera. Images of neurites were acquired with 1.6X Optovar., while synaptosomes images were obtained with 1X Optovar. The settings applied for samples were determined in a random field of a control sample and ensuring no intensity saturation. Images from five random fields per coverslip were acquired with ZEN 2 (blue edition) version 2.0.0.0. software (Zeiss). Quantifications performed in neurites (being a neurite a process that extends from the neuronal soma) were performed choosing neurites with an average length of 70 µm.

For survival assessment, images were adjusted for the best fit. Pyknotic nuclei identified by DAPI staining were quantified in 5–10 fields from each sample. Live cells were calculated subtracting the number of apoptotic nuclei to the total amount of cells stained with DAPI and are represented as percentage.

Figure representation has been performed adjusting contrast and background settings. For all images, the setting of the staining of interest was set identically for all conditions, while the pre- and postsynaptic markers used as counterstain were adjusted to obtain an optimal visualization in figures.

2.14 Statistical analysis

All the statistical analyses have been carried out with Prism 8 and 10 (GraphPad Software, San Diego, CA, United States). No normality tests were performed prior to statistical analyses. Typically, two-way analyses of variance (ANOVAs) were performed as more than one variable are analyzed. Otherwise, one.way ANOVA or *t*-test analyses were used. Sample size and statistical analyses are specified in the Figure Legends or throughout the Results section.

3 Results

3.1 Neuron–astrocyte communication through secreted factors induces local translation in synaptic compartments

In this study, we aimed at addressing whether neuron–astrocyte communication regulates local protein synthesis in neurons, specifically in synaptic compartments. Before determining a potential role of astrocytes in synaptic translation, we characterized neuronal monocultures to determine their purity and survival (Figure 1A^{i}). Primary hippocampal cultures at 24 days *in vitro* (DIV) exhibited 79.15% of survival (Figure 1A^{ii}), with 93.53% of live cells being positive for the neuronal marker MAP2 (Figure 1A^{iii}). We identified a 5.38% of GFAP-positive cells and 0.22% of Iba1-positve cells (Figure 1A^{iv}). These results indicate a high enrichment of neurons in our primary cultures. On the other hand, 14 DIV primary glial cultures (Figure 1B^{ii}) showed 83.01% of survival (Figure 1B^{iii}). 85.99% of living cells expressed the astroglial marker S100β, and 82.11% were GFAP-positive (Figure 1B^{v}; Supplementary Figure 1A). In addition, based on the percentage of S100β- or GFAP-expressing cells, we estimated an average of 10.32% of cells being Iba1-positive microglia, and 3.70–7.57% could not be identified either as astrocytes or microglia (Figure 1B^{iv}, lower and upper pie charts, respectively). Finally, 62.68% of cells were positive for both S100β and GFAP.

After characterizing our primary cultures, we performed neuron–astrocytes co-cultures in modified Boyden chambers, which consist of inserts with a 1 µm-diameter-pore polyethylene terephthalate (PET) membrane, that enable communication between two cell types through secreted factors (Figure 1B^{i}). Primary hippocampal neurons were seeded onto coverslips and cultured for 21 DIV, time in which 11 DIV astrocytes were seeded onto the membrane and co-cultured with the neurons for 3 days. Neuron-only cultures were used as controls. To visualize local translation in synaptic compartments, cells were exposed to a 2-min puromycin pulse. Puromycin is an aminoacyl-tRNA analog that incorporates into nascent polypeptide chains during elongation in a ribosome-catalyzed reaction (Schmidt et al., 2009), and specific anti-puromycin antibodies can be used to detect *de novo* protein synthesis. Cells were counterstained with antibodies against synaptophysin-1 (Syn) and Homer-1 (Homer) to visualize pre- and postsynaptic compartments, respectively (Figure 1C^{i}). Our results indicated a significant increase in relative puromycin levels in areas covered by the colocalization between pre- and postsynaptic markers (Syn-Homer ROIs; Figure 1C^{ii}) in co-cultures compared to neuronal monocultures. These results were likely attributed to the effect of astrocytes on both post- and presynaptic translation, as increased puromycin labeling was observed separately in Homer and Syn ROIs (Figures 1C^{iii,iv}). To verify puromycin incorporation was indeed translation-dependent, some cultures were pre-treated with the protein synthesis inhibitor anisomycin 30 min prior to the puromycin pulse. We confirmed that the puromycin labeling observed in synaptic compartments in the presence of astrocytes was in fact a result of increased protein synthesis (Figure 1C). These results suggest that neuron–astrocyte communication promotes local translation in neurons.

We next wanted to investigate whether we were able to find specific proteins whose local synthesis in neurons could be modulated by the presence of astrocytes. Based on the literature, we identified two potential candidates, namely, SNAP25 and Rpl26. On the one hand, there is evidence showing that the synaptosomal protein SNAP25 is locally synthesized in presynaptic terminals during synapse formation *in vitro* (Batista et al., 2017). On the other hand, the mRNA encoding ribosomal protein Rpl26 is known to be locally translated in dendrites (Fusco et al., 2021). Thus, to determine whether these proteins were modulated by the presence of astrocytes, we performed proximity ligation assays (PLA) combining antibodies against puromycin and the protein of interest. We again treated neuronal monocultures or neuron–astrocyte co-cultures with puromycin for 2 min, and we quantified the triple colocalization between the PLA signal, Syn, and Homer (to visualize newly synthesized proteins in synapses) or the double colocalization between the PLA signal and either Homer or Syn (to visualize newly synthesized proteins in post- or presynaptic compartments, respectively). The results were accordingly normalized to the total number of puncta stained with both Syn and Homer, or with either Syn or Homer. No significant SNAP25-PLA signal was detected in synaptic compartments neither in neuronal cultures nor in neuron–astrocytes co-cultures as the identified percentage of positive puncta were similar in cells incubated with puromycin alone or with puromycin and anisomycin (Figures 2A^{ii-iv}). Levels of no-puromycin negative controls are shown for descriptive purposes, although one-way ANOVA comparing this column to all other columns confirmed no detection of SNAP25 synthesis: $p = 0.55$ for Syn-Homer+ compartments; $p = 0.43$ for Homer+ compartments; $p = 0.47$ for Syn + compartments). We then wondered whether a neurodegenerative stimulus would uncover an effect of astrocytes on local SNAP25 synthesis. We treated cultures with Aβ oligomers, main drivers of Alzheimer's disease, which are known to induce local translation in axons (Baleriola et al., 2014; Gamarra et al., 2021). However, we were again unable to detect SNAP25-PLA puncta in neither experimental condition (Supplementary Figure 1B). Conversely, Rpl26 synthesis was readily visible in neurons co-cultured with astrocytes compared to neuronal monocultures (Figure 2B), although only in Homer-Syn-positive synapses (Figures 2B^{i-iii}). Thus, we could not attribute this effect to dendritic spines or presynaptic terminals. No effect of astrocytes could be detected in cultures treated with Aβ oligomers (Supplementary Figure 1C). Thus far, our results indicate that communication between astrocytes and neurons through secreted factors enhances the local synthesis of at least Rpl26 in basal conditions, and this effect might be impaired in pathological conditions.

3.2 Isolated synaptosomes are functionally competent to incorporate puromycin in a protein synthesis-dependent manner

Puromycin assays described thus far were performed by feeding cultures with puromycin for only 2 min. Given this short exposure, it is unlikely that newly synthesized puromycilated peptides arise in synapses as a result from the transport of somatically produced proteins. However, one of the potential limitations of exposing neurons to such a short pulse is that, depending on the translation rate of localized transcripts, signals arising from the PLA approach might be below detection levels. Hence, we decided to isolate synaptosomes devoid from somatic inputs, and once isolated, we exposed them to puromycin for a longer period to improve the detection of newly synthesized proteins. Our aim was 2-fold: first, to determine whether isolated synaptosomes still retained their translation capacity; second, to confirm that astrocytes modulate synaptic translation in neurons.

We first characterized synaptosomes isolated from hippocampal neurons with Syn-PER buffer by cryo-electron microscopy (Cryo-EM). Cryo-EM evidenced electrodense presynaptic terminals, some of them containing visible synaptic vesicles, with a nearby postsynaptic density, both separated by the synaptic cleft (Figure 3A^{i}). Moreover, we identified synaptic proteins by immunoblotting and observed that postsynaptic markers PSD95 and NR2A, although not enriched in synaptosome preparations compared to the whole lysate, they were decreased in the cytosolic fraction (Figures 3B^{i-iii}). Conversely, the presynaptic marker Syn was enriched in crude synaptosome preparations compared to both the whole lysate and the cytosol (Figure 3B^{v}). Actin was used as a cytoskeletal marker and remained unchanged in all fractions (Figure 3B^{vi}). These results were not unexpected as previous publications have reported the enrichment of presynaptic terminals compared to postsynaptic densities using alternative synaptosome isolation methods (Hafner et al., 2019). Finally, we also characterized our synaptosome preparation by conventional immunocytochemistry. To that end, we attached freshly resuspended synaptosomes to poly-D-lysine-treated coverslips by centrifugation. After fixation, synaptosomes were immunostained with antibodies against Syn and Homer, following the same approach as in neuronal cultures (Figures 1, 2). Despite Homer not being enriched in synaptosomes based on our results from immunoblotting (Figure 3B^{iv}), we did observe the colocalization of both markers (Figure 3C^{ii}), in line with the results obtained by Cryo-EM.

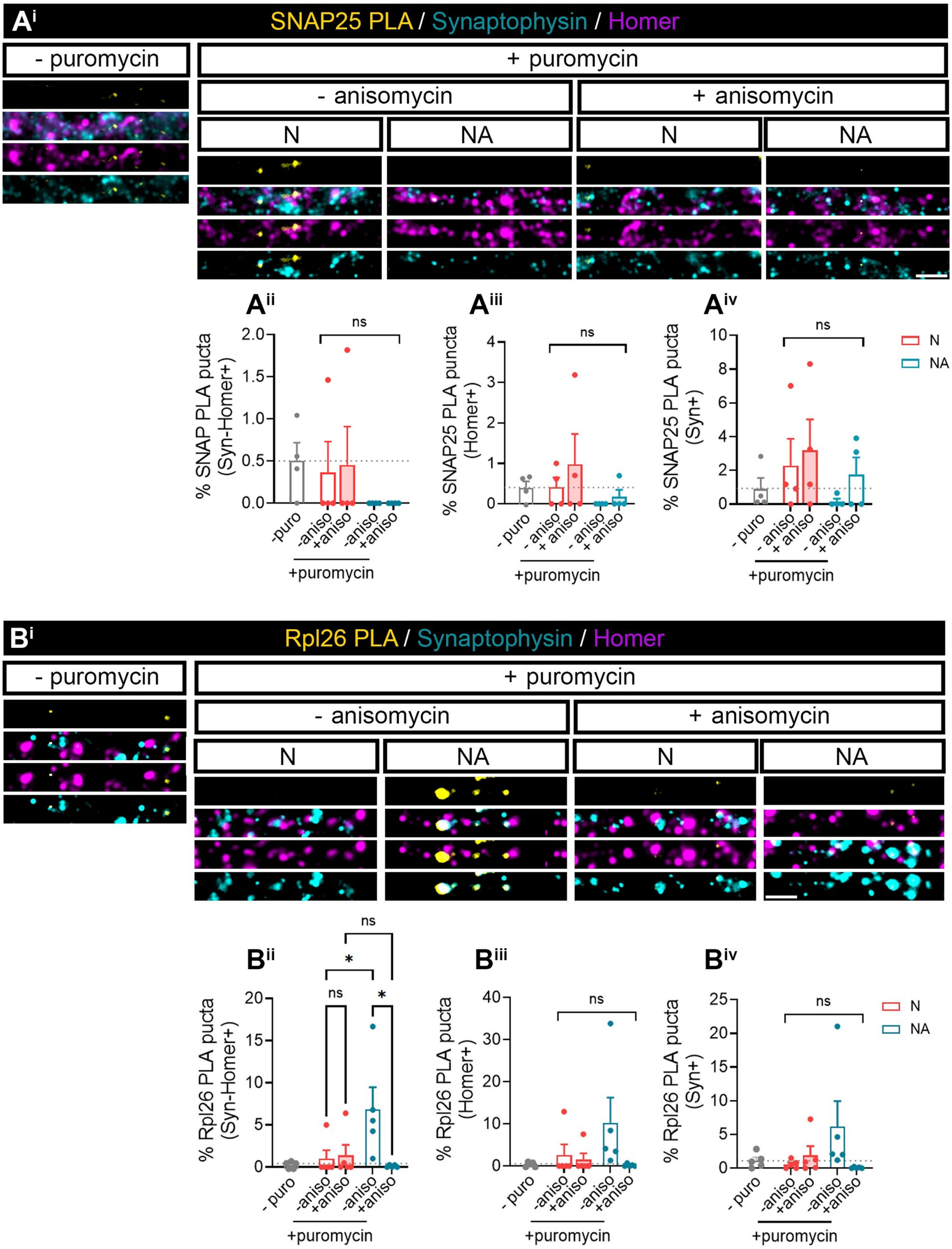


FIGURE 2
Factors secreted in neuron–astrocyte co-cultures induce *Rpl26* synaptic local translation in basal conditions. Twenty four DIV neuron cultures (N) or neuron–astrocyte co-cultures (NA) in modified Boyden chambers in basal condition were exposed 2-min with 2 μM puromycin. The translation was blocked with 40 μM anisomycin. Cells were treated with vehicle (– puromycin) as negative control. Puromycin proximity ligation assay (Puro-PLA) was performed of **(Ai)** SNAP25 protein (SNAP25 PLA) and **(Bi)** Rpl26 protein (Rpl26 PLA). Scale bar: 2 μm. Percentage (%) of the triple colocalization analysis obtained from **(Aii)** SNAP25 PLA or **(Bii)** Rpl26 PLA puncta with synaptophysin-Homer+ synapses (Syn-Homer+, obtained as a result of the double

(Continued)

FIGURE 2 (Continued)
colocalization of Syn + and Homer+ puncta), normalized to the total Syn-Homer+ synapse for each individual condition. Percentage of the double colocalization of **(A[iii])** SNAP25 PLA or **(B[iii])** Rpl26 PLA puncta with Homer postsynapses (Homer+), normalized to the total Homer+ for each individual condition. Percentage of the double colocalization of **(A[iv])** SNAP25 PLA or **(B[iv])** Rpl26 PLA puncta with Syn + presynapses (Syn+), normalized to the total Syn + for each individual condition. In all graphs, levels of no-puromycin negative controls are shown for descriptive purposes. Two-way ANOVA test was carried out, and whenever the ANOVA was significant (ns when the ANOVA was not significant), Holm-Sidak's *post-hoc* analysis for selected pairs of columns was performed, *$p < 0.05$ and ns: not significant. SNAP25 PLA graphs represent mean ± SEM of four independent experiments, whereas Rpl26 PLA graphs represent mean ± SEM of five independent experiments.

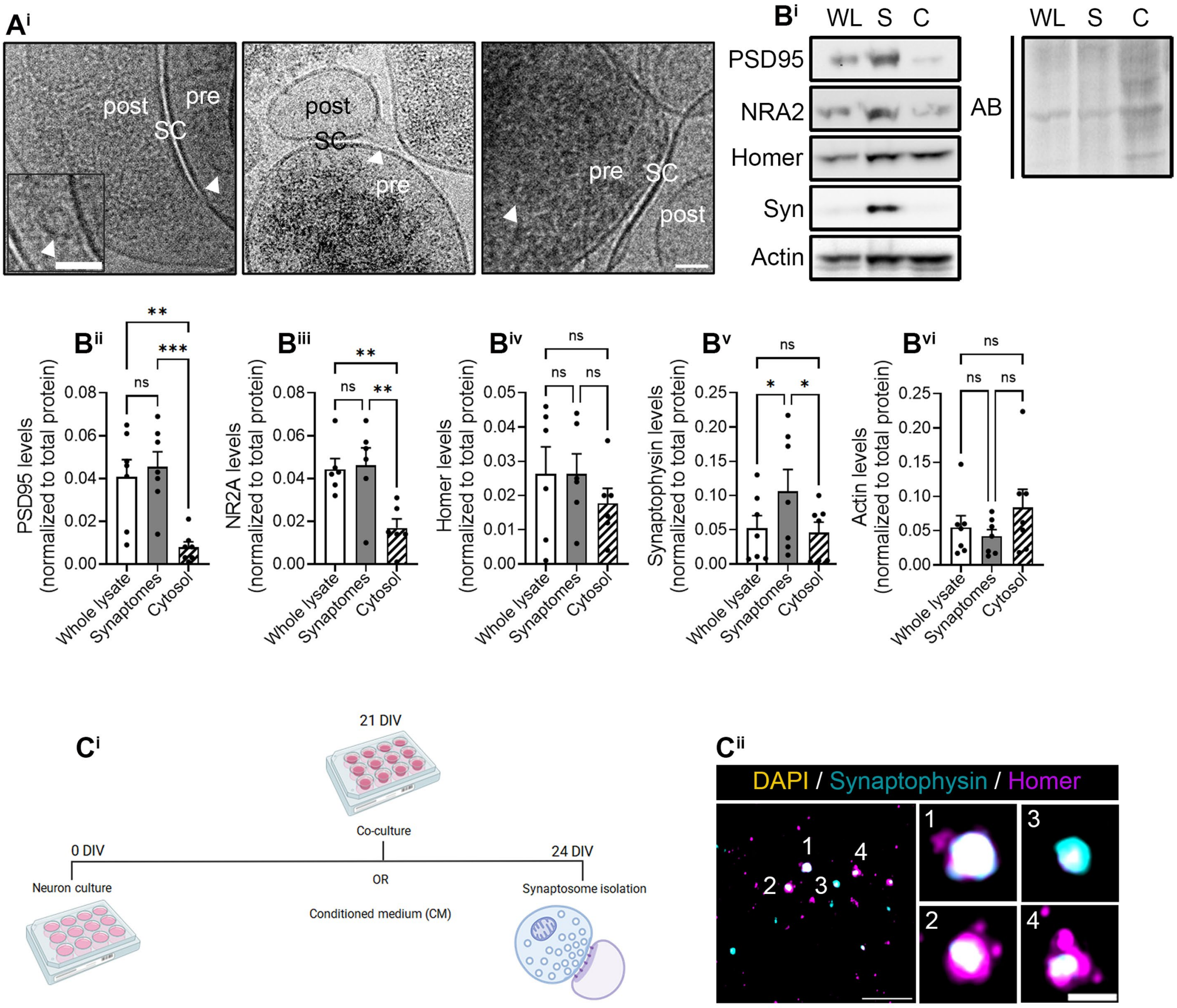


FIGURE 3
Synaptosome characterization. Synaptosome characterization by **(A[i])** Cryo-EM image of isolated synaptosomes, where presynapses (pre) and postsynapses (post) can be distinguished by the synaptic cleft (SC). Some synaptic vesicles (indicated by arrowheads in the images) can be observed within presynaptic compartment. Scale bar 50 nm (25 nm in inset). **(B[i])** Representative images of the Western blot (WB). All proteins were normalized to the total amount of protein detected with amido black (AB) staining solution. WL, whole lysate; S, synaptosomal fraction; C, cytosolic fraction. WB quantification of **(B[ii])** postsynaptic marker PSD95, **(B[iii])** N-methyl-D-aspartate (NMDA) receptor (NRA2), **(B[iv])** postsynaptic marker Homer-1 (Homer), **(B[v])** presynaptic protein synaptophysin-1 (Syn), and **(B[vi])** the cytoskeletal marker actin. All proteins were normalized to the total amount of protein. RM one-way ANOVA followed by Holm-Sidak's *post-hoc* test for selected pairs of columns, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ns: not significant. **(C[i])** Experimental protocol to perform the puromycilation assay in isolated synaptosomes. The representative figure has been created with BioRender. **(C[ii])** Immunocytochemistry of isolated synaptosomes represented by synaptophysin-positive pre-synapses (in cyan) and homer-positive postsynapses (in magenta). Synaptosomes were counterstained with DAPI (in yellow) to confirm the absence of the somatic input. Scale bar 10 μm; 2 μm in insets.

Next, we addressed if isolated synaptosomes were functionally competent to incorporate puromycin into newly synthesized polypeptide chains. Thus, we performed control experiments exposing cells to Aβ oligomers, which induce local translation in axons and presynaptic terminals (Baleriola et al., 2014). Synaptosomes were isolated from vehicle- or Aβ-treated neurons, and, once attached to a coverslip, they were exposed to 2 µM puromycin and/or to the translation inhibitor anisomycin for 30 min. Puromycilation assays revealed a higher colocalization between puromycin and synaptic markers in Aβ-treated cells compared to anisomycin-treated synaptosomes, whereas no differences were observed in synaptosomes isolated from control cells (Supplementary Figure 2A). These results indicate that local translation can be measured in isolated synaptosomes that do not receive somatic input, at least under certain conditions.

3.3 Astrocyte-conditioned medium induces local translation in isolated synaptosomes

Our previous results (Figure 1) indicated that neuron–astrocyte communication via secreted factors enhances local protein synthesis in synaptic compartments in neurons. Next, we wanted to determine whether our results could be validated in isolated synaptosomes. To that end, we performed neuronal monocultures on neuron–astrocytes co-cultures in modified Boyden chambers as before. Synaptosomes were then isolated and treated with puromycin for 10 min (Figure $4A^{i}$). In accordance with our previous observations, the presence of astrocytes increased newly synthetized proteins in Syn-Homer synapses (Figure $4A^{ii}$), as well as in post- (Figure $4A^{iii}$) and presynaptic compartments (Figure $4A^{iv}$). In all cases, this effect was blocked by anisomycin. We also wondered whether factors secreted solely by astroglia would elicit the same effect on synaptic translation such as neuron–astrocyte co-cultures. We therefore exposed neurons to astrocyte-conditioned medium (CM) for 3 days and observed a strong trend toward an increase of puromycin puncta in Syn-Homer synaptosomes ($p = 0.05$; Figure $4A^{ii}$), which became significant in post- (Figure $4A^{iii}$) and presynaptic compartments (Figure $4A^{iv}$). Finally, we wanted to determine whether synaptic translation was selectively enhanced by astrocytes, or whether other cell types would drive a similar response in neurons. Thus, we performed experiments on neurons cultured in Boyden chambers, but we now co-cultured them with fibroblasts or treated them with fibroblast-conditioned medium (Supplementary Figure 2B). Synaptosomes were isolated and exposed to puromycin for 10 min. In this case, neither the presence of fibroblasts in culture nor their conditioned medium had any effect on local translation in Syn-Homer synaptosomes or in postsynaptic compartments (Figures $4B^{i–iii}$). However, we did observe a significant increase in presynaptic compartments form neurons co-cultured with fibroblasts or exposed to conditioned medium. Interestingly, puromycin incorporation was blocked by anisomycin in the latter but not in synaptic terminals isolated from co-cultured neurons (Figure $4B^{iv}$). A potential explanation for these results will be discussed later in this report, but we can affirm that fibroblast-conditioned medium enhances local protein synthesis in presynaptic terminals.

In summary, astrocyte-secreted factors regulate synaptic local translation. This effect is not exclusively driven by astrocytes since secreted factors from other cell types, such as fibroblasts, can also modulate newly synthetized proteins at least within the presynaptic compartment. However, both cell types do show differences in their effect on local protein synthesis in synapses and postsynaptic density, which seem to be modulated selectively by astrocytes.

3.4 Astrocyte-secreted factors enhance Rpl26 local synthesis in postsynaptic compartments

In this study, we aimed at addressing if detection of local SNAP25 and Rpl26 synthesis was improved by exposing isolated synaptosomes with puromycin for 10 min. Like in previous experiments, Puro-SNAP25-PLA puncta were not detected in any synaptic compartment analyzed neither in the present or absence of astrocytes in basal conditions (Figure $5A^{i}$) nor in response to Aβ treatments (Supplementary Figure 3A). Conversely, Rpl26 synthesis was enhanced by the presence of astrocytes in culture in Syn-Homer synapses (Figures $5B^{i,ii}$), in line with previous observations. Importantly, the 10-min exposure of isolated synaptosomes with puromycin uncovered the modulation of newly produced Rpl26 in postsynaptic compartments (Figures $5B^{i,iii}$) although not in presynaptic terminals (Figures $5B^{i,iv}$). Fibroblasts, on the other hand, did not affect the local synthesis of Rpl26 in Syn-Homer synapses (Figure $5B^{v}$, Two-way ANOVA, column factor p=0.002. No diferences dcetected in *post hoc* test) or in post- and presynaptic compartments (Figures $5B^{vi,vii}$). Thus, local Rpl26 production is likely selectively boosted by astrocytes in basal conditions. Nevertheless, in response to Aβ treatment Rpl26 local translation was not detected in any synaptic compartment (Supplementary Figure 3B).

Finally, we wanted to determine whether Rpl26 synthesis was also enhanced by astroglial-conditioned medium. Surprisingly, after 3 days of astrocyte-CM treatment, Puro-Rpl26 PLA puncta were not detected in any synaptic compartment. We obtained similar results with fibroblast CM (Figure 6A). We reasoned that increased synaptic translation observed in synaptosomes in neurons co-cultured with astrocytes could be a result of constant communication between both cell types, in which astroglia continuously secrete molecules to the medium, whereas signals potentially responsible for *de novo* Rpl26 production could be depleted from the conditioned medium over time, hence diminishing their effect. To test this possibility, we acutely exposed synaptosomes to astrocyte-CM while performing puromycin labeling for 10 min. Interestingly, we found that upon direct treatment with astrocyte-CM, Syn-Homer synapses showed a trend toward increasing locally synthesized Rpl26 when compared to synaptosomes co-incubated with anisomycin (Figures $6B^{i,ii}$, $p = 0.09$). Differences between both conditions were significant when only focusing on double colocalization of Puro-Rpl26 PLA signal with Homer in postsynaptic densities (Figure $6B^{iii}$), while no changes were detected in presynaptic terminals (Figure $6B^{iv}$). These results strongly suggest that astrocyte-conditioned medium positively regulates local Rpl26 production at the postsynaptic level in an acute manner.

3.5 Puro-PLA labeling of isolated synaptosomes might help identify the global or local origin of synaptic proteins

Finally, we wanted to address if levels of identified proteins (namely, Rpl26 and SNAP25) were changed overall in synaptosomes isolated from neuron–astrocyte co-cultures compared to neuronal monocultures. To our surprise, no changes were detected (Supplementary Figure 4). With these results, it is tempting to speculate that Puro-PLA labeling of isolated synaptosomes might help dissect the relative contribution of locally synthesized proteins versus preexisting protein pools in synaptic compartments.

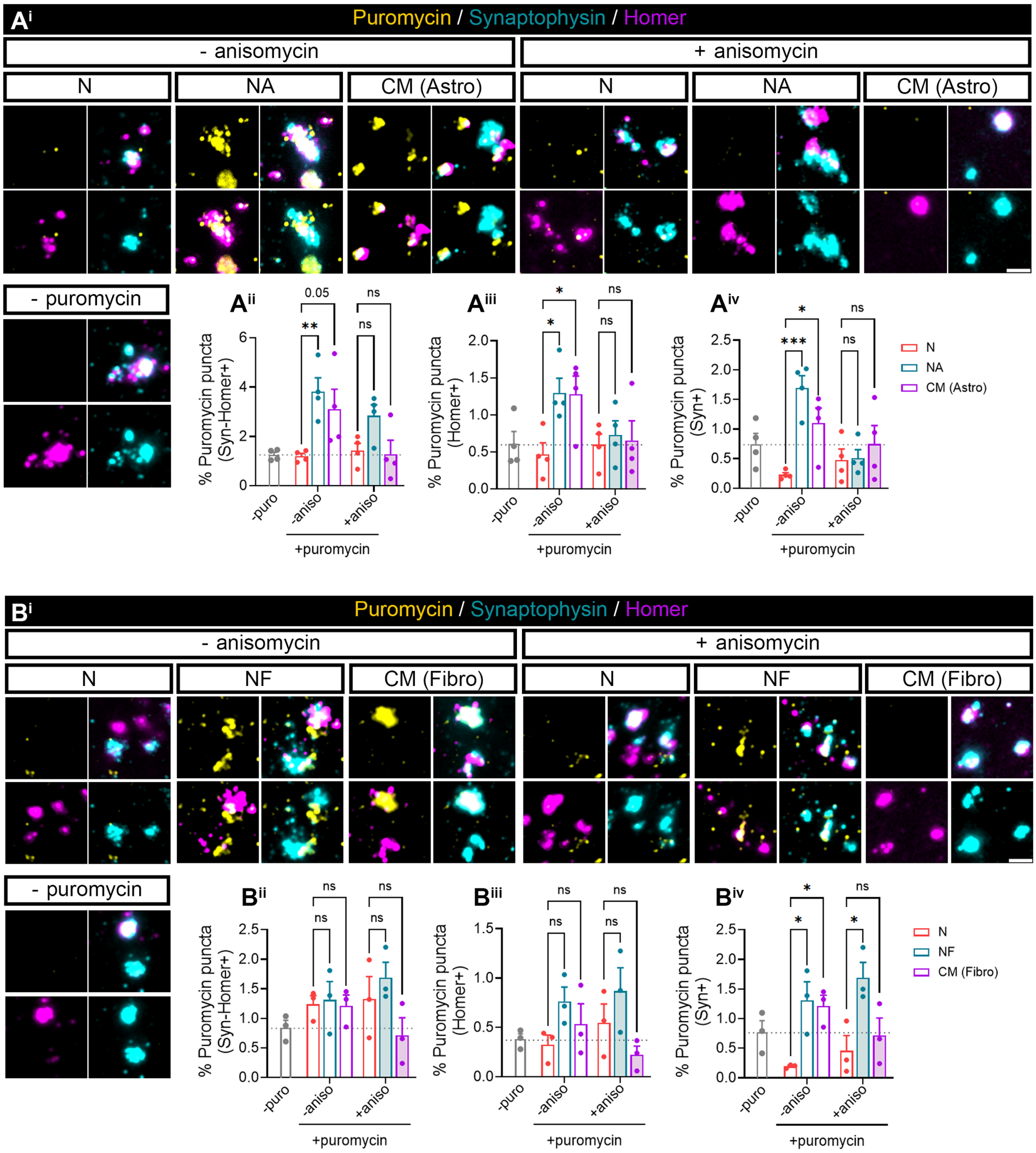


FIGURE 4
Puromycilation assay detection in isolated hippocampal synaptosomes. Twenty four DIV hippocampal neurons were cultured or co-cultured with astrocytes or astrocytic-conditioned medium **(A)** or co-cultured with fibroblast or fibroblast derived conditioned medium **(B)** for 3 days. **(A^{i})** Puromycin treatment was carried out in isolated synaptosomes coming from neurons exposed to astrocyte-secreted factors. Scale bar 2 μm. Percentage (%) of the colocalization analysis obtained from puromycin puncta with **(A^{ii})** synaptophysin-Homer+ synapses (obtained as a result of the double colocalization of Syn + and Homer+ puncta), **(A^{iii})** Homer postsynapses (Homer+), and **(A^{iv})** Syn + presynapses (Syn+). Each individual condition was normalized to the total amount of Syn-Homer+ synapses, Homer+ postsynapses, or Syn + presynapses, respectively. Two-way ANOVA followed by Holm-Sidak's *post-hoc* test for selected pairs of columns, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and ns: not significant ($p = 0.05$). All graphs represent mean ± SEM of four independent biological experiments. **(B^{i})** Puromycin treatment was performed in isolated synaptosomes coming from neurons in the presence of fibroblast-secreted factors. Scale bar 2 μm. Percentage (%) of the colocalization analysis obtained from puromycin puncta with **(B^{ii})** synaptophysin-Homer+ synapses (obtained as a result of the double colocalization of Syn + and Homer+ puncta), **(B^{iii})** Homer postsynapses (Homer+), and **(B^{iv})** Syn + presynapses (Syn+). Each individual condition was normalized to the total amount of Syn-Homer+ synapses, Homer+ postsynapses, or Syn + presynapses, respectively. Two-way ANOVA statistical test was performed, $*p<0.05$, and ns: not significant. All graphs represent mean ± SEM of three independent biological experiments.

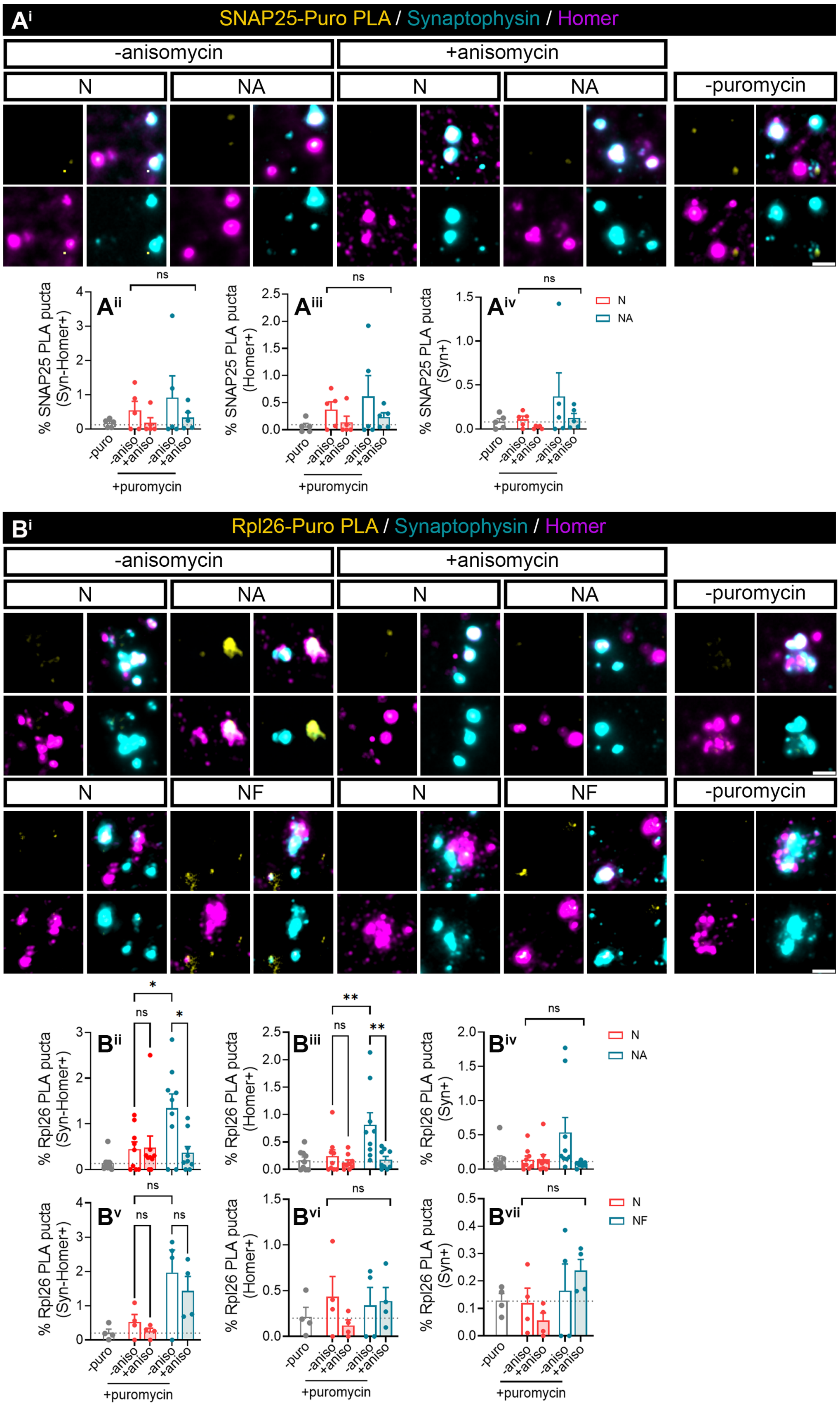


FIGURE 5

Puromycin direct treatment in isolated synaptosomes confirmed the role of astrocytic-secreted factors to promote *Rpl26* synaptic local translation in basal conditions. Synaptosomes isolated from 24 DIV neuron cultures (N) or neuron–astrocyte co-cultures (NA) in modified Boyden chambers in basal

(Continued)

FIGURE 5 (Continued)
condition were exposed 10-min with 2 μM puromycin. The translation was blocked with 40 μM anisomycin. Cells were treated with vehicle (– puromycin) as negative control. **(A^{i})** Puro-PLA of SNAP25 (SNAP25 PLA) was performed. Scale bar 2 μm. Percentage (%) of the colocalization analysis obtained from SNAP25 PLA puncta with **(A^{ii})** synaptophysin-Homer+ synapses (obtained as a result of the double colocalization of Syn + and Homer+ puncta), **(A^{iii})** Homer postsynapses (Homer+), and **(A^{iv})** Syn + presynapses (Syn+). Each individual condition was normalized to the total amount of Syn-Homer+ synapses, Homer+ postsynapses, or Syn + presynapses, respectively. In all graphs, levels of no-puromycin negative controls are shown for descriptive purposes. Two-way ANOVA, ns: not significant. All graphs represent mean ± SEM of five independent biological experiments. **(B^{i})** Puro-PLA of Rpl26 (Rpl26 PLA) was performed in isolated synaptosomes derived from 24 DIV neuron cultures (N) or co-cultures of neuron–astrocyte (NA) or neuron–fibroblast (NF) in modified Boyden chambers in basal condition. Synaptosomes were exposed to a 10-min puromycin pulse and anisomycin for 30 min. Non-puromycin treatment (– puromycin) was used as negative control. Scale bar 2 μm. Percentage (%) of the colocalization analysis obtained from Rpl26 PLA puncta with **(B^{ii},B^{v})** synaptophysin-Homer+ synapses (obtained as a result of the double colocalization of Syn + and Homer+ puncta), **(B^{iii},B^{vi})** Homer postsynapses (Homer+), and **(B^{iv},B^{vii})** Syn + presynapses (Syn+). Each individual condition was normalized to the total amount of Syn-Homer+ synapses, Homer+ postsynapses, or Syn + presynapses, respectively. In all graphs, levels of no-puromycin negative controls are shown for descriptive purposes. Two-way ANOVA test was carried out, and whenever the ANOVA was significant (ns when the ANOVA was not significant), Holm-Sidak's *post-hoc* test for selected pairs of columns was performed, $^{*}p < 0.05$, $^{**}p < 0.01$, and ns: not significant. All graphs represent mean ± SEM of nine independent biological experiments for NA condition, whereas for NF condition four independent biological experiments have been used.

4 Discussion

4.1 Potential regulation of local mRNA translation in neurons by cell non-autonomous mechanisms

To our knowledge, this is the first report that directly addresses astroglial regulation of local translation in neurons. We have demonstrated that neuron–astrocyte communication and astrocytic-seceted factors upregulate local protein synthesis in synaptic compartments. Interestingly, fibroblast-conditioned medium also enhanced local protein synthesis in presynaptic terminals. These results are not unexpected, as fibroblast growth factor (FGF) has been historically used to maintain neuronal survival in culture and supports neuritic outgrowth (see Walicke et al., 1986 as example), and local translation is known to promote axon elongation (Leung et al., 2006). One surprising result, however, was that enhanced puromycin labeling in presynapses in neuron–fibroblast co-cultures compared to neuronal monocultures was not inhibited by anisomycin. It has been reported that stalled ribosomes are able to incorporate puromycin, while this reaction is independent of translation elongation inhibitors, such as anisomycin and others (Graber et al., 2013). Thus, one possibility is that, through unknown mechanisms, fibroblast–neuron communication leads to presynaptic ribosome stalling. The functional significance of this phenomenon should be further explored. Overall, our results point toward a cell non-autonomous modulation of the synaptic proteome through local translation in subneuronal compartments. The molecular mechanisms leading to the effect of non-neuronal cells on neurons deserve future investigation. In the case of neuron–astrocyte communication, our results open new exciting venues for the understanding of how astrocytes regulate synaptic function, which is (at least) partially regulated by mRNA localization and local protein synthesis.

4.2 Isolated synaptosomes maintain a functional translation machinery

In this study, we implemented a simple methodology to measure local translation in isolated synaptosomes, which lack somatic input, by basic immunocytochemistry. We found that isolated synaptosomes are capable of puromycin incorporation in a protein synthesis-dependent manner. With this approach, we aimed at determining whether isolated synaptosomes still retained their translational capacity. In addition, we wanted to corroborate the influence of astrocytes on local translation in neurons. Our first approaches used 2-min puromycin pulses in neurons (Figures 1, 2) to minimize the diffusion from somatic-derived proteins. However, puromycin assays used to detect local translation have been recently criticized (Enam et al., 2020) even when using short exposures to the drug. Moreover, one of the potential limitations of exposing neurons to such a short pulse is that, depending on the translation rate of localized transcripts, some newly synthesized proteins might not be efficiently detected. Indeed, our own results indicate that Rpl26 localized synthesis was not conclusive in postsynapses when neurons were exposed to puromycin for 2 min in neuron–astrocyte co-cultures compared to monocultures. Conversely, 10-min puromycin treatments of isolated synaptosomes revealed an effect of astroglia on Rpl26 local production in postsynaptic densities. Hence, our approach on isolated synaptosomes uncovered the influence of astrocytes on Rpl26 local postsynaptic synthesis.

4.3 Advantages of puromycin labeling of isolated synaptosomes

Neuronal local translation has been addressed in many instances by labeling neurons with non-canonical amino acids or by puromycin tagging of nascent polypeptides (to mention but a few approaches) (Gamarra et al., 2021; Holt et al., 2019), followed by the detection of these molecules in distal neuronal compartments. Short exposure of neurons to such molecules minimizes the potential "contamination" of somatically synthesized proteins transported (or diffused) toward the periphery of neurons. However, recent evidence suggests that even short treatments with puromycin, for instance, might not accurately distinguish local translation from other events (Enam et al., 2020). Synaptosome isolation followed by protein synthesis detection might be a powerful tool to identify local translatomes in synaptic compartments, given that they are disconnected from somatic inputs, which enables labeling of newly synthesized proteins at a *de bona fide* local level. In addition, having the opportunity to treat isolated synaptosomes with puromycin (and similar molecules) at different exposure times might help distinguish fast translating

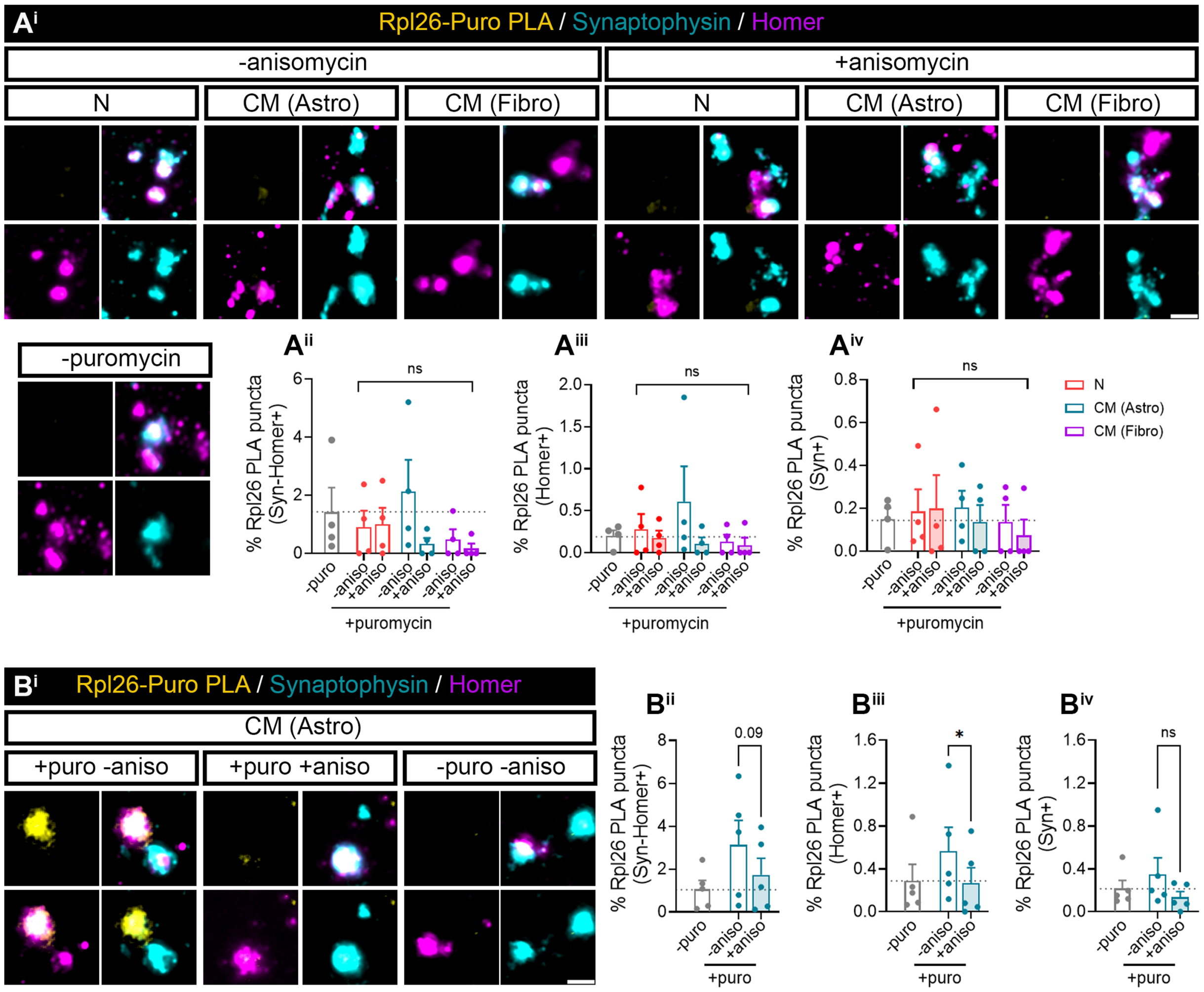


FIGURE 6
Rpl26 synaptic local translation is translated in a fast speed manner after astrocyte-conditioned medium treatment in basal conditions. Twenty four DIV hippocampal neurons were treated with neuronal (N), astrocytic (astro), or fibroblast (fibro) conditioned medium (CM). Isolated synaptosomes were treated with 2 μM puromycin for 10 min. The translation was blocked with 40 μM anisomycin for 30 min. Cells were treated with vehicle (– puromycin) as negative control. **(A^i)** Puromycin proximity ligation assay (Puro-PLA) was performed of Rpl26 protein (Rpl26 PLA). Scale bar 2 μm. Percentage (%) of the colocalization analysis obtained from Rpl26 PLA puncta with **(A^ii)** synaptophysin-Homer+ synapses (obtained as a result of the double colocalization of Syn + and Homer+ puncta), **(A^iii)** Homer postsynapses (Homer+), and **(A^iv)** Syn + presynapses (Syn+). Each individual condition was normalized to the total amount of Syn-Homer+ synapses, Homer+ postsynapses, or Syn + presynapses, respectively. Two-way ANOVA statistical test was performed, ns: not significant. All graphs represent mean ± SEM of four independent biological experiments. **(B^i)** Rpl26 PLA was carried out in isolated synaptosomes directly treated with astrocyte-CM for 30 min. 10 min prior to the end of the treatment, puromycin was added to synaptosomes. Anisomycin was used to inhibit the translation. Scale bar 2 μm. Percentage (%) of the colocalization analysis obtained from Rpl26 PLA puncta with **(B^ii)** synaptophysin-Homer+ synapses (obtained as a result of the double colocalization of Syn + and Homer+ puncta), **(B^iii)** Homer postsynapses (Homer+), and **(B^iv)** Syn + presynapses (Syn+). Each individual condition was normalized to the total amount of Syn-Homer+ synapses, Homer+ postsynapses, or Syn + presynapses, respectively. One-way ANOVA test with Holm-Sidak's *post-hoc* analysis, **p* < 0.05, and ns: not significant (*p* = 0.09). All graphs represent mean ± SEM of five independent biological experiments.

versus slow translating localized transcripts. Indeed, mRNAs encoding ribosomal proteins have been identified as highly translated yet fast decaying mRNAs in axons (Jung et al., 2023). This could explain why long exposure on neurons to astrocyte-conditioned medium enhances overall puromycin incorporation in synaptic compartments, yet Rpl26 local synthesis is only detected upon acute exposure to astroglial-secreted factors.

Although in many instances, mRNA localization in subneuronal compartments has been used a proxy for local translation, local transcriptomes are unlikely a reflection of local proteomes (Jung et al., 2023). In this context, we believe our methodology might help identify which transcripts are locally translated in certain experimental conditions and perform correlation analyses with preexisting localized mRNAs and proteins and even evaluate mRNA local decay and protein half-lives at subcellular levels.

Although this methodology can be perfectly explored in synaptosomes isolated from an entire brain *in vivo*, we believe our *in vitro* approach has the advantage of controlling the cell types that influence neurons (and vice versa) in a controlled environment. The intricate brain connectivity is often difficult to dissect, and our method enables us to test the uni- and bidirectional communication between cell type "pairs." Obviously, cell-to-cell communication is much more complex in the brain, and this complexity should be taken into consideration.

In addition, our results can be associated with excitatory synapses as we have focus on Homer+ postsynapses, which are enriched in glutamatergic postsynapses. Indeed, astrocyte-secreted factors have been known not only to regulate excitatory synapses but also inhibitory synaptogenesis, where astrocyte-secreted neurocan controls inhibitory synaptogenesis and functions (Irala et al., 2024). Hence, as reported in previous articles, in our synaptosomal preparation, we observed 2.15 times synapses in neuron–astrocyte co-cultures (19,888,027 synaptosomes/mL; from an average of 3,314,617 synaptosomes/cm^2 obtained in 1/6 mL) compared to the synapses coming from neuron cultures (9,229,510 synaptosomes/mL; from an average of 1,538,241 synaptosomes/cm^2 in 1/6 mL). Overall, these findings suggest that if astrocyte-secreted factors promote synaptogenesis in excitatory and inhibitory synapses, leading to synaptic proteomic changes, astrocyte-secreted factors could be driven synaptogenesis by local translation in excitatory and inhibitory synapses.

5 Conclusion

In this article, we have demonstrated for the first time that astrocyte-secreted factors enhance synaptic mRNA local translation in synaptic compartments. In addition, we have defined a method to visualize local protein synthesis in isolated synaptosomes. We believe this method can be implemented to perform high-throughput analyses of local translatomes in diverse experimental conditions by capturing puromycilated peptides in isolated synaptosomes at different timepoints, as well as to verify locally synthesized proteins by conventional immunocytochemistry. Crude synaptosome lysates can be further separated into presynaptic and postsynaptic fractions, thus distinguishing local presynaptic and postsynaptic translatomes. Both our main finding of neuronal local translation regulation by potential cell-non-autonomous mechanisms and our methodology to measure local protein synthesis in isolated synaptosomes might open new venues in the field of local translation.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors upon reasonable request.

Ethics statement

The animal study was approved by Comisión de Ética en la Investigación y Docencia (CEID/IIEB), University of the Basque Country (UPV/EHU). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

AC-G: Validation, Writing – original draft, Writing – review & editing, Conceptualization, Data curation, Formal analysis, Investigation, Methodology. JB: Validation, Writing – original draft, Writing – review & editing, Conceptualization, Supervision, Funding Acquisition, Project administration.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This study was supported by grants SAF2016-76347-R and by PID2022-139451OB-I00 funded by MICIU/AEI/10.13039/501100011033 with FEDER funds (EU. To JB), a start-up grant RYC-2016-19837 (to JB) and two Alzheimer's Association grants AARG-19-618303 and AARG-19-618303-RAPID. (to JB). AdC-G is a predoctoral fellow funded by the Basque Government.

Acknowledgments

We acknowledge the Electron Microscopy and Crystallography Platform from the Center for Cooperative Research in Biosciences (CIC bioGUNE) for the synaptosomal characterization by cryo-electron microscopy.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2025.1427036/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Astrocyte-secreted factors effect on *Rpl26* synaptic local translation is inhibited in Aβ conditions. The expression of S100β and GFAP from cultures used for these experiments is shown **(A^{i})**. Scale bar, 100 μm. 24 DIV vehicle- or Aβ-treated neuron cultures (N) or Aβ-treated neuron-astrocyte co-cultures (NA) in modified Boyden chambers were exposed 2-minutes with 2 μM puromycin. Anisomycin was used to block translation and no-puromycin treatment (- puromycin) as negative control. Percentage (%) of the colocalization analysis obtained from SNAP25 PLA puncta with **(B^{i})** synaptophysin-Homer+ synapses (obtained as a result of the double colocalization of Syn+ and Homer+ puncta), **(B^{ii})** Homer postsynapses (Homer+) and **(B^{iii})** Syn+ presynapses (Syn+). Each individual condition was normalized to the total amount of Syn-Homer+ synapses, Homer+ postsynapses or Syn+ presynapses, respectively. Two-way ANOVA, ns not

significant. All graphs represent mean ± SEM of 4 independent biological experiments. Representative micrographs are shown in **(B[iv])**. Scale bar 2 µm. Percentage (%) of the colocalization analysis obtained from Rpl26 PLA puncta with **(C[i])** synaptophysin-Homer+ synapses, **(C[ii])** Homer+ postsynapses and **(C[iii])** Syn+ presynapses. Each individual condition was normalized to the total amount of Syn-Homer+ synapses, Homer+ postsynapses or Syn+ presynapses, respectively. Representative micrographs are shown in **(C[iv])**. Scale bar 2 µm. Two-way ANOVA, ns not significant. All graphs represent mean ± SEM of 5 independent biological experiments. In all graphs levels of no-puromycin negative controls are shown for descriptive purposes.

SUPPLEMENTARY FIGURE 2
Puromycin incorporation within isolated synaptosomes in basal and Aβ conditions. **(A[i])** Synaptosomes were isolated from 24 DIV hippocampal neurons treated with vehicle or Aβ. Puromycilation assay was performed within isolated synaptosomes exposing them to 30 minutes of puromycin or puromycin co-incubated with anisomycin treatment. Scale bar 10 µm and 2 µm in insets. **(A[ii])** Percentage (%) of puromycin puncta colocalization with Syn-Homer+ synapses, normalized to the total Syn-Homer+ synapses. Levels of no-puromycin are shown as negative control. Two-way ANOVA analysis with Holm-Sidak's *post hoc* test for selected pairs of columns was performed, $^{**}p<0.01$ and ns not significant. The graph represent the mean ± SEM of 5 independent biological experiments. **(B[i])** Primary rat fibroblast cultures were characterized by staining with vimentin to label intermediate filaments (magenta), phalloidin for F-actin filaments (green) and DAPI (gray). Scale bar 50 µm. **(B[ii])** Experimental protocol followed to performed puromycilation assay in isolated synaptosomes coming from neurons, neuron-fibroblast co-cultures or fibroblast conditioned medium (CM). The representative figure has been created with Biorender.

SUPPLEMENTARY FIGURE 3
Puro-Rpl26 PLA performed in synaptosomes from neuron-astrocyte co-cultures confirmed the inhibition of *Rpl26* synaptic local translation in Aβ conditions. Synaptosomes were isolated from 24 DIV vehicle- or Aβ-treated neuron cultures (N) or Aβ-treated neuron-astrocyte co-cultures (NA) in modified Boyden chambers were exposed 2-min with 2 µM puromycin. Anisomycin was used to block translation and no-puromycin treatment (- puromycin) as negative control. Puro-PLA of SNAP25 (SNAP25 PLA) representative micrographs are shown in **(A[i])**. Scale bar 2 µm. Percentage (%) of the colocalization analysis obtained from SNAP25 PLA puncta with **(A[ii])** synaptophysin-Homer+ synapses (obtained as a result of the double colocalization of Syn+ and Homer+ puncta), **(A[iii])** Homer postsynapses (Homer+) and **(A[iv])** Syn+ presynapses (Syn+). Each individual condition was normalized to the total amount of Syn-Homer+ synapses, Homer+ postsynapses or Syn+ presynapses, respectively. Puro-PLA of Rpl26 (Rpl26 PLA) representative micrographs are shown in **(B[i])**. Scale bar 2 µm. Percentage (%) of the colocalization analysis obtained from Rpl26 PLA puncta with **(B[ii])** synaptophysin-Homer+ synapses, **(B[iii])** Homer+ postsynapses and **(B[iv])** Syn+ presynapses. Each individual condition was normalized to the total amount of Syn-Homer+ synapses, Homer+ postsynapses or Syn+ presynapses, respectively. Data were analyzed in 5 independent cultures and analyzed by two-way ANOVA, ns not significant. All graphs represent mean ± SEM and the levels of no-puromycin negative controls are shown for descriptive purposes.

SUPPLEMENTARY FIGURE 4
Astrocyte-secreted factors do not have an impact on global synaptic SNAP25 and Rpl26 proteins. Total protein of SNAP25 and Rpl26 has been quantified within isolated synaptosomes coming from 24 DIV neuron or neuron-astrocyte co-cultures in basal condition. Percentage (%) of the colocalization of SNAP25 protein puncta with **(A[i])** synaptophysin-PSD95+ synapses (obtained as a result of the double colocalization of Syn+ and PSD95+ puncta), **(A[ii])** PSD95 postsynapses (PSD95+) and **(A[iii])** Syn+ presynapses (Syn+). Each individual condition was normalized to the total amount of Syn-Homer+ synapses, PSD95+ postsynapses or Syn+ presynapses, respectively. Representative micrographs are shown in **(A[iv])**. Scale bar 2 µm. Percentage (%) of the colocalization of Rpl26 protein puncta with **(B[i])** synaptophysin-PSD95+ synapses, **(B[ii])** PSD95+ postsynapses and **(A[iii])** Syn+ presynapses. Each individual condition was normalized to the total amount of Syn-PSD95+ synapses, PSD95+ postsynapses or Syn+ presynapses, respectively. Representative micrographs are shown in **(B[iv])**. Scale bar 2 µm. Data were analyzed in 5 independent cultures and analyzed by unpaired *t*-test, ns not significant.

References

Baleriola, J., Walker, C. A., Jean, Y. Y., Crary, J. F., Troy, C. M., Nagy, P. L., et al. (2014). Axonally synthesized ATF4 transmits a neurodegenerative signal across brain regions. *Cell* 158, 1159–1172. doi: 10.1016/j.cell.2014.07.001

Batista, A. F. R., Martínez, J. C., and Hengst, U. (2017). Intra-axonal synthesis of SNAP25 is required for the formation of presynaptic terminals. *Cell Reports* 20, 3085–3098. doi: 10.1016/j.celrep.2017.08.097

Bernard, C., Exposito-Alonso, D., Selten, M., Sanalidou, S., Hanusz-Godoy, A., Aguilera, A., et al. (2022). Cortical wiring by synapse type–specific control of local protein synthesis. *Science* 378:eabm7466. doi: 10.1126/science.abm7466

Dahlgren, K. N., Manelli, A. M., Blaine Stine, W., Baker, L. K., Krafft, G. A., and Ladu, M. J. (2002). Oligomeric and fibrillar species of amyloid-β peptides differentially affect neuronal viability. *J. Biol. Chem.* 277, 32046–32053. doi: 10.1074/jbc.M201750200

Enam, S. U., Zinshteyn, B., Goldman, D. H., Cassani, M., Livingston, N. M., Seydoux, G., et al. (2020). Puromycin reactivity does not accurately localize translation at the subcellular level. *eLife* 9, 1–34. doi: 10.7554/ELIFE.60303

Farizatto, K. L. G., and Baldwin, K. T. (2023). Astrocyte-synapse interactions during brain development. *Curr. Opin. Neurobiol.* 80:102704. doi: 10.1016/j.conb.2023.102704

Fusco, C. M., Desch, K., Dörrbaum, A. R., Wang, M., Staab, A., Chan, I. C. W., et al. (2021). Neuronal ribosomes exhibit dynamic and context-dependent exchange of ribosomal proteins. *Nat. Commun.* 12:6127. doi: 10.1038/s41467-021-26365-x

Gamarra, M., Blanco-Urrejola, M., Batista, A. F. R., Imaz, J., and Baleriola, J. (2020). Object-based analyses in FIJI/ImageJ to measure local RNA translation sites in neurites in response to Aβ1-42 oligomers. *Front. Neurosci.* 14:547. doi: 10.3389/fnins.2020.00547

Gamarra, M., de la Cruz, A., Blanco-Urrejola, M., and Baleriola, J. (2021). Local translation in nervous system pathologies. *Front. Integr. Neurosci.* 15:689208. doi: 10.3389/fnint.2021.689208

Garrett, A. M., and Weiner, J. A. (2009). Control of CNS synapse development by γ-protocadherin-mediated astrocyte-neuron contact. *J. Neurosci.* 29, 11723–11731. doi: 10.1523/JNEUROSCI.2818-09.2009

Graber, T. E., Hébert-Seropian, S., Khoutorsky, A., David, A., Yewdell, J. W., Lacaille, J. C., et al. (2013). Reactivation of stalled polyribosomes in synaptic plasticity. *Proc. Natl. Acad. Sci. USA* 110, 16205–16210. doi: 10.1073/pnas.1307747110

Hafner, A.-S., Donlin-Asp, P. G., Leitch, B., Herzog, E., and Schuman, E. M. (2019). Local protein synthesis is a ubiquitous feature of neuronal pre-and postsynaptic compartments. *Science* 364:eaau3644. doi: 10.1126/science.aau3644. PMID: 31097639

Holt, C. E., Martin, K. C., and Schuman, E. M. (2019). Local translation in neurons: visualization and function. *Nat Struct Mol Biol* 26, 557–566. doi: 10.1038/s41594-019-0263-5

Irala, D., Wang, S., Sakers, K., Nagendren, L., Ulloa Severino, F. P., Bindu, D. S., et al. (2024). Astrocyte-secreted neurocan controls inhibitory synapse formation and function. *Neuron* 112, 1657–1675.e10. doi: 10.1016/j.neuron.2024.03.007

Jung, J., Ohk, J., Kim, H., Holt, C. E., Park, H. J., and Jung, H. (2023). mRNA transport, translation, and decay in adult mammalian central nervous system axons. *Neuron* 111, 650–668.e4. doi: 10.1016/j.neuron.2022.12.015

Khan, M., and Gasser, S. (2016). Generating primary fibroblast cultures from mouse ear and tail tissues. *J. Vis. Exp.* 2016:53565. doi: 10.3791/53565

Leal, G., Comprido, D., and Duarte, C. B. (2014). BDNF-induced local protein synthesis and synaptic plasticity. *Neuropharmacology* 76, 639–656. doi: 10.1016/j.neuropharm.2013.04.005

Leung, K. M., Van Horck, F. P. G., Lin, A. C., Allison, R., Standart, N., and Holt, C. E. (2006). Asymmetrical β-actin mRNA translation in growth cones mediates attractive turning to netrin-1. *Nat. Neurosci.* 9, 1247–1256. doi: 10.1038/nn1775

Liu-Yesucevitz, L., Bassell, G. J., Gitler, A. D., Hart, A. C., Klann, E., Richter, J. D., et al. (2011). Local RNA translation at the synapse and in disease. *J. Neurosci.* 31, 16086–16093. doi: 10.1523/JNEUROSCI.4105-11.2011

Lynn, C. W., Holmes, C. M., and Palmer, S. E. (2024). Heavy-tailed neuronal connectivity arises from Hebbian self-organization. *Nat. Phys.* 20, 484–491. doi: 10.1038/s41567-023-02332-9

Martin, K. C. (2004). Local protein synthesis during axon guidance and synaptic plasticity. *Curr. Opin. Neurobiol.* 14, 305–310. doi: 10.1016/j.conb.2004.05.009

Murai, K. K., Nguyen, L. N., Irie, F., Yu, Y., and Pasquale, E. B. (2003). Control of hippocampal dendritic spine morphology through ephrin-A3/EphA4 signaling. *Nat. Neurosci.* 6, 153–160. doi: 10.1038/nn994

Murai, K. K., and Pasquale, E. B. (2011). Eph receptors and ephrins in neuron-astrocyte communication at synapses. *Glia* 59, 1567–1578. doi: 10.1002/glia.21226

Perea, G., Navarrete, M., and Araque, A. (2009). Tripartite synapses: astrocytes process and control synaptic information. *Trends Neurosci.* 32, 421–431. doi: 10.1016/j.tins.2009.05.001

Pyka, M., Busse, C., Seidenbecher, C., Gundelfinger, E. D., and Faissner, A. (2011). Astrocytes are crucial for survival and maturation of embryonic hippocampal neurons in a neuron-glia cell-insert coculture assay. *Synapse* 65, 41–53. doi: 10.1002/syn.20816

Schmidt, E. K., Clavarino, G., Ceppi, M., and Pierre, P. (2009). SUnSET, a nonradioactive method to monitor protein synthesis. *Nat. Methods* 6, 275–277. doi: 10.1038/nmeth.1314

Sun, W., Liu, Z., Jiang, X., Chen, M. B., Dong, H., Liu, J., et al. (2024). Spatial transcriptomics reveal neuron–astrocyte synergy in long-term memory. *Nature* 627, 374–381. doi: 10.1038/s41586-023-07011-6

Ullian, E. M., Sapperstein, S. K., and Christopherson, K. S. (2001). Control of synapse number by glia. *Science* 291, 657–661. doi: 10.1126/science.291.5504.657. PMID: 11158678

Walicke, P., Cowan, W. M., Ueno, N., Baird, A., and Guillemin, R. (1986). Fibroblast growth factor promotes survival of dissociated hippocampal neurons and enhances neurite extension. *Proc. Natl. Acad. Sci. USA* 83, 3012–3016. doi: 10.1073/pnas.83.9.3012

Wong, H. H. W., Watt, A. J., and Sjöström, P. J. (2024). Synapse-specific burst coding sustained by local axonal translation. *Neuron* 112, 264–276.e6. doi: 10.1016/j.neuron.2023.10.011

Yoon, B. C., Jung, H., Dwivedy, A., O'Hare, C. M., Zivraj, K. H., and Holt, C. E. (2012). Local translation of extranuclear Lamin B promotes axon maintenance. *Cell* 148, 752–764. doi: 10.1016/j.cell.2011.11.064

Zhang, X.-H., and Poo, M.-M. (2002). Localized synaptic potentiation by BDNF requires local protein synthesis in the developing axon. *Neuron* 36, 675–688. doi: 10.1016/S0896-6273(02)01023-1