

Autism spectrum disorder: insights into convergent mechanisms from transcriptomics

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Abstract | Heredity has a major role in autism spectrum disorder (ASD), yet underlying causal genetic variants have been defined only in a fairly small subset of cases. The enormous genetic heterogeneity associated with ASD emphasizes the importance of identifying convergent pathways and molecular mechanisms that are responsible for this disorder. We review how recent transcriptomic analyses have transformed our understanding of pathway convergence in ASD. In particular, deep RNA sequencing coupled with downstream investigations has revealed that a substantial fraction of autistic brains possess distinct transcriptomic signatures. These signatures are in part a consequence of altered neuronal activity and have a particular impact on pre-mRNA alternative splicing patterns.

Autism spectrum disorder (ASD) encompasses a highly heterogeneous set of neurodevelopmental conditions that are semantically and clinically grouped because they share common behavioural hallmarks, including abnormal social interactions, impaired communication skills and repetitive or stereotypical behaviour. A high concordance rate of ASD in monozygotic twins^{1,2} and increased recurrence among siblings³ confirmed that the aetiology of ASD bears a strong genetic component, with heritability estimated to be ~50%^{4,5}. However, the genetic causes of ASD are varied and often complex, even in individual cases. This genetic complexity, paired with variable presentation, including between monozygotic twins, can complicate diagnosis and has hindered the development of therapies. Moreover, even for disorders such as fragile X syndrome and Rett syndrome, for which ASD-causing genetic alterations have been identified, a successful treatment has not yet been developed.

On the basis of clinical presentation, 4–5% of ASD cases can be defined as carrying mutations in known ASD-causing loci, such as fragile X or Rett syndrome⁶ (BOX 1). About 25% of ASD individuals have been found to carry variations that can be detected cytogenetically, such as chromosome 15q duplications (Dup15q syndrome; BOX 1) or by sequence-based analyses of known or candidate ASD genes. However, for ~75% of patients with ASD the causes are unknown⁶. This majority of cases, referred to as having nonsyndromic idiopathic ASD, is the focus of this Review.

Despite the profound genetic complexity of ASD, enrichment analyses have revealed that genes significantly

associated with this disorder contribute to brain development, neuronal activity, signalling and transcription regulation^{7–11}. Moreover, synaptic function, translation and WNT signalling have been identified as three major cellular pathways commonly affected by mutations in diverse ASD-related genes^{12–14} (FIG. 1), and altered WNT pathway signalling is considered to have a key role in the aetiology of autism^{15,16}. The convergence of dozens of risk genes on common molecular pathways can help explain how a genetically heterogeneous population of individuals displays similar behavioural features. Moreover, during fetal and childhood development, and throughout adult life, environmental factors and neuronal activity modulate all three of these pathways^{9,17}. Thus, in ASD, altering these sensitive, dynamic processes that normally establish and sustain neuronal functions can activate feedback and feedforward mechanisms that further exacerbate any disturbances in cellular regulatory networks and contribute to the progression and pathogenesis of ASD.

These observations raise the following question: which processes controlled by these pathways might contribute to ASD pathogenesis? Indeed, the three main pathways implicated control large programmes of cellular events. For example, on their own or together, defective synaptogenesis or synaptic function, altered WNT signalling during brain development and misregulated translation in neurons can all influence neuronal circuit formation and activity^{18–20}, which in turn can trigger signalling cascades by transmitting action potentials. Ultimately, changes in neuronal activity modulate specific transcriptional programmes comprising

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Box 1 | Syndromic ASD and transcriptomic analyses

Limited transcriptomic analyses of human cultured neurons or post-mortem brains have been performed for the following syndromes associated with autism spectrum disorder (ASD).

Fragile X syndrome

Fragile X syndrome (FXS) is responsible for 1–2% of all diagnosed cases of ASD¹²⁹. FXS occurs when a CCG triplet repeat exceeds 200 or more copies in the 5' untranslated region of the *FMR1* gene. This triplet repeat expansion causes epigenetic silencing of *FMR1* gene expression as well as RNA toxic gain of function, toxic repeat-associated non-ATG translation and somatic instability. The *FMR1* gene encodes fragile X mental retardation protein 1 (FMRP), an RNA-binding protein that regulates the transport, stability and local protein synthesis of hundreds of mRNAs in the mammalian brain¹³⁰. Analyses of *Fmr1* loss-of-function mouse models have confirmed the importance of FMRP in neuronal development and function as well as behaviour but cannot assess the possible additional contribution of trinucleotide repeat expansion mechanisms¹³¹.

Analyses of the human FXS neural transcriptome have been limited. A microarray-based analysis of the transcriptomes of neurons differentiated from induced pluripotent stem cells (iPSCs) from patients with FXS revealed aberrant regulation of neural differentiation and axon guidance genes¹³².

Rett syndrome

X-Linked Rett syndrome is typically caused by mutations in methyl-CpG binding protein 2 (MECP2)¹³³ and leads to ASD in 61% of affected girls¹³⁴. Boys with Rett syndrome frequently die shortly after birth. MECP2 binds DNA at methylated cytosines and is thought to have a role in chromatin remodelling and thus transcriptional regulation^{135,136}. The consequences of MECP2 loss have been explored extensively in mice lacking *Mecp2*, which display the neurobiological deficits seen in patients with Rett syndrome¹³⁷.

Transcriptomic analyses of post-mortem brain samples of patients with Rett syndrome using RNA sequencing (RNA-seq) and microarrays have revealed downregulation of neurodevelopmental genes and overexpression of neuroinflammatory genes in the forebrain and cerebellum^{138,139}. One study reported an overexpression of neuroinflammatory genes¹³⁸, which was not observed in the other study¹³⁹.

Chromosome 15 deletion and duplication syndromes

Deletion and duplication events that affect an imprinted region on chromosome 15q11–13 are responsible for a group of neurodevelopmental disorders associated with ASD, including Prader–Willi syndrome and Angelman syndrome. Of patients with Prader–Willi syndrome¹⁴⁰, 26.7% meet diagnostic criteria for ASD, as do 34% of patients with Angelman syndrome¹³⁴. Duplications of the chromosome 15q11–13.1 region (Dup15q) are associated with an estimated 1–3% of all autism cases¹⁴¹.

Prader–Willi syndrome is thought to be largely due to loss of the paternal SNORD116 small nucleolar RNA (snoRNA) cluster, whereas maternal loss of the UBE3A ubiquitin ligase causes Angelman syndrome¹⁴². Deletion of the *Snord116* cluster in a mouse model causes anxiety¹⁴³ and hyperphagia (excessive hunger)^{143,144}, whereas mice inheriting a mutant *Ube3a* allele from their mother have defective synaptic development¹⁴⁵. A mouse model for paternal Dup15q shows poor social interaction, behavioural inflexibility, abnormal ultrasonic vocalizations and correlates of anxiety^{146,147}.

Because Prader–Willi syndrome causes severe hyperphagia, transcriptomic studies have focused on RNA-seq analyses of the post-mortem hypothalamus, which has key roles in the regulation of appetite and energy homeostasis¹⁴⁸. Consistent with hyperphagia, upregulated expression of orexigenic (appetite-stimulating) genes and decreases in the expression of anorexigenic (appetite-inhibitory) genes were observed. Aside from anorexigenic genes, downregulated genes were largely neuron-specific and were involved in neurogenesis, neurotransmitter release and synaptic plasticity, whereas upregulated genes were normally expressed in microglia and were involved in inflammatory responses. Disruption of alternative splicing, possibly due to roles of snoRNAs in splicing, was also seen¹⁴⁸. RNA-seq analysis of mRNA from differentiated neurons derived from patients with Angelman syndrome or Dup15q found downregulated expression of genes involved in neuronal development, including many autism candidate genes¹⁴⁹.

Glutamate signalling
Molecular pathway that includes the excitatory neurotransmitter glutamate and its synaptic receptors; largely responsible for driving neuronal activity.

hundreds of genes, either by affecting transcription factors directly²¹ or through chromatin remodelling²². Although transcriptional programmes control the overall expression levels of genes relevant to ASD, steps in post-transcriptional RNA processing, particularly alternative splicing, which considerably expands the

repertoires of gene products expressed in the nervous system, are also highly responsive to changes in synaptic function and neuronal activity^{23–27}. Thus, although genetic studies have identified specific cellular pathways that are targeted by mutations in diverse ASD-related genes, complementary approaches are required to capture the consequent changes that occur in downstream processes, such as transcription and pre-mRNA splicing.

Transcriptomic analyses can provide insights into how diverse ASD-causing genetic mechanisms or risk factors might result in shared behavioural outcomes. The advent of microarrays, followed by high-throughput RNA sequencing (RNA-seq), has enabled whole-transcriptome analysis (FIG. 2). These approaches yield information about transcript abundance and can be applied to mRNA and small or long non-coding RNA (lncRNA) transcripts. RNA-seq data can also be used to interrogate the nature and frequency of alternative splicing events in different samples.

In this Review, we highlight how transcriptome profiling in different brain regions has revealed ASD-associated signatures of mRNA and non-coding RNA expression as well as alternative splicing. We also highlight the importance of neuronal activity in the control of these regulatory programmes and discuss how they fit within current models of ASD pathogenesis stemming from genomic investigations. Finally, we discuss new mechanistic insights into convergent molecular mechanisms that have emerged from transcriptomic investigations of ASD and how these mechanisms link major pathways underlying neurodevelopmental disorders.

Gene expression in the ASD brain

Transcriptomic analyses have focused mostly on three brain regions suggested to be implicated in ASD²⁸: the prefrontal cortex, the superior temporal cortex and the cerebellum (FIG. 1; TABLE 1).

mRNA expression

Misregulation of neuronal genes. Microarrays were used first to detect genome-wide differential expression of genes relevant to ASD pathogenesis. The earliest targeted study comparing gene expression in the cerebellum of individuals with ASD and control cases provided evidence for the upregulation of genes involved in glutamate signalling²⁹. In this study, gene expression changes validated by reverse transcription (RT)-PCR and immunoblotting confirmed increased expression of the glutamate transporter excitatory amino acid transporter 1 (EAAT1; also known as SLC1A3) and the AMPA receptor component glutamate receptor 1 (GRIA1) in the cerebellum. Altered glutamate signalling was later functionally linked to ASD³⁰, and excitatory-to-inhibitory imbalance in general has long been hypothesized to underlie ASD³¹. This early study demonstrated that transcriptomic analyses can provide insight into the underlying biology of ASD.

Misregulation of immune genes. Whereas the dysregulation of genes relevant for neuronal function is an expected outcome of transcriptomic analysis of the brains of individuals with ASD, upon surveying a

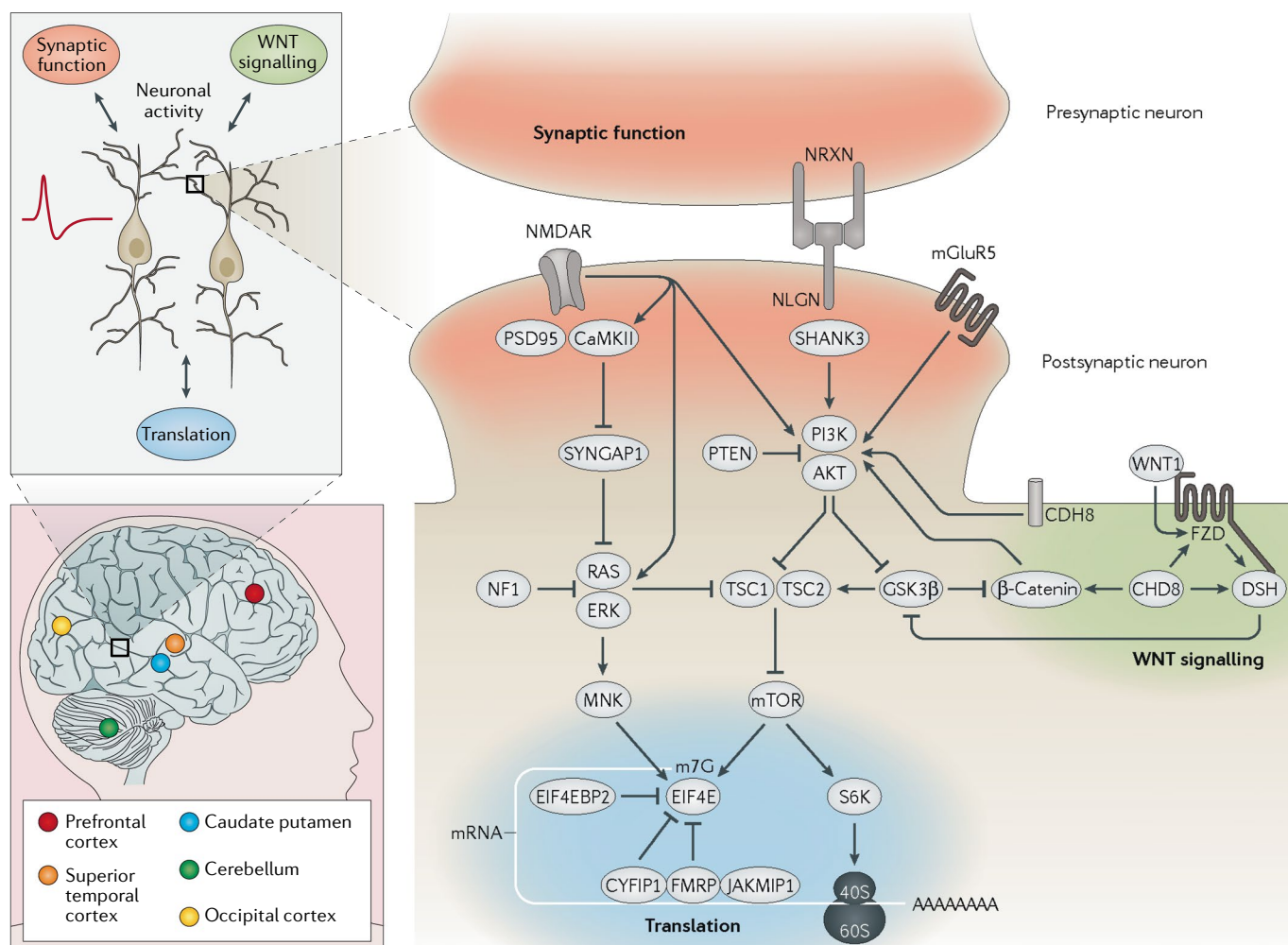


Fig. 1 | Major cellular pathways underlying ASD are interconnected through neuronal activity. Well-established autism spectrum disorder (ASD) risk variants are found in genes that function in three critical cellular pathways: synaptic function, WNT signalling, and translation. These three pathways are highly integrated: WNT signalling controls key transcriptional programmes that affect neuronal maturation and neural circuit formation, which are also dependent on synaptic activity during development; localized translation at the synapse underlies synaptic plasticity and cognition, whereas synaptic translation is stimulated by synaptic activity. Importantly, the three pathways respond to and are affected by neuronal activity. Brain regions that have been surveyed in transcriptomic studies are represented in the bottom left panel. FMRP, fragile X mental retardation protein 1; m7G, 7-methylguanosine.

larger number of genes expressed in the temporal cortex, an early profiling study identified over 150 genes with differential expression in ASD versus control individuals³²; 85% of these genes were upregulated and enriched for pathways mostly involved in the immune response. Subsequent microarray and RNA-seq analyses confirmed this finding but also demonstrated that downregulated genes were enriched for neuronal functions^{33,34}. Although genes genetically associated with ASD generally relate to neuronal rather than immune functions, microglia and astroglia activation was observed in the cerebellum of individuals with ASD, and elevated levels of pro-inflammatory cytokines were found in the cerebrospinal fluid and blood of ASD samples^{35–40}. It is conceivable that upregulation of genes with functions in the immune response corresponds to a secondary effect of neurobiological defects that trigger inflammation in the brain^{33,34}. However, it is also

possible that, in some idiopathic cases, environmental factors causing inflammation might trigger ASD. Indeed, maternal infection during pregnancy, especially in the second trimester, has been evoked as a risk factor for autism^{41–43}. Thus, analysis of transcript levels has revealed that two different biological systems thought to participate in autism pathogenesis — that is, neuronal development and maturation and immune system activation — are both misregulated in the brains of individuals with ASD.

Loss of regional gene expression signatures. In addition to a decrease in the expression of neuronal genes that substantially overlap with those previously identified³², differences in gene expression levels observed between the frontal and the temporal cortex in control individuals were significantly attenuated in ASD³⁴. A later study comparing larger cohorts of ASD and control

Excitatory-to-inhibitory imbalance
Change in the ratio between excitatory (primarily glutamate) and inhibitory (primarily GABA) synaptic transmission that regulates normal brain activity and behaviour.

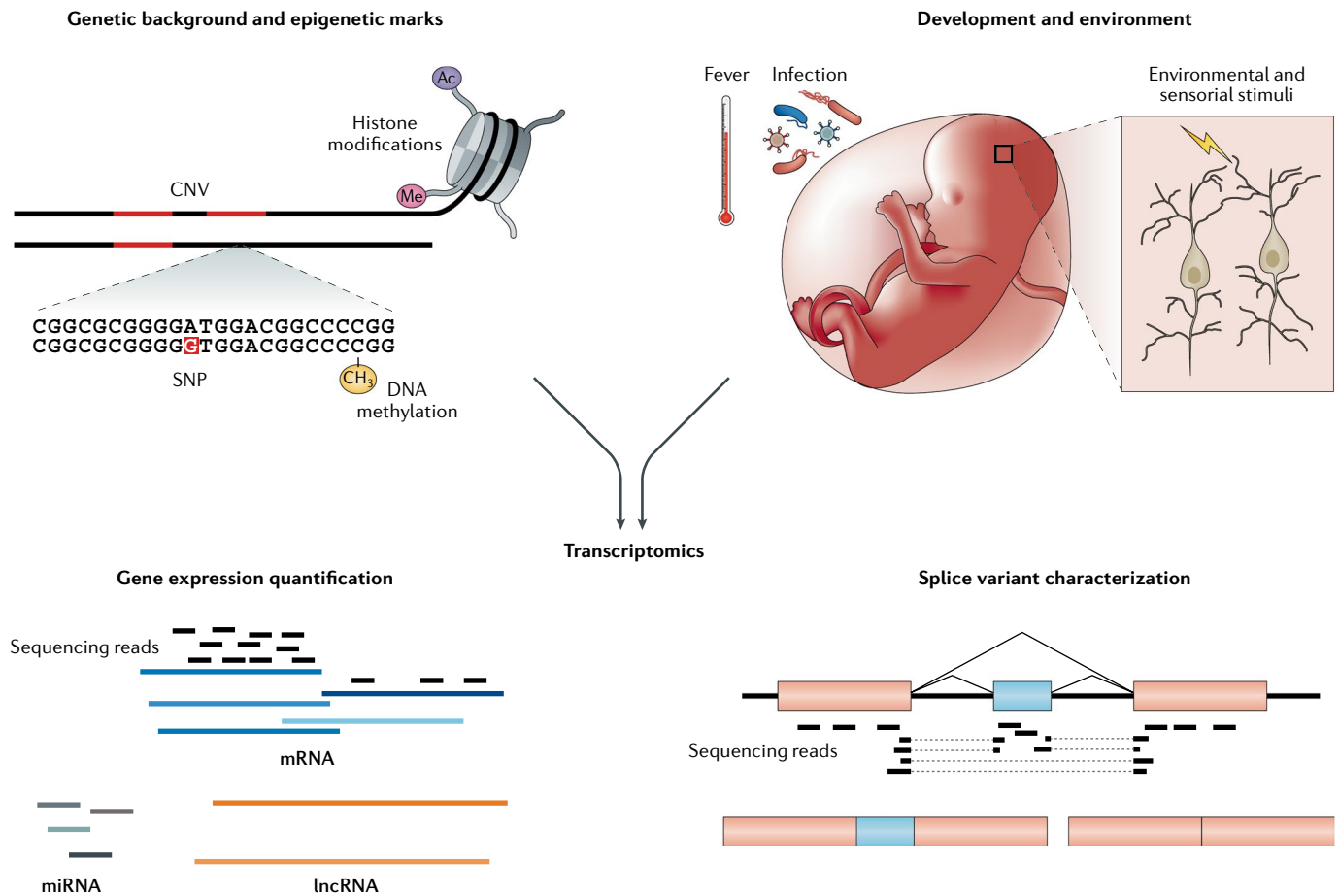


Fig. 2 | Using transcriptomics to assess molecular changes underlying ASD. Transcriptomic analyses measure changes in gene expression and post-transcriptional processing that lie downstream of genetic, epigenetic, environmental and cellular variations. This information can reveal molecular pathways on which distinct and independent factors may converge. Ac, acetylation; ASD, autism spectrum disorder; CNV, copy number variation; lncRNA, long non-coding RNA; Me, methylation; miRNA, microRNA; SNP, single-nucleotide polymorphism.

Glutamatergic neurons
Glutamate-expressing neurons that potentiate neuronal activity.

GABAergic neurons
GABA-expressing neurons that act as inhibitors of neuronal activity.

Corticogenesis
Process during which neural stem cells proliferate and differentiate into glial cells and neurons to generate the cortex during brain development.

Interneurons
GABAergic interneurons are a type of inhibitory neuron that filters neuronal activity and maintains the excitatory-to-inhibitory balance.

individuals confirmed this finding³³. The list of genes displaying a loss in regional expression specificity in ASD comprises markers of neuronal subtype identity (for example, glutamatergic neurons (*SLC17A6*, *CPLX2*, *MET* and *SLC6A7*) and GABAergic neurons (*PVALB* and *SYT2*)), which suggests that cortical neurons may differentiate improperly to acquire an erroneous identity or fail to mature altogether. These observations are particularly important for three reasons: first, disruption of corticogenesis, impaired differentiation of interneurons and miswiring of the neuronal circuitry were observed using functional imaging and post-mortem anatomical analysis of ASD brains^{44,45}; second, anatomical imbalance between the number and/or the positioning of excitatory and inhibitory neurons agrees with the idea that the excitatory-to-inhibitory signalling ratio is often altered in autism⁴⁶; and third, genes with loss of regional specificity are enriched in WNT signalling³³.

Cerebral organoids. One caveat of transcriptomics studies is that the number of cases that can be analysed is several orders of magnitude lower than in genomic analyses, because transcriptomic analyses rely on the availability of post-mortem brain samples. These tissues are

difficult to obtain and reflect only a specific time point in the life of an individual, usually after most developmental processes have been completed. In order to circumvent these challenges, induced pluripotent stem cells were derived from fibroblasts and used to generate cerebral organoids from a small number of individuals exhibiting idiopathic autism and macrocephaly, a trait associated with increased symptom severity, as well as their unaffected parents⁴⁷. This approach allowed functional and transcriptional profiling of brain-like tissues at different time points during development. The study revealed altered production of interneurons (FIG. 3), a process thought to underlie ASD⁴⁵, in organoids derived from individuals with ASD. Gene expression profiling by RNA-seq implicated overexpression of the transcription factor-encoding gene *FOXP1* in this developmental defect. Intriguingly, the number of differentially expressed protein-coding genes in developing organoids derived from this subset of individuals with ASD was much greater than in any post-mortem analysis of postnatal tissues to date (2,433 differentially expressed genes in organoids versus 1,097 differentially expressed genes for all post-mortem studies when considered together). This finding may be

Table 1 | Brain regions and approaches used to survey transcriptome changes in ASD

Brain region	Method	Refs
mRNA expression		
Prefrontal cortex	Microarray	29,34,150
Prefrontal cortex	RNA-seq	33,72,151
Superior temporal cortex	Microarray	32,34
Superior temporal cortex	RNA-seq	33
Cerebellum	Microarray	10,29
Cerebellum	RNA-seq	33
Caudate putamen	Microarray	29
Occipital cortex	RNA-seq	151
Cerebral organoids	RNA-seq	47
MicroRNA expression		
Prefrontal cortex	RNA-seq	68
Superior temporal cortex	Microarray	66,67
Superior temporal cortex	RNA-seq	68
Cerebellum	RT-qPCR	64
Non-coding RNA expression		
Prefrontal cortex	Microarray	80
Prefrontal cortex	RNA-seq	33,68
Superior temporal cortex	Microarray	66
Superior temporal cortex	RNA-seq	33
Cerebellum	Microarray	80
Cerebellum	RNA-seq	33
Alternative splicing		
Prefrontal cortex	RNA-seq	33,151
Superior temporal cortex	RNA-seq	33,34,109
Cerebellum	RNA-seq	33
Occipital cortex	RNA-seq	151

RNA-seq, RNA sequencing; RT-qPCR, reverse transcription quantitative PCR.

in line with the critical importance of developmental stages in the aetiology of autism and reflect in particular the observation that organoids model early embryonic development⁴⁸ whereas post-mortem samples are generally collected from adults. Technical issues such as organoid heterogeneity, especially in the number of forebrain neurons detected in cultures^{49,50}, and differences between methods for profiling gene expression patterns may also contribute to variability in the detection of transcriptomic changes between (and within) organoids compared with post-mortem samples. Nevertheless, these emerging techniques will likely complement profiling of post-mortem tissues in the future and provide important information on the cellular defects accompanying changes in gene regulatory programmes.

MicroRNA expression

During nervous system development, some microRNAs (miRNAs) are dynamically regulated and display cell type or regional specificity in the brain^{51–53}. Examples of this include miR-124 and miR-9, which are enriched in differentiating and mature neurons⁵³. Functional studies have shown that both of these miRNAs^{54–57}, as well as

others^{58,59}, are important for neurogenesis and neuronal maturation. Therefore, miRNAs are involved in developmental processes that are thought to be disrupted in autism^{60,61}. More generally, the recent observation that local maturation of miR-181a at the synapse reduces translation of one of its target transcripts in response to neuronal activity⁶² strongly suggests that miRNAs are involved in synaptic function. Most genome-wide association study-based analyses have focused on protein-coding regions of the genome and have therefore largely overlooked miRNA genes. miRNA analysis in transcriptomic studies may thus help reveal whether their misregulation contributes to or underlies ASD.

Initially assessed by quantitative RT-PCR (RT-qPCR) using probes against 466 miRNAs, which correspond to approximately 25% of the total number of currently annotated human miRNAs⁶³, the levels of 28 miRNAs were shown to markedly differ in cerebellar tissue from individuals with ASD relative to control individuals⁶⁴. Interestingly, one of these is the brain-specific miR-128, which controls cortical neurogenesis⁶⁵. However, the signature of misregulated miRNAs was not uniform across cases, and no miRNA displayed consistent expression changes in more than 2 of 13 ASD samples; most were differentially expressed only in a single individual with ASD⁶⁴.

A more comprehensive survey using microarrays revealed a consistent pattern of misregulation for six miRNAs across ten individuals with ASD⁶⁶. Interestingly, predicted targets of miRNAs that were differentially expressed in many of the ASD cases compared with control individuals were enriched for genes with functions related to neuronal biology, cell cycle and cell signalling. The pathways in which these target genes are involved substantially overlap those previously identified when analysing differential mRNA expression^{32,34}, as well as in genomic studies⁶⁶. Another study in which microarrays were employed found that miRNAs that are differentially expressed between two regions of the temporal cortex in control individuals do not display detectable differences in expression levels between the same regions in autistic brains⁶⁷, similar to the aforementioned observation of attenuated region-differential expression patterns involving mRNAs^{33,34}.

A recent study, in which small RNA species were enriched before sequencing, uncovered 58 miRNAs that displayed significant expression changes in the frontal and temporal cortex of individuals with ASD⁶⁸. Predicted targets of differentially expressed miRNAs were significantly enriched in genes associated with ASD (for example, *CUL3*, *DYRK1A* and *TSC1*) and implicated in neuronal functions (for example, *DLGAP1*, *RGS4* and *UNC13A*). RNA-seq analysis of polyadenylated RNAs from this cohort revealed a negative correlation between differentially expressed miRNAs and the transcript levels of their predicted targets^{33,68}, suggesting that observed changes in miRNA levels are likely functional. The fact that miRNAs misregulated in ASD are functionally related suggests that a common mechanism underlies the control of their expression and that differential miRNA expression profiles could define subsets of ASD cases in a manner similar to that proposed

Cerebral organoids

Tissues grown in vitro from embryonic stem cells or induced pluripotent stem cells under conditions that promote the generation of differentiated, cortex-like structures (including neurons) to model brain development.

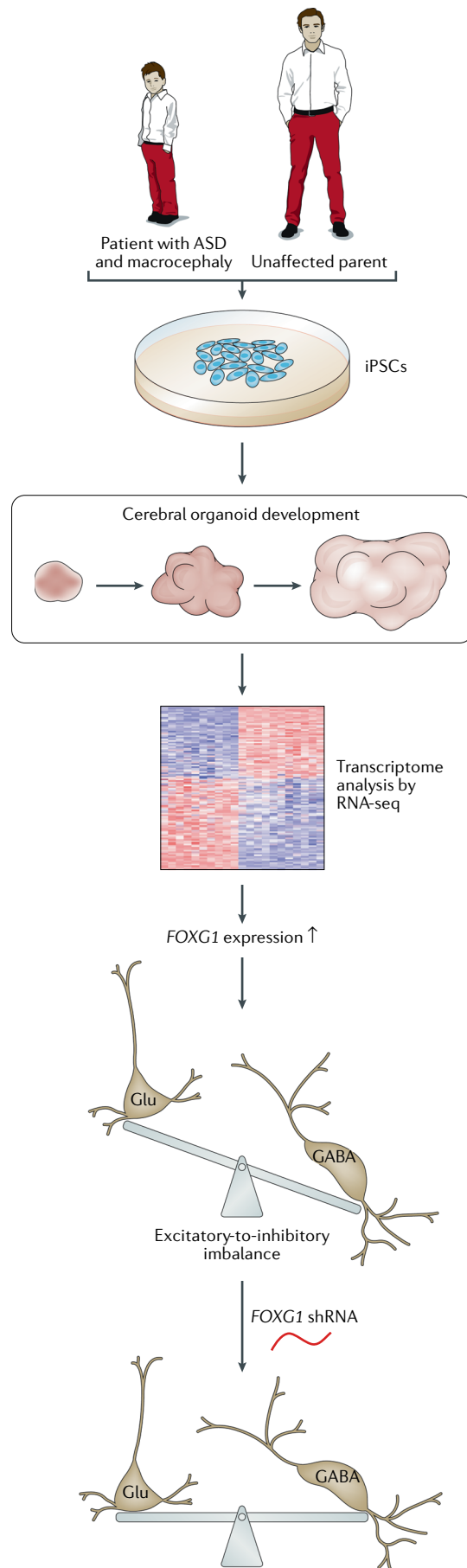


Fig. 3 | Cortical organoids grown in vitro to model human brain development reveal an excitatory-to-inhibitory imbalance in ASD. Induced pluripotent stem cells (iPSCs) produced from patients with autism spectrum disorder (ASD) and macrocephaly as well as from unaffected parents were differentiated into telencephalic neurons using a free-floating 3D culture method. Transcriptome analysis of organoids at different stages of development revealed early increased expression of *FOXG1* in samples from patients with ASD. Increased proliferation and differentiation of GABAergic neurons was observed without changes in glutamatergic (Glu) neurons, causing an imbalance in the Glu:GABA neuron ratio. Overproduction of GABAergic cells was partially attributable to elevated *FOXG1* gene expression, as evidenced by knockdown of *FOXG1*, which rescued the phenotype. RNA-seq, RNA sequencing; shRNA, short hairpin RNA. Figure adapted with permission from REF.⁴⁷, Cell.

for mRNAs. However, because none of the ASD-related miRNAs reported in the four studies overlapped^{64,66–68}, even though there was some overlap between the brain regions analysed and clinical parameters were comparable, it is likely that the current picture for differential miRNA expression in ASD is only partial. Future assessment using a more thorough annotation of miRNAs, and consistent and sensitive detection methods, will be required to properly define ASD-associated miRNA expression signatures.

Small nucleolar RNA expression

Non-coding RNAs include several classes of transcripts other than miRNAs, including small nucleolar RNAs (snoRNAs). To date, snoRNAs are best known for their roles in RNA modification, in particular in the maturation of other non-coding RNAs, but a contribution to brain-specific processes has also been suggested⁶⁹. Several snoRNAs are enriched in the brain, including *SNORD115* and *SNORD116* (REF.⁷⁰). Both of these snoRNAs are associated with Prader–Willi syndrome, which is characterized by cognitive impairment, absence of speech and other behavioural manifestations, such as abnormally increased appetite (hyperphagia). In mice, deficiency of *Snord116* leads to motor learning deficiencies, anxiety-related behaviour and increased food intake, whereas loss of *Snord115* alters serotonin-mediated behaviour⁷¹.

In light of emerging roles for snoRNAs in neuronal processes, Ander et al. surveyed the expression of 2,216 snoRNAs in the temporal cortex of ASD and control brains and found that the levels of 6 snoRNAs changed markedly. Of these, only *SNORA11c* is enriched in the brain (according to GeneCard), but it is expressed at low levels and its function has not yet been established. Interestingly, in an RNA-seq analysis of gene expression changes from ribosomal RNA (rRNA)-depleted total RNA samples, six of eight of the most significantly differentially expressed transcripts between ASD and control samples were snoRNAs⁷². One of these, *SNORA74B*, which suppresses mTOR signalling⁷³, was found to be downregulated in ASD samples. This observation is of considerable interest because increased mTOR signalling has been observed in the context of ASD⁷⁴.

Long non-coding RNA expression

A large fraction of the transcribed genome comprises lncRNAs. Although the possible functional significance of the vast majority of these transcripts is unclear, a growing number of studies have provided evidence that specific lncRNAs have important roles in diverse cellular processes, including transcription and translational control⁷⁵. lncRNAs exhibit a higher degree of tissue-specific expression than mRNAs, and a large number of them are restricted to the brain⁷⁶. The evolution of primate-specific lncRNAs and the presence of gene expression modules enriched for synaptic functions⁷⁷ that include lncRNAs are additional indications that this class of RNA may have widespread roles in the brain and contribute to the aetiology of ASD^{33,78,79}.

An initial study using microarrays on a limited number of samples showed that gene expression changes in ASD were not limited to mRNAs or miRNAs, as the levels of 225 lncRNA transcripts showed pronounced changes in the cerebellum or the cortex of individuals with ASD⁸⁰. Intriguingly, the number of differentially expressed lncRNAs between the cortex and cerebellum was almost six times lower in the brain of individuals with ASD than in controls. This loss of region-differential expression of lncRNAs mirrors that reported for miRNAs and mRNAs³⁴. RNA-seq analysis of a larger cohort confirmed this loss in regional patterning, specifically between the frontal and temporal cortex³³. This latter study also reported ASD-associated changes in 60 lncRNAs in the cortex but, in contrast to the microarray results⁸⁰, no changes in the cerebellum of ASD samples³³. The observation that regional patterning is lost for mRNAs, miRNAs and non-coding RNAs likely reflects that fundamental developmental processes are deregulated in ASD. Important neurodevelopmental defects such as those mentioned above (for example, altered cortical layering and interneuron maturation, discussed above) that lead to changes in neuronal subtype identity, total numbers, ratios and distributions could account for the attenuated transcriptomic signatures detected between brain regions.

Globally, lncRNAs altered in ASD are expressed at higher levels in the brain relative to other tissues, and many are primate-specific. The lower number of lncRNAs with differential expression detected by RNA-seq³³ compared with microarray⁸⁰, and the lack of overlap between differentially expressed lncRNAs reported by the two studies, might be due, at least in part, to differences in detection sensitivities and calling stringencies between the methods used⁷⁶. In addition, lncRNAs are often expressed at low levels, which complicates their annotation and quantification⁷⁶. Further investigation using deeper sequencing and improved analysis methods, as well as orthogonal methods such as RT-qPCR and northern blotting to validate RNA-seq analyses, are needed to confirm possible links between lncRNAs and ASD pathogenesis.

Nevertheless, these analyses illuminate possible cross-talk between different layers of gene regulation. Indeed, 9 of the differentially expressed lncRNAs in ASD interact with the fragile X mental retardation protein 1 (FMRP; encoded by *FMR1*) and 20 interact with miRNA–protein

complexes³³. Two of the lncRNAs that interact with miRNA processing components are primate-specific and developmentally regulated³³, but their function remains to be defined. Interestingly, at least one miRNA (*hsa_can_1002-m*), the expression of which is disrupted in ASD, is also specifically expressed in primate brains^{33,68}. *hsa_can_1002-m* targets are enriched for the epidermal growth factor (EGF) and fibroblast growth factor (FGF) signalling pathways, which contribute to nervous system development and neural plasticity and are involved in psychiatric disorders^{81,82}.

The recently identified non-coding RNA genes with differential expression and possible functional implications in the context of ASD likely represent the tip of the iceberg. Indeed, the roles of most brain-specific snoRNAs and lncRNAs remain to be elucidated, and the discovery of new, possibly tissue-specific and/or species-specific non-coding RNA genes with important functions can be expected. A few reports also describe genomic regions from which non-coding antisense transcripts are encoded as genetic risk loci for ASD^{83,84} or as being differentially expressed in ASD⁸⁵. As such, it will be important to monitor sense and antisense non-coding transcript expression in future transcriptome-wide analyses of autistic brains, and detailed investigation of the molecular functions of specific altered non-coding RNAs will be needed in order to assess whether they have possible mechanistic links to the aetiology of ASD.

Alternative splicing in ASD

Alternative splicing affects the processing of transcripts from at least 95% of human protein-coding genes^{86,87} and provides a critical orthogonal layer of gene regulation that is especially prevalent in the nervous system^{88–91}. Major classes of alternative splicing that function in development and that are misregulated in human diseases and disorders, including ASD, are shown in FIG. 4a. The importance of investigating RNA processing, and in particular alternative splicing, in ASD is underscored by the fact that several genes implicated in neurological disorders have RNA-binding activity⁹². These genes include those encoding FMRP, RNA-binding protein fox-1 homologue 1 (RBFOX1; also known as A2BP1), methyl-CpG binding protein 2 (MECP2) and the neural-specific serine/arginine repetitive matrix protein 4 (SRRM4; also known as nSR100). Although FMRP is mostly known for its role in translation and RNA transport, it has also been shown to regulate the splicing of its own transcripts⁹³. Additionally, FMRP has been reported to interact with the RNA-binding protein RBM14 to regulate alternative splicing of at least two genes with important neuronal functions (*Zfyve27* and *Mapt*) in mice⁹⁴. This finding indicates that FMRP could have a more widespread role in the regulation of splicing, although at the time of writing, possible changes in alternative splicing profiles have not yet been addressed at the transcriptomic level in patients with fragile X syndrome or in *Fmr1*-mutant mice. Similarly, although MECP2 is mostly known for its roles in chromatin remodelling, it also binds to RNA with high affinity⁹⁵ and has been reported to interact with Y-box-binding protein 1 (YB1; also known as YBX1) to regulate splicing⁹⁶. In fact,

Cortical layering

Anatomical distribution of neurons into six molecularly distinct layers in the cortex during brain development; this layering is conserved in mammals.

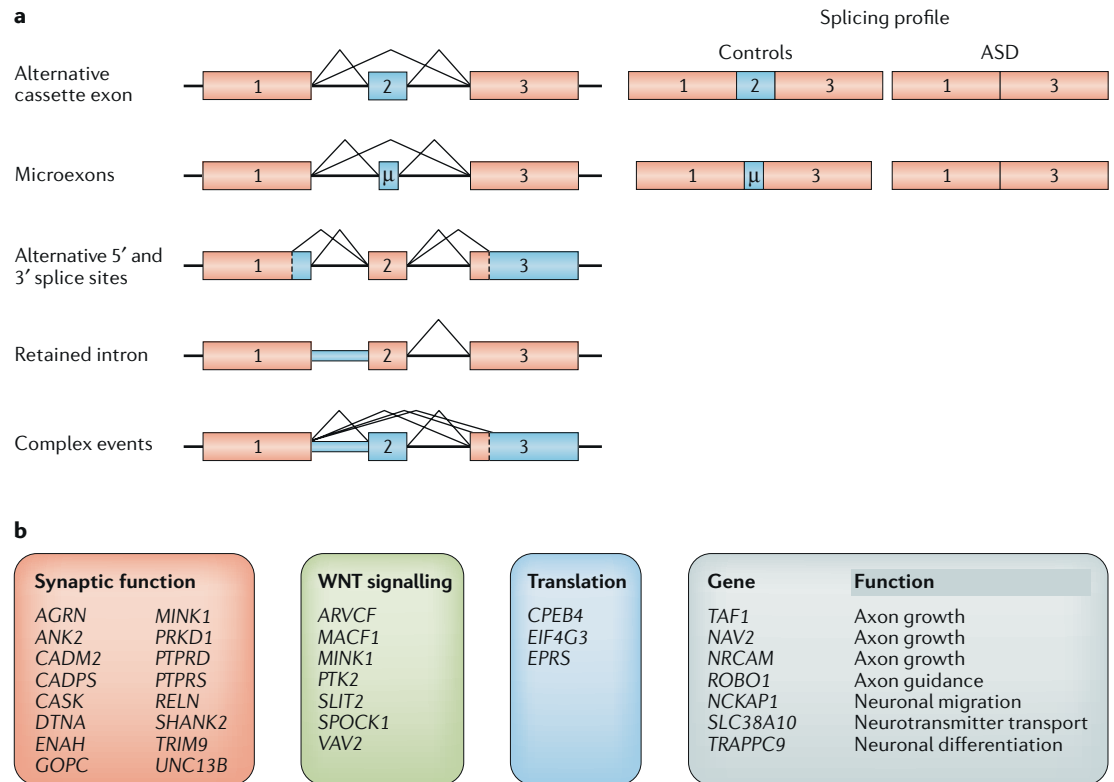


Fig. 4 | Splicing changes in ASD. a | RNA sequencing analyses revealed that cassette exons and microexons (3–27 nucleotides) display more skipping in individuals with autism spectrum disorder (ASD)^{33,34,109}. Alternative 5' and 3' splice sites represent a minority of differential events (205 of 1,127 events) in ASD³³. Changes in other classes of alternative splicing events (retained introns and complex events) have not yet been assessed. **b** | Genes containing microexons with differential inclusion in ASD are listed in the context of molecular hubs of ASD pathogenesis (coloured boxes) or their roles in neuronal biology (grey box).

deletion of *Mecp2* was recently shown to drive changes in the splicing patterns of dozens of genes in the rat brain⁹⁷. Furthermore, another recent study reported that loss of *Mecp2* in mice results in changes in activity-dependent splicing⁹⁸. Collectively, these observations emphasize the need for a careful analysis of splicing events that are disrupted in autism.

Cassette exon misregulation

RBFOX1 is a splicing regulator that controls hundreds of cassette exons^{99–101} and has been genetically associated with ASD^{10,102–104}. In addition to its role in alternative splicing, it is also known to regulate RNA stability and transport, and all these functions are likely perturbed in individuals in whom this factor is disrupted^{105,106}. Initial transcriptome analyses revealed that *RBFOX1* expression is downregulated in a subset of ASD cases³⁴. Furthermore, RNA-seq analysis of three ASD cases with reduced levels of *RBFOX1* mRNA revealed changes in 212 alternative splicing events; these events are enriched for proximal *RBFOX1* binding sites and therefore many of them are likely direct targets of this factor³⁴. The fact that *RBFOX1* targets are also enriched among ASD risk genes¹⁰ provides independent support for the role of *RBFOX1* in the aetiology of autism. Deletion of *Rbfox1* in the mouse brain causes spontaneous seizures¹⁰⁷, a condition that frequently accompanies ASD¹⁰⁸, but its effect on core behavioural domains of autism, such as social

and repetitive behaviour, which would lend support for a causal role for *RBFOX1* loss in autism, has yet to be investigated.

RBFOX1 targets account for only a small fraction of the total number of splicing events disrupted in autism cases³⁴. Thus, the expression of other splicing regulators is also expected to be altered in ASD. Recently, surveys of alternative splicing in larger sets of ASD and control samples showed that splicing is disrupted in transcripts from a much larger number of genes than initially reported^{33,109}. Genes with alternative splicing changes differed from genes with differential expression, consistent with the observation that these processes represent distinct regulatory layers that have an impact on primarily non-overlapping sets of genes in the nervous system¹¹⁰. Interestingly, although miRNAs were similarly misregulated in the cortex and cerebellum⁶⁸, ASD-associated changes in mRNA, lncRNA and alternative splicing levels were most significant in the cortex^{33,34}. This finding may indicate that regulatory programmes in the cortex are more susceptible to genetic or environmental variations than those in the cerebellum.

Importantly, exons that are skipped in autism are often differentially regulated in neurons, and a significant correlation was found between changes in splicing programmes and the expression levels of specific splicing regulators, including *SRRM4*, *RBFOX1*, the RNA-binding protein *NOVA1* and polypyrimidine

Cassette exons

Exons that can be skipped or included in mRNA transcripts through alternative splicing.

tract-binding protein 1 (PTBP1), in the cortex of individuals with ASD^{33,109}. These analyses thus reveal that expression changes in specific splicing factors that regulate large programmes of alternative splicing in the brain may constitute a common feature of ASD and represent a molecular mechanism by which multiple genes with genetic links to ASD, but that are seemingly functionally disconnected, are coordinated.

Microexon misregulation

The programmes of misregulated splicing events described above largely comprise alternative cassette exons (FIG. 4a). Among this class are microexons (3–27 nucleotides (nt) or <50 nt in length, depending on the definition). Remarkably, the large majority of 3–27 nt alternative microexons are neuron-specific, frame preserving and often conserved throughout the vertebrate lineage^{109,111}. Indeed, alternative microexons represent the most highly conserved class of alternative splicing event detected to date in vertebrate species¹⁰⁹. Most alternative microexons are positively regulated by SRRM4. Others are governed by RBFOX1 and PTBP1, and combinations of all three factors regulate different microexon subsets^{109,111}. Interestingly, alternative microexons are enriched in genes with neuronal-related functions (FIG. 4b), as well as in genes linked to ASD. Strikingly, we have observed that 126 microexons, representing 25% of all detected microexons in the data set, are misregulated in approximately one-third of analysed individuals with ASD¹⁰⁹. By contrast, 5% of cassette exons >27 nt, corresponding to ~800 events, displayed altered splicing levels in the same ASD cases, although in general the degree of misregulation of exons >27 nt was found to be less pronounced than for microexons¹⁰⁹. Moreover, consistent with its role in promoting microexon inclusion, *SRRM4* transcript levels were reduced in these ASD samples, and the degree to which its levels decreased correlated significantly with reduced microexon splicing levels. This correlation suggests that loss of SRRM4 has a causative role in ASD. Importantly, supporting this proposal, *Srrm4* haploinsufficient mice, in addition to recapitulating microexon misregulation, display key hallmark ASD-like features, including altered social behaviours such as avoidance of social contact, as well as sensory hypersensitivity, altered synaptic transmission and cortical layering^{27,112}.

Given their frame-preservation potential, evolutionary conservation and strong dependence on SRRM4 for regulation, it is possible that microexon misregulation has a disproportionately larger effect on brain function than misregulation of neural exons >27 nt. This concept is supported by the observation that a significantly greater fraction of microexons display neuronal activity-dependent changes than longer cassette exons or other classes of alternative splicing events, a finding that is consistent with the additional observation that SRRM4 protein levels rapidly decrease in response to induced neuronal activity²⁷. Strikingly, 61.8% (34 of 55) and 57.1% (48 of 84) of mouse and human activity-regulated microexons, respectively, are also detected as mis-spliced in the brains of individuals with ASD^{27,113}. By contrast, a smaller fraction of longer alternative cassette exons that

change upon depolarization are also mis-spliced in the brains of individuals with ASD (29.2%; 119 of 407 in human; M.Q.-V., R.J.W., S.P.C. and B.J.B., unpublished observations). Misregulation of microexons in individuals with ASD may therefore arise in large part as a consequence of altered neuronal activity. Supporting their important biological roles, disruption of single microexons can lead to pronounced phenotypes¹¹⁴. As examples, loss of a single SRRM4-dependent microexon in the *Unc13* gene has been linked to a neuritogenesis defect¹¹², and loss of another SRRM4-regulated microexon in the lysine demethylase gene *Kdm1a* results in altered neurite formation, hypoexcitability and decreased seizure susceptibility¹¹⁵.

It is important to note that the transcriptomic analyses described in this Review have focused on five brain regions: the prefrontal cortex, the occipital cortex, the superior temporal cortex, the cerebellum and the caudate putamen (FIG. 1). Therefore, it will be of considerable interest to expand these analyses to additional brain regions that have been strongly implicated in autism, including the amygdala, the striatum or basal ganglia and the hippocampus^{28,116}. Moreover, given emerging evidence that individual microexons contribute to neuronal and behavioural phenotypes, it will be very important in the future to more systematically characterize the functions of additional microexons, as well as other alternative splicing events, to determine their individual and combinatorial functions in determining neurological disorder-associated and disease-associated phenotypes.

RNA-binding proteins in ASD aetiology

Considering the fact that the transcriptomic studies presented here all point to a widespread involvement of RNA processing in ASD and that neuronal activity directly regulates RNA levels and splicing of genes associated with ASD, we propose to add RNA processing to the list of molecular pathways unifying ASD pathogenesis (FIG. 5). Three of the best known genes for which mutations are causally linked to or constitute strong risk factors for ASD, *FMRI*, *MECP2* and *RBFOX1*, encode proteins that have RNA-binding activity and functions in RNA processing. These proteins are involved in the other molecular pathways commonly implicated in ASD through the large translational, transcriptional, RNA splicing and/or RNA transport programmes that they regulate (see, for example, REFS^{105,106}; FIG. 5). SRRM4, which also binds to RNA, is downregulated in the brain of many individuals with ASD; this causes the misregulation of a large number of target alternative splicing events in ASD, in particular 3–27 nt microexons, which are enriched in ASD genes¹⁰⁹. Although RBFOX1 function was shown to affect neuronal activity¹⁰⁷, it is not clear whether its transcript or protein levels are modulated by neuronal activity. By contrast, in addition to controlling neuronal activity, MECP2 and FMRP are also regulated by neuronal activity^{117,118}. The same applies to SRRM4 (REF.²⁷), making it a protein of interest in the quest to find molecular hubs of autism (FIG. 5). Finally, it is also worth mentioning that other RNA-binding proteins,

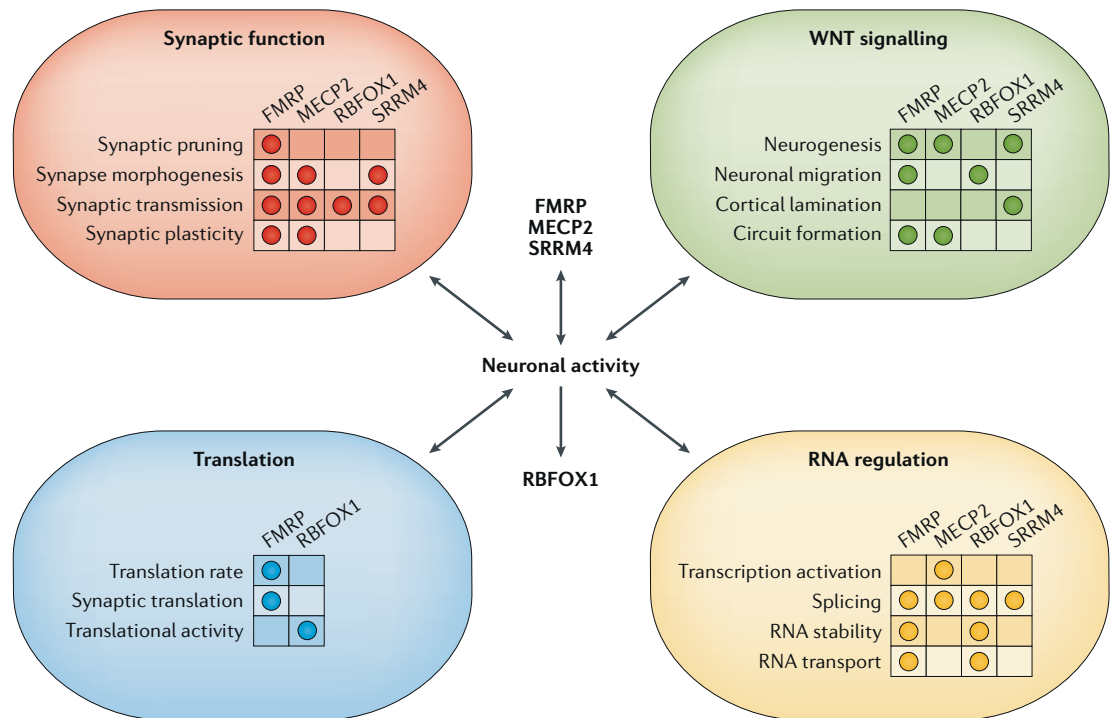


Fig. 5 | **Misregulated RNA processing is a central component of ASD aetiology.** Transcriptomic studies reveal that RNA regulation is tightly connected to autism spectrum disorder (ASD) pathogenesis and that key RNA-binding proteins may act as molecular hubs for ASD through their reciprocal regulatory interaction with neuronal activity. FMRP, fragile X mental retardation protein 1.

including KH domain-containing, RNA-binding, signal transduction-associated protein 2 (also known as SLM1; encoded by *KHDRBS2*) and SLM2 (encoded by *KHDRBS3*), ELAV-like protein 2 (*ELAVL2*) and *ELAVL3*, CUGBP Elav-like family member 4 (*CELF4*), integrator complex subunit 6 (*INTS6*), heterogeneous nuclear ribonucleoprotein H2 (*HNRNPH2*) and *HNRNPU* and cytoplasmic activation and proliferation-associated protein 1 (*CAPRIN1*), have also been genetically associated with ASD¹¹⁹.

Genetic studies have brought further evidence to support the claim that RNA processing is involved in the pathogenesis of ASD. For example, de novo synonymous mutations predicted to affect splicing are enriched in ASD cases¹²⁰. This finding is important because splice variants shape protein–protein interactions in networks of ASD risk factor genes¹²¹. In addition, splicing quantitative trait loci, which contribute to complex traits to an extent comparable to expression quantitative trait loci¹²², are enriched in schizophrenia risk loci, another complex brain disorder sharing some similarities with ASD¹²³.

Conclusions — beyond transcriptomics

The lack of a unifying picture emerging from genetic analyses in the quest for understanding common mechanisms underlying ASD has called for alternative approaches to better describe the pathogenesis of these disorders. Transcriptome analysis affords the opportunity to survey changes resulting from genetic, physiological or environmental insults that may be involved in ASD pathogenesis. Alterations in gene regulatory

programmes and/or feedback loops involving key neuronal processes during and after brain development can thus be detected. These studies have already brought important advances in our understanding of ASD. Some of the most notable findings resulting from transcriptome analyses include that cortical patterning of gene expression is lost in autism; mRNA, lncRNA, miRNA and other non-coding RNA programmes are disrupted in ASD; subsets of mRNAs regulating neuronal processes are consistently downregulated in individuals with ASD from different cohorts; splicing programmes — in particular a highly conserved neuronal microexon programme — are commonly disrupted in ASD; and RNA splicing regulatory programmes disrupted in ASD are responsive to neuronal activity and, conversely, affect neuronal activity.

Transcriptome analysis thus represents a powerful complement to genomic studies. This observation is further exemplified by a recent comparison of the expression profiles of several neuropsychiatric disorders, which revealed a partial overlap of the molecular signatures in ASD, schizophrenia and bipolar disorder¹²⁴, three conditions known to share common genetic risk factors¹²⁵. At present, RNA-seq data from autistic individuals and ASD models are still fairly scarce, but the greater availability of sequencing platforms, the creation of larger tissue banks and the development of increasingly advanced computational tools will become valuable resources in the quest to find additional connections between cellular pathways involved in ASD. In particular, larger sample sizes will help address whether the observations described here also apply to

Splicing quantitative trait loci
Genomic regions that are associated with variations in splicing patterns.

women with ASD. Indeed, only 24.5% (53 of 216) of individuals included in transcriptomic studies were women, owing to the higher prevalence of ASD in men in the general population¹²⁶. Finally, single-cell sequencing, a technique that has now been used in several studies of gene expression in the brain¹²⁷, could also generate insight into the identity and activity of neurons in the brains of individuals with ASD¹²⁸.

In time, complementary approaches to survey the transcriptome may reveal that changes in additional

processes such as RNA editing, transport, localization and stability, as well as functional interactions between RNA transcripts and DNA or proteins, contribute to autism. Future efforts should continue to aim to establish functionally relevant links between transcriptomic changes and genomic analyses so that it is possible to better assess the effects of risk variants on gene expression, splicing and, ultimately, neuronal biology.

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