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The role of regulatory RNA in cognitive evolution

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The evolution of the human brain has resulted in the emergence of higher-order cognitive abilities, such as reasoning, planning and social awareness. Although there has been a concomitant increase in brain size and complexity, and component diversification, we argue that RNA regulation of epigenetic processes, RNA editing, and the controlled mobilization of transposable elements have provided the major substrates for cognitive advance. We also suggest that these expanded capacities and flexibilities have led to the collateral emergence of psychiatric fragilities and conditions.

The path to cognition

Since the divergence of the human and chimpanzee lineages approximately 5 million years ago, the human brain has tripled in size, largely scaling with respect to numbers of neurons and a roughly equal number of glia [1]. Cortical expansion has involved a number of permitting mechanisms including increased cranial capacity (e.g., through genetic alterations [2]), human-specific metabolic changes [3], and an increased number of neuronal cells, possibly through an enhanced capability of progenitor cell divisions in the outer subventricular zone (OSVZ; [4]). Humans are not, however, the only species to possess an enlarged OSVZ [5] and the regulation of this process, if different from other species, is not known. Interestingly, the human prefrontal cortex has disproportionately increased in size and it is this area of the brain that is associated with many of the higher-order cognitive abilities [6,7], which suggests region- and cell-specific advances contributing to cognitive evolution.

Cognitive evolution has likely required not only structural changes to the brain driven by altered developmental programs, but also the evolution of novel mechanisms to enable neural circuitry to be structured and re-wired by experience. This suggests that, in addition to a larger brain with greater complexity and capacity, a concomitant expansion of novel functional components and regulatory systems was necessary to successfully execute these adaptive functions. Here we suggest that the RNA-directed epigenetic mechanisms (see Glossary) that evolved to enable human development – the precise, relatively hard-wired placement of ~100 trillion cells in different muscles, bones and organs – have been expanded and rendered plastic in the human brain by the superimposition of controlled RNA editing and transposon mobilization (Figure 1). We also suggest that this expanded and malleable regulatory architecture is likely perturbed in human neuropsychiatric disorders.

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Glossary

Alternative splicing: a regulatory mechanism by which multiple protein-coding RNA isoforms of the same gene are generated by variations in exon usage. This process can lead to increased genetic diversity by increasing the products derived from a single locus.

***Alu* element**: *Alu* elements are repetitive ~300 bp DNA elements that invaded the primate lineage early in its development. A subset of them are still active and capable of inserting into new genomic locations by relying on the LINE retrotransposon machinery. An *Alu* element harbors a single recognition site for the restriction enzyme *Alu*I, from which these elements have obtained their name. *Alu*s are derived from the 7SL RNA gene and are present only in the primate lineage.

Chromatin: the nuclear complex of DNA, RNA, and protein (histones) that condense to form the chromosome. Chromatin is a multifaceted complex whose roles include facilitating DNA packaging to allow cell division and the regulation of gene expression.

Epigenetic processes: processes that create a chromosomal state change, and thereby frequently alter gene expression, without altering the underlying DNA sequence. These processes include DNA methylation, histone modification, and RNA editing. The effects of many epigenetic processes are heritable.

Hard-wired developmental processes: neurological and behavioral processes that are innate (i.e., instinctual), and therefore, by definition, not learned. In healthy humans these processes include pain avoidance and neurological control of heartbeat and respiration. These processes are critical to development, and disturbances (genetic or otherwise) may result in non-viability of the organism.

Non-coding RNA: non-coding RNAs (ncRNAs) are typically defined as RNA transcripts that lack protein-coding capacity. They can be broadly divided into small (<200 bp) and long ncRNAs (>200 bp). Previously thought to be 'junk', ncRNAs have now been shown to be involved in nearly all fundamental biological pathways.

Proteome: the complete catalogue of proteins that are encoded by a given organism's genome. The proteome is often difficult to completely define, given that many products are produced in specific cellular and environmental contexts.

RNA editing: a process by which the nucleotide sequence of an RNA molecule is post-transcriptionally modified. In humans, there are two major forms of RNA editing, both involving base deamination (removal of an amino group): cytidine to uridine modifications are carried out by the APOBEC enzymes, while adenosine to inosine changes are catalyzed by the ADAR enzymes.

RNA transcription: is the method by which an RNA copy of a DNA sequence is synthesized, either to be utilized as a protein-coding intermediate or as a regulatory non protein-coding RNA.

Soft-wired developmental processes: neurological and behavioral processes that are learned. These processes are adaptable, or 'plastic', to allow experience-dependent development. Such behaviors include the acquisition of fine motor skills associated with artistry.

Transposition (retrotransposition): transfer of a segment of DNA from one chromosomal location to another. In humans, transposition is most frequently mediated by endogenous retroviral (retrotransposition) or DNA transposon molecular machinery. Nearly 50% of the human genome is derived from transposed repetitive elements.

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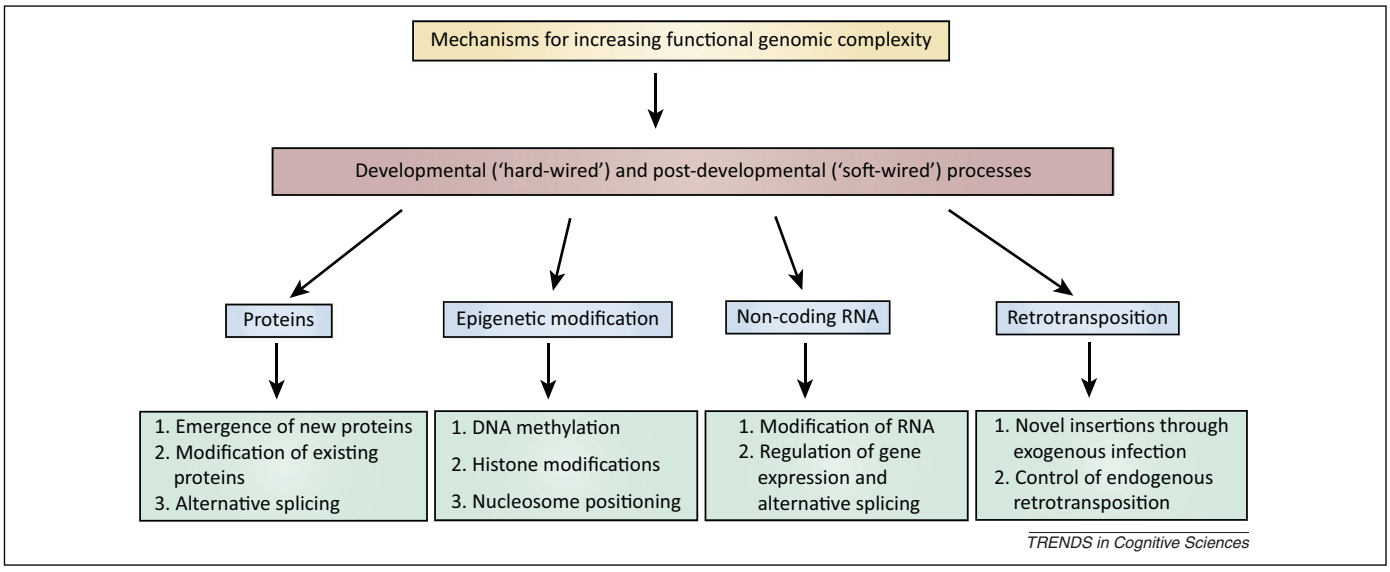

Figure 1. Potential mechanisms for increasing functional genomic complexity. The human brain may have evolved rapidly through a number of mechanisms, including protein innovations, altered epigenetic programs, expansion of regulatory RNAs that direct chromatin modifications, and retrotransposition. Especially relevant for the evolution of higher-order cognition is the dramatic increase in RNA editing of primate-specific Alu sequences and the human-specific isoforms of APOBEC3 that mediate retrotransposition during post-developmental cellular responses.

The proteome: evolving through adaptation

Analysis of the human genome sequence indicates that, contrary to expectations, the number and repertoire of encoded proteins are similar to a wide range of other animals [8,9], although there are significant differences that undoubtedly account for some of the observed variation between species. New protein-coding genes can evolve through duplication, loss, and rearrangement processes [10] and, as studies investigating orphan genes indicate, *de novo* from non-coding sequences [11]. In the case of gene loss, particular genes may have lost their original function either due to a lack of necessity, such as various olfactory-related genes, or to allow relaxation of limiting functions, such as *MYH16* [12], which is involved in cranial expansion.

It is perhaps unsurprising that many protein-coding genes and promoter regions that have undergone recent and accelerated positive selection are involved in neural function [12–14]. For example, accelerated regions include those that have been shown to influence brain size (e.g., microcephaly-related genes [12]), neural development (e.g., *AHI1* and *SHH* [12]) and language (e.g., FOXP2 [12]). Others are involved in enigmatic forms of RNA editing [15]. Gene duplications have led to, among other things, functional diversification of key components of the synaptic receptor and signal transmission complexes that are clearly related to cognitive advances [16]. Furthermore, copy number variations (CNVs) in humans may have contributed to accelerated evolution [17]: compared with other primates, CNVs are biased toward genes of neuronal function and may account for subtle gene expression changes resulting in human cognitive disturbances such as autism [18,19].

Alternative splicing is a powerful means of generating multifunctional proteins and has been suggested to be a significant contributor to functional neuronal changes in humans as compared to other primates [20]. Although this could account for an increase in functional complexity, and no doubt contributes, it requires a concomitant increase in regulatory control. Moreover, as we will argue, these processes are ultimately controlled by epigenetic mechanisms that are themselves controlled by non-coding RNA.

The programming of developmental complexity: non-coding RNA (r)evolution and regulation of epigenetic processes

In contrast to the relatively modest changes in the proteome through evolution, the amount of non-protein-coding DNA has increased dramatically and accounts for >98% of the human genome sequence [21]. The expansion of the non-coding genome in mammals, and particularly humans, may have been the consequence of the expansion of a regulatory RNA network required not simply for placental reproduction and development but also for brain function, processes that may themselves be closely linked. Although specific examples of the involvement of non-coding RNA in the recent increases in size and complexity of the human brain have been demonstrated [22,23], the full extent of the importance of non-coding RNA in higher-order cognition may not be fully appreciated.

It is now clear that the vast majority of the human genome is dynamically transcribed [24,25], especially in the brain [26,27], to produce a myriad of small and long non-coding RNAs (lncRNAs; >200 bp) that appear to constitute previously hidden layers of gene regulation ([28–30]; Figure 2). Moreover, it has been shown that a major function of lncRNAs, and perhaps some subclasses of small RNAs, is to direct relatively generic chromatin-modifying complexes to their sites of action [31,32], thereby controlling epigenetic processes that regulate chromatin architecture and the epigenetic trajectories that supervise human differentiation and development [33,34].

Epigenetic processes are, perhaps not surprisingly, central not only to development but also to learning and memory [35]. lncRNAs form a crucial layer of regulation in neuronal function, including learning and memory [36]. lncRNAs are widely expressed in dynamic temporal and

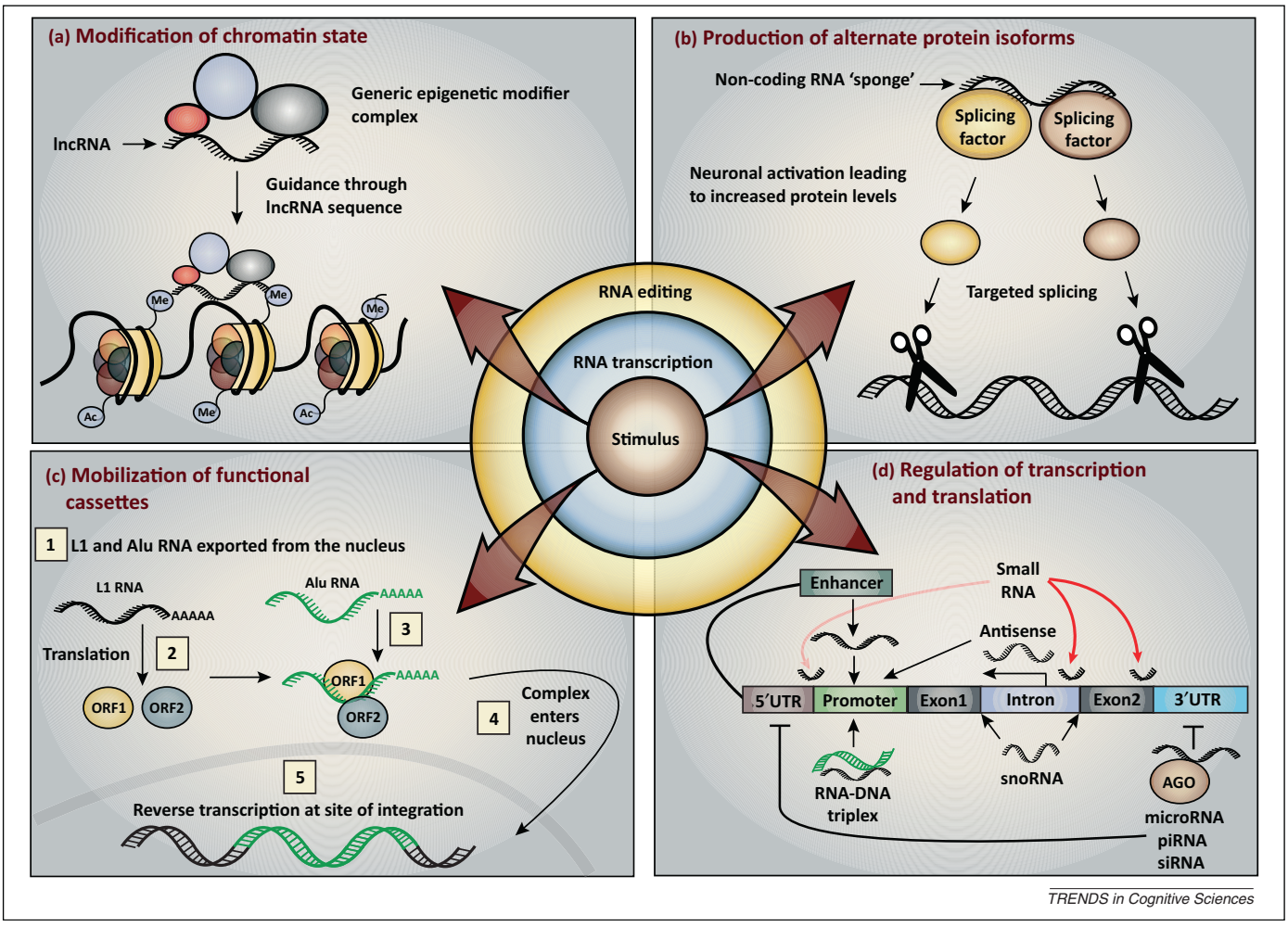

Figure 2. Non-coding RNAs are involved in diverse pathways that affect neuronal function. In response to neuronal activation, coding and non-coding RNAs are transcribed and potentially modified post-transcriptionally through mechanisms such as RNA editing. This early RNA editing step is likely crucial in determining sequence-dependent RNA guidance. Following RNA editing, non-coding RNAs direct multiple functions to allow cellular response. **(a)** Long non-coding RNA (lncRNA) guide generic epigenetic complexes to their sites of activity [91]. **(b)** Alternative splicing is an essential response following neuronal activation and is disrupted in cognitive disorders [82]. lncRNAs are emerging as key factors through their ability to sequester splicing factors thereby regulating their levels at active sites [81]. **(c)** Insertion rates of Alu elements into genes of neuronal function have increased in the human lineage [76] and may prove to have essential functions in cognition through their ability to mobilize in the human brain [90] through an LINE-1 (L1)-dependent mechanism [63]. Step 1: L1 and Alu elements are transcribed and exported to the cytoplasm. Step 2: L1-derived proteins ORF1 and ORF2 are translated into the L1 enzyme complex. Steps 3–5: This complex then combines with the Alu RNA and re-enters the nucleus to perform reverse transcription and site-specific integration. **(d)** In addition to lncRNAs discussed above, a wide range of non-coding RNAs has been demonstrated to regulate gene expression such as enhancer RNA [92], antisense RNA [93], various small RNAs [40–42] and RNA-DNA triplexes [94].

spatial patterns in the brain [26] and are involved during neuronal cell differentiation and fate determination [37]. Accumulating evidence of the extensive range of regulatory RNA mechanisms coupled with the scaling of non-coding sequence and species complexity [30] provides a compelling argument for non-coding RNA as the basis for the evolution of higher-order cognition. Studies investigating the underlying genetic basis responsible for human evolution since branching from the chimpanzee have shown that non-coding RNA is a major target for these changes through sequence deletions [22] and accelerated nucleotide substitution rates [38].

Small non-coding RNAs also regulate gene expression through a number of different mechanisms. Various classes of small RNAs, such as microRNAs [39,40], small interfering RNAs [40], piwi-interacting RNAs [40], and small nucleolar RNAs [41], have all been shown to regulate cell differentiation and function. More recently, new classes of small RNA have been described [42], some of which may regulate nucleosome positioning [43,44], thus potentially directing alternative splicing [45], and it is reasonable to assume that there may be other, yet to be identified, classes of small RNA with significant functions in mammalian cells. Small regulatory RNAs have been implicated in normal and pathological brain function [46,47] and it seems likely that they will be shown to have wider and essential roles in neural processes.

These data strongly suggest that classes of non-coding RNAs are temporally and spatially regulated to control both feedback ('hard-wired') processes during development and feed-forward ('soft-wired') processes during post-developmental cellular function.

Evolution of RNA plasticity in the brain

As discussed above, non-coding RNA-based regulatory networks may underpin epigenetic trajectories that control development and thereby ensure the cogent assembly of a functional multicellular organism. It also appears that

evolution has superimposed plasticity on these processes to provide the epigenetic flexibility required for learning and memory, primarily by innovation and expansion of enzymes involved in nucleotide editing, which is emerging as the key basis of molecular plasticity in the primate brain [31,48].

The major form of RNA editing is the conversion of adenosine to inosine (A>I) by base deamination. There are three enzymes (ADARs; adenosine deaminases that act on RNA) involved, the third of which (ADAR3) is vertebrate-specific and brain-enriched [49], although little is known about its RNA targets or its role in cognitive function. Single nucleotide changes and consequent codon changes in particular neurotransmitter receptors through the process of RNA editing, ostensibly to change their structure-function relationships and the electrophysiological properties of the synapse, were demonstrated almost two decades ago [50,51]. However a number of recent papers comparing large-scale cDNA libraries with genomic DNA have indicated that A>I editing is far more widespread than previously suspected, and occurs in thousands of transcripts [52–55]. Most of the edited sites occur in non-coding regions, implying that editing is not only modifying the structure-function properties of neuronal proteins, but also RNA-based regulatory circuits, and therefore potentially epigenetic processes.

There is a massive increase (~35×) in the intensity of RNA editing in humans compared to mouse [54]. Most (>90%) of this editing occurs in primate-specific Alu sequences [52–55], which invaded the primate lineage in three successive waves, and now comprise ~1.2 million copies that collectively occupy ~10.5% of our genome [8,56]. Moreover, the amount of editing has also increased during primate evolution, correlating with new human-specific Alu insertions enriched in genes of neuronal function [57], strongly suggesting that the expansion of RNA editing in the human lineage was central to the molecular events underpinning increased cognitive capacity.

Evolution of genomic plasticity in the brain

A second editing mechanism deaminates cytosine to produce uracil, and is carried out by vertebrate-specific enzymes called APOBECs, which may act on RNA or DNA or both. There are 5 families of APOBECs, two of which (APOBEC 1 and 3) are mammal-specific [58,59]. The best characterized is AID, which is involved in somatic rearrangements and hypermutation of immunoglobulins in the immune system [58]. Interestingly, there are many parallels between the nervous and adaptive immune systems, including the presence of immunoglobulin domains in many neuronal cell surface receptors [60,61], indicating that both may use similar mechanisms to tune cell receptor interactions. Moreover, the existence of many unusual DNA repair enzymes, many of which appear to be linked to reverse transcriptase activity, suggests that RNA-directed DNA recoding may play a role in long-term memory formation [48]. Intriguingly, APOBECs have also been shown to deaminate methylated cytosine to thymine, a mechanism important in developmental processes [62], suggesting that cytosine methylation and conversion to thymine by deamination may not simply occur in evolutionary time, but be a pre-programmed aspect of real time human developmental biology.

The APOBEC3 family appears to be especially important in human evolution. It originated after the divergence of the marsupial and placental lineages and has greatly expanded in the primate lineage, with very strong signatures of positive selection [15,58]. At least some appear to be involved in the control of exogenous and endogenous retrotransposition [63,64].

Sequences derived from transposable elements encompass almost half of the human genome. Retrotransposons are pervasively transcribed and may be of critical importance in genome-wide gene regulation [65]. Although most are rendered immobile through various mechanisms [66,67], active mobile elements nevertheless remain widespread in the human genome [68]. This activity has been traditionally regarded as a problem, which enzymes such as APOBECs have evolved to control.

However, recent evidence suggests that this system may constitute a strategy for generating somatic mosaicism in neuronal cells, to generate another level of complexity in the brain. ABOBEC-mediated editing is involved in the modification and likely domestication of retrotransposons [69]. Active retrotransposition has been observed in neuronal precursor cells in culture [70] and has very recently been shown to occur in the human brain [71]. Retrotransposition is in part controlled by methyl-CpG-binding protein 2 (MeCP2; [66]), a protein involved in widespread methylation and is the causative genetic defect in Rett syndrome [72]. These results indicate that epigenetic regulatory systems are involved in retrotransposition and that non-coding RNA may also underpin retrotransposition in the brain, given that non-coding RNA directs generic epigenetic machinery. These considerations lead to the prediction that each neuron will have a unique genomic, epigenomic, and transcriptomic signature depending on intrinsic and extrinsic experiences, and that all will modulated by non-coding RNA-mediated plasticity. This supposition can be tested by exposing individual groups of neurons to various stimuli prior to performing single cell sequencing and determining whether unique experiences are reflected in a common genomic response.

There is mounting evidence that transposition events cause a significant number of heritable disorders [73], but these are more likely to be detected than non-pathological, but nonetheless significant, events. Positive events are likely to be widespread and could result in positive outcomes, such as activation and strengthening of a system. For example, retrotransposition events were detected in the mouse brain upon voluntary exercise [74], suggesting that these events may occur as a specific response to a positive stimulus as opposed to being solely random and ultimately pathogenic. Retrotransposons have undoubtedly shaped our genome and continue to do so through active elements, mainly Alu sequences [75], that have markedly increased in insertion rate in the human genome since the split from chimpanzees [76] (Box 1).

New flexibility, new fragility

Although the increase in mammalian cognitive ability has provided unique mechanisms to evolve exceptional skills,

Box 1. What makes human brains unique?

To determine how humans differ cognitively from all other primates, including chimpanzees (our closest relatives), we need to establish key human-specific variation. We hypothesize that four mechanisms have combined to afford humans the ability to respond to extrinsic stimuli in a heightened and multifarious manner (Figure I):

(i) There are thousands of unique Alu insertions in genes of neuronal function present in the human genome [57] that may result in enhanced brain functionality.

(ii) RNA editing has increased dramatically in humans, especially in the brain and predominantly in Alu elements [57] amplifying the potential repertoire of human-specific Alu transcripts.

(iii) Recent studies have suggested that active retrotransposition occurs in the human neurons [70] and likely in the brain [71], displaying individual-specific patterns most likely reflecting responses to individual-specific experiences.

(iv) Examples of both gain [23] and loss [22] of neural human-specific functional non-coding RNAs have been demonstrated although the true extent of human-specific non-coding transcripts has yet to be uncovered.

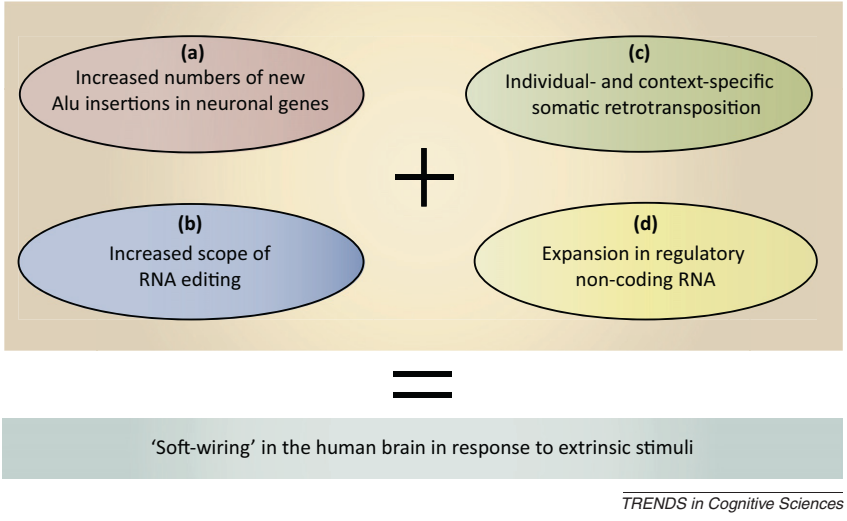

Figure I. Four mechanisms that may have combined to make human brains unique.

such as reasoning and awareness, it would also seem likely that a relatively new and increasingly complex regulatory system would have weaknesses and be vulnerable to stressors. Drug abuse, for example, is an example of an environmental stressor that exposes cognitive vulnerability, especially as epigenetic mechanisms have been demonstrated to be dysregulated in the brain following chronic drug use [77]. However, external pressures may not be the entire reason for exposing fragilities. More simplistically, primates and especially humans may currently be in a stage of evolution where the advancement of cognitive processing, and related pathways, is the primary focus. This could underlie psychiatric disorders, such as autism spectrum disorder (ASD) that have a broad range of genetic variation and cognitive phenotypes. Indeed, ASD arises from an extensive suite of genomic variants [78], but may encompass common pathways [79], providing mechanistic insight to the manner in which cognitive evolution is taking place.

Proteins form the core of basic cellular functioning, but we suggest that it has been the increased sophistication, complexity, and plasticity of the regulatory RNA superstructure that has been at the heart of human cognitive advance. By contrast, we suggest that the trade-off has been an increased fragility and spectrum of neural disorders, including schizophrenia, anxiety, depression, and ASD, induced by genetic defects, environmental stressors, or a combination thereof. For example, lncRNAs are involved in regulating widespread alternative splicing [80,81] and this process is commonly dysregulated in psychiatric disorders [82]. Furthermore, microRNAs have been linked with anxiety pathways [83] and psychiatric disorders [84–86], and RNA editing has recently been linked with psychiatric disorders [87] and is an efficient method of altering genome-wide function rapidly upon external stimuli.

Future directions

RNA-mediated mechanisms are attractive candidates for underpinning the rapidly evolving plastic brain. However, the considerations above make several predictions and suggest several important directions for future research that only upon testing will ultimately reveal the true extent of the role of regulatory RNA in cognitive adaptation and function. In summary, it is known that cognitive processes are dependent on epigenetic mechanisms. Evidence is accumulating that the site-specificity of epigenetic modifications is controlled by regulatory RNAs that are, in turn, subject to context-dependent A>I editing, which occurs at high levels in the brain and increases in intensity with cognitive evolution, reaching its zenith in primates. We conclude that RNA editing is the primary source of the molecular plasticity that underpins the epigenomic and ultimately network plasticity in the brain. It has been shown in a *Drosophila* model that RNA editing is a plausible mechanism for inter-individual experience-dependent cognitive differences [88], but investigations in higher species are required for any conclusions to be drawn. We predict that RNA editing is involved in cognitive processes and this can be explored in mammalian systems using mouse genetics. ADAR3 may have a critical role in brain

cognition, but formal testing of cognitive abilities in *ADAR3* knockout mice has not yet been completed. Similarly, *ADAR1* and *ADAR2* knockout mice are lethal, but the use of cell- and region-specific conditional knockout mice could reveal their involvement in cognition. Moreover, if cognitive defects can be detected in mice lacking ADAR1, 2 and/or 3, the nature of the defect and the genetic background in which it is observed should enable identification of the region(s) most critically affected, allowing focused deep sequencing analyses to compare the nature of the editing changes observed in the transcriptome under conditions of ADAR deficiency in the presence or absence of the relevant cognitive stimulus or task.

The other type of editing (C>U) also shows a strong link with cognitive evolution. The enzymes involved are vertebrate-specific, with two new families emerging in mammals, one of which (APOBEC3) expands enormously in primates under positive selection. These enzymes are more enigmatic and it is uncertain whether they act on RNA and/or DNA, but they are involved in complex genomic rearrangements and, in the case of APOBEC3, in the control of exogenous and endogenous retrotransposition. Our interpretation is that these processes have not evolved simply to protect against retrotransposition, but rather to domesticate it as part of neuronal plasticity [31]. This notion is supported by the demonstrations of active retrotransposition in neuronal cells in culture [89] and in human brains *in vivo* [90], a process that is in part regulated by MeCP2, the protein whose function is impaired in Rett syndrome [66]. This leads to the prediction that interference with APOBEC activity, especially APOBEC3s, will impair cognitive function. Testing this prediction is more difficult, as mice only have one orthologue of APOBEC3, but could provide a lead in the right direction.

Concluding remarks

We regard the observations and suggestions made here as the tip of a very large iceberg, as human-specific neural disorders will most likely include evolutionarily recent, or enhanced versions of more established, mechanisms (see also Box 2). Only by understanding the molecular basis of these newly developed systems will we be able to accurately diagnose and appropriately treat patients with disturbances in specifically affected neural pathways. We predict that a focus on RNA regulatory systems and the transcriptomic and genomic plasticity that underpin brain function will significantly advance our understanding of neuropsychiatric disorders.

Box 2. Outstanding questions

- Are human-specific Alu element-derived transcripts involved in cognition?
- Does Alu retrotransposition occur in the human brain in response to neuronal activity and, if so, does it target specific genomic regions?
- Does ADAR3 play a role in human cognition?
- Does dysregulation of RNA editing and retrotransposition, during development or in response to extrinsic stimuli, underlie aspects of psychiatric illness?

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