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Lineage divergence of activity-driven transcription and evolution of cognitive ability

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Abstract | Excitation–transcription coupling shapes network formation during brain development and controls neuronal survival, synaptic function and cognitive skills in the adult. New studies have uncovered differences in the transcriptional responses to synaptic activity between humans and mice. These differences are caused both by the emergence of lineage-specific activity-regulated genes and by the acquisition of signal-responsive DNA elements in gene regulatory regions that determine the extent to which transcription can be induced by synaptic activity. Such evolutionary divergence may have contributed to lineage-related advancements in cognitive abilities.

The basic brain structures and anatomical connectivities that enable perception, motor function, social behavior and cognitive skills are similar in mice and humans. However, in the 80–100 million years since the last common ancestor of these two species, human brain complexity and circuit organization has diverged substantially from that of mice, coinciding with the development of superior intellectual abilities in humans¹⁻³. The prevailing view is that, in mammals, such phenotypic divergence has been driven primarily by changes in the relative expression levels of genes⁴, rather than by differences in their coding regions. This hypothesis, which is over 40 years old⁵, is grounded in the observation that there are substantial biological differences between species, despite conservation of protein sequences⁵ and is supported by recent genome-wide comparative transcriptome analyses that have demonstrated human-specific gene expression networks in the brain and elsewhere⁶⁻⁸.

The evolutionary changes in gene expression that have supported progression towards an increased cognitive repertoire in humans may have been caused by alterations in regulatory DNA regions, such as promoters and enhancers. Indeed, one study showed that forebrain-specific enhancers that are active in early development show high levels of evolutionary

conservation of function, but enhancers that are active after mid-gestation do not⁹. While this genome-wide study addressed the functional preservation of activity, rather than conservation of specific sequences, it nevertheless suggested that conserved core gene expression patterns (such as those that control cortical neuron identity¹⁰) may give way to later, species-specific specializations in gene expression.

Signal-regulated transcription is a basic feature of synaptically-activated neurons and allows the conversion of short-lasting electrical events into long-lasting structural and functional alterations. For example, synaptic activity-induced gene expression supports the stabilization of synapses, allows for maintenance of changes in synaptic efficacies, leads to adjustments of metabolism and boosts neuronal survival. The principal mechanisms underlying signal transduction from the synapse to the nucleus in rodent neurons are well understood and the most crucial target DNA regulatory elements and their cognate transcription factors, which that are responsible for mounting the gene expression responses, have been identified (BOX 1). However, the impact of these signal-regulated changes in the transcriptome in the context of evolutionary divergence and mammalian brain development has received less attention.

Whole transcriptome studies using rodent neurons have identified several hundred activity-responsive genes¹¹⁻¹⁴. However, these results are difficult to extrapolate to humans because 80 million years is sufficient time to have allowed significant divergence of transcription factor binding sites in gene promoters¹⁵. Given that experimental alterations in a gene's regulatory region can dramatically change its responsiveness to neuronal activity¹⁶⁻¹⁹, it is expected that these naturally occurring, lineage-related promoter variations may also translate into altered signal-evoked transcriptional responses and, thus, are likely to change the nature of the neurons' adaptive response to synaptic activity. This article will provide an overview of recent evidence²⁰⁻²² that the responsiveness to neuronal activity of the genome has indeed changed during evolution. We will discuss both the underlying mechanisms and potential functional implications for the evolution of cognitive abilities.

[H1] Activity-regulated transcription

[H3] Comparing mouse and human neurons

Three recent studies set out to probe the transcriptional responses to neuronal activity of human neurons and to compare this to that of mouse neurons²⁰⁻²². Albeit using different cell sources and activation paradigms, all three studies demonstrated the conserved activity-responsiveness of human orthologues of many well-studied activity-regulated mouse genes, including classical immediate early genes. This is in line with the concept of a largely generic

and conserved neuronal activity-dependent transcriptional program²³. However, all three studies also uncovered evolutionary divergence of activity-regulated transcriptomes.

In their study, Qiu *et al.* compared cortical neurons derived from human embryonic stem cells (ESCs) with mouse primary cortical neurons or cortical neurons derived from mouse ESCs and showed that the extent to which the mRNA levels of orthologous human and mouse genes changed in response to membrane depolarization was similar²⁰. However, the correlations were far from perfect, suggesting that there are quantitative differences in activity-dependent transcriptional responses between the two species, with some genes being more (and others less) responsive to activity in human neurons than in mouse neurons.

As a means of evoking neuronal activity, Ataman *et al.* also induced membrane depolarization, in this case in primary cells derived from human fetal brain and in mouse or rat neurons²¹. Pruunsild *et al.*, on the other hand, triggered excitatory synaptic activity within networks of human neurons derived from induced pluripotent stem cells (iPSCs) or within networks of co-cultured human iPSC-derived neurons and mouse primary neurons, by the administration of a GABA type A receptor antagonist together with a weak K⁺ channel blocker²². This stimulation protocol is often used for gene expression analyses in rodent neuronal networks²⁴. In both studies, the comparison of transcriptional responses in human and mouse neurons revealed remarkable differences in the kinetics of transcriptional induction for a number of human and mouse gene orthologues, even though the mechanisms that mediate synapse-to-nucleus communication and regulate the genomic response in human neurons were shown to involve the evolutionarily highly conserved nuclear Ca²⁺/calmodulin kinases pathway^{22,23}.

In addition to these quantitative findings, all three studies revealed qualitative differences between the human and mouse activity-regulated transcriptomes²⁰⁻²². For example, some human activity-regulated genes, such as the protein-coding gene *ZNF331*^{21,22} and the noncoding RNAs *LINC00473*^{21,22} and *BRE-AS1*²², lack a mouse orthologue, either because it has been lost in the mouse (in the case of *ZNF331*) or because it has been acquired in humans (in the cases of *LINC00473* and *BRE-AS1*). Other genes, such as *OSTN*²¹, *CAMTA1*^{20,22} and *TUNAR*^{20,22}, are present both in the human genome and in the mouse genome, but are activity-regulated in human neurons only²⁰⁻²² (Table 1).

[H3] Divergent promoter architectures

The lineage-specific differences in responses to neuronal activity of gene orthologues described above could arise either due to divergence in the genes' regulatory regions or due to differences in the cellular environment and signal-processing machinery of the neuron. A

compelling experiment by Qiu *et al.* strongly supports the idea that the divergence in genes' regulatory regions is the predominant factor²⁰. By using neurons derived from the Tc1 transchromosomal mouse strain, which carries a copy of human chromosome 21 (HSA21), they studied HSA21 genes and their mouse orthologues side-by-side in the same cellular environment. The results of this experiment broadly recapitulated the differences that were observed in separate human and mouse neuron preparations²⁰, providing strong evidence that changes in DNA sequence must, at least partly, underlie the differential activity responsiveness of the human and mouse orthologues. However, the existence of as yet undiscovered activity-responsive factors that are unique to the Primates order or to the human lineage cannot be ruled out as an additional contributor to this evolutionary divergence.

The three studies described above²⁰⁻²² suggested that acquisition of signal-regulated DNA elements in gene regulatory regions underlies the evolutionary divergence of activity responsiveness. Among the regulatory elements that were shown to have been acquired by human promoters during evolution are binding sites for AP-1, myocyte enhancer factor 2 (MEF2) and early growth response protein 1 (EGR1)²⁰⁻²². MEF2 is regulated directly by Ca²⁺ signaling pathways, whereas EGR1 and AP-1 (heterodimers of the FOS and/or JUN families) are themselves transcriptionally induced by electrical activity. Thus, these DNA elements can confer direct or indirect activity-responsiveness onto a gene. The promoter of human *ETS2*, which was found to be more strongly induced by activity than its mouse counterpart, was revealed to have gained three activity-responsive AP-1 sites in evolution, two being primate-specific and one hominid-specific²⁰. The promoter of *OSTN*, a gene robustly induced in human neurons, but not in mouse or rat neurons, was shown to have become activity-responsive in the primate lineage by gaining binding sites for MEF2²¹. The *HIC1* gene displayed sustained induction by activity in human neurons but only transient induction in mouse as a result of the presence of an EGR1 binding site in the human promoter that is absent in the mouse promoter²². Thus, transcriptional inducibility by synaptic activity has changed during evolution through the emergence of signal-regulated DNA elements in the genes' regulatory regions.

Although it is unknown whether these divergent promoter architectures evolved through natural selection or genetic drift, there is good evidence that remodeling of gene regulatory regions is a general feature of molecular evolution²⁵. Gains, and perhaps also losses, of activity-responsive regulatory elements probably occurred in other lineages and contributed to the divergence of activity-dependent transcriptomes between species. The extent to which there are human lineage-specific differences remains a matter of further investigation. As noted above, the critical activity-responsive promoter element in *OSTN* is present in other primates, as are two of the sites in the *ETS2* promoter. In addition to targeting specific

examples, however, it would be instructive to perform side-by-side analyses of neurons derived from human, monkey (such as rhesus macaque) and ape (such as chimpanzee) iPSCs. Assessment of the activity-dependent transcriptomes across these closely related primate species, as well as in rodents, would clarify whether any leap in divergence occurred after the human lineage separated from that of other primates or happened earlier, after primates and rodents diverged.

[H3] Functional impact of divergent gene inducibility

Studies of the *OSTN* gene (which encodes osteocrin (also known as musclin)) have provided insight into the potential functional consequences of a lineage-specific gain of neuronal activity-responsive DNA motifs in gene regulatory regions. *OSTN* was shown to be induced by activity *in vivo* in the primate lineage in experiments that manipulated the sensory input to the macaque visual cortex by monocular deprivation²¹. Activity-dependent expression of *OSTN* in layer IV of the primary visual cortex as well as across the multimodal parietal cortex pointed towards a potentially widespread role for this activity responsiveness in development. Indeed, its cortical expression in human peaks in mid-late gestation and remains high through childhood²⁶. Overexpression and knock-down studies *in vitro* identified *OSTN*, previously known to have functions in muscle and bone in mice^{27,28}, as a negative regulator of dendritic growth and arborization in primate neurons. This suggests that *OSTN* has been repurposed, through a primate-specific remodeling of its regulatory region, to serve a regulatory role in the experience-dependent organization of cortical networks in primates.

The functions of several other genes regulated by neuronal activity in human but not in mouse (Table 1) suggest further mechanisms by which synaptically-activated human neurons may mount a physiological response that is distinct from that of mouse neurons. One example is *HIC1*, which has been shown to repress the transcription of the reelin receptor genes *LRP8* (also known as *APOER2*) and *VLDLR*²⁹. The sustained induction of *HIC1* expression by synaptic activity in human neurons²² could provide control of reelin signaling³⁰ that is different from that in mouse neurons, where *Hic1* is induced transiently²². Another example is *CAMTA1*, a Ca²⁺-sensitive transcription factor that is involved in cognitive functions. *CAMTA1* polymorphisms in humans correlate with performance levels in psychological tests³¹ and haploinsufficiency of *CAMTA1* results in non-progressive cerebellar ataxia with mental retardation³². In mice, *Camta1* knock-down leads to impairments of long-term memory formation³³ and loss of *Camta1* causes ataxia and cerebellar atrophy³⁴. The function of *CAMTA1* in both human and mouse neurons is regulated through its interaction with Ca²⁺ and/or calmodulin³⁵. However, in human neurons activity can also alter the rate of *CAMTA1* transcription²², adding another level of *CAMTA1* regulation in human neurons.

[H1] Implications for brain function

The discovery of operational changes in activity-regulated transcription in evolution caused by lineage-related divergence in promoter architectures provides the mechanistic basis for a new conceptual framework that may help us to understand the evolution of cognitive abilities. According to this hypothesis, species-specific activity-regulated transcriptomes may both specify the construction of neuronal networks during development and determine their capacity for structural and functional plasticity in the adult. This could contribute to the advancement of cognitive abilities during evolution and the development of the intellectual power of humans.

Many neuronal activity-regulated genes are expressed with a human-specific temporal profile in the brain³⁶. Moreover, disturbance of this human-specific gene expression program is associated with autism spectrum disorder (ASD)³⁷, consistent with experience-dependent gene expression playing a role in both circuit development and cognition. These observations support the view that activity-regulated transcription may be a source of the dissimilarities in brain development and function of humans compared to other species.

It is known that activity-induced control over brain development begins prenatally. For example, cortical neuron migration and probably also gyrogenesis is regulated by synaptic inputs and N-methyl-D-aspartate (NMDA) receptor-induced Ca^{2+} signaling^{38,39}, which also strongly activates gene expression¹⁶. Evolutionary divergence of the activity-dependent transcriptome could therefore affect sensory input-mediated network maturation of the brain and may represent a major functional consequence of promoter divergence, in addition to its impact on the responses of mature circuits to neuronal activity. The neuronal structure-related function of particular genes, such as *OSTN*²¹, provides an example of how species-dependent activity-driven gene regulation could impact on dendritic arborization and network architecture, which have been linked to cognitive abilities⁴⁰. However, the evolutionary adjustments that bring about the progression of the brains' intellectual capacities are probably the result of the combined effects of alterations in activity-responsiveness of many genes.

Until now, the genetic bases of vocal learning, neuronal metabolism⁴¹⁻⁴³ and, especially, expansion and convolution of the neocortex⁴⁴⁻⁴⁷ have been the center of attention in searches for the evolutionary changes that underlie the cognitive capacity of primates and the pre-eminence of the human brain. There is plenty of evidence that alterations in developmental gene regulation programs and gene expression patterns in the brain are major sources of this phenotypic divergence^{1,8,48}. Evolutionary advances, such as the emergence of lineage-specific

genes or gene copies⁴⁹⁻⁵¹, species-dependent gene regulatory changes^{52,53} and cortical region-specific patterns of gene expression⁵⁴, are likely to have provided a basis for the increase in cortex size and the concurrent elaboration of its structure that, in the primate lineage, are in part driven by the evolution of neuronal progenitor proliferative capacity^{55,56}. The new findings described above²⁰⁻²² provide an additional mechanism for human brain evolution, in which the evolution of a particular regulatory property of neuronal genes (namely the responsiveness of the genome to synaptic activity) may have lifted the capacity of the brain from being able to execute core cognitive tasks, such as learning and memory, to permits highly advanced functions, such as language and theory of mind (Figure 1). Progression towards a superior performance level may rest on altered (perhaps increased) network complexity that is driven by the expansion of brain size⁵⁷ as well as by the implementation of particular structural measures and plasticity capacities. Such features of neuronal circuitries have been implicated in the evolution of the human brain⁵⁸⁻⁶¹ and, moreover, are the prototypical targets for regulation by neuronal activity-induced genes^{23,62}.

Finally, it should also be noted that changes in the architecture of gene regulatory regions may not only affect intellectual capacities, but may also increase the vulnerability of neurons to conditions that initiate degenerative processes. A common pathology in a broad spectrum of excitotoxicity-associated neurodegenerative diseases, including stroke, Alzheimer disease, Huntington's disease and amyotrophic lateral sclerosis, is a transcriptional shut-off mechanism that is triggered by increased extrasynaptic NMDA receptor signaling^{63,64}. The cAMP response element (CRE) binding protein CREB, the prototypical activity-regulated transcription factor^{23,62}, is a prime target of this shut-off mechanism⁶⁵. Thus, acquisition by the human genome of new CREs that bind CREB and may confer new responses to synaptic activity could also render the human transcriptome more susceptible to deregulation in disease.

[H1] Concluding remarks

The coupling of neuronal excitation to gene transcription is mechanistically highly conserved from invertebrates to mammals and triggers largely generic gene expression responses. However, during evolution, the responsiveness of certain promoters has evolved through the acquisition of DNA motifs that serve as genomic targets of transcription-regulating synapse-to-nucleus signaling pathways. The specificity of transcriptional profiles evoked by synaptic inputs in different species, genera or other taxonomic ranks may contribute to differences in the construction and functioning of neuronal networks, providing a mechanism that fuels the advancement of cognitive abilities in evolution. This hypothesis could be tested; for example, mutations could be experimentally introduced into putative activity-responsive promoter

elements that are present in the human genome but not mouse and the impact of these mutations on functional responses to electrical activity in human neurons probed. Alternatively, mouse promoters could be altered to resemble the architecture of their human orthologues. Such promoter editing studies may shed light on facets of brain development, cognitive function and disease that are unique to humans.

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Author Contributions

H.B. and P.P. contributed equally to researching the data for the article and making substantial contributions to discussion of content. H.B. and G.E.H. wrote the article. H.B, G.E.H., P.P. and M.E.G. contributed equally to reviewing and/or editing of the manuscript before submission.

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BOX 1 Mechanisms and functions of neuronal activity-dependent transcription

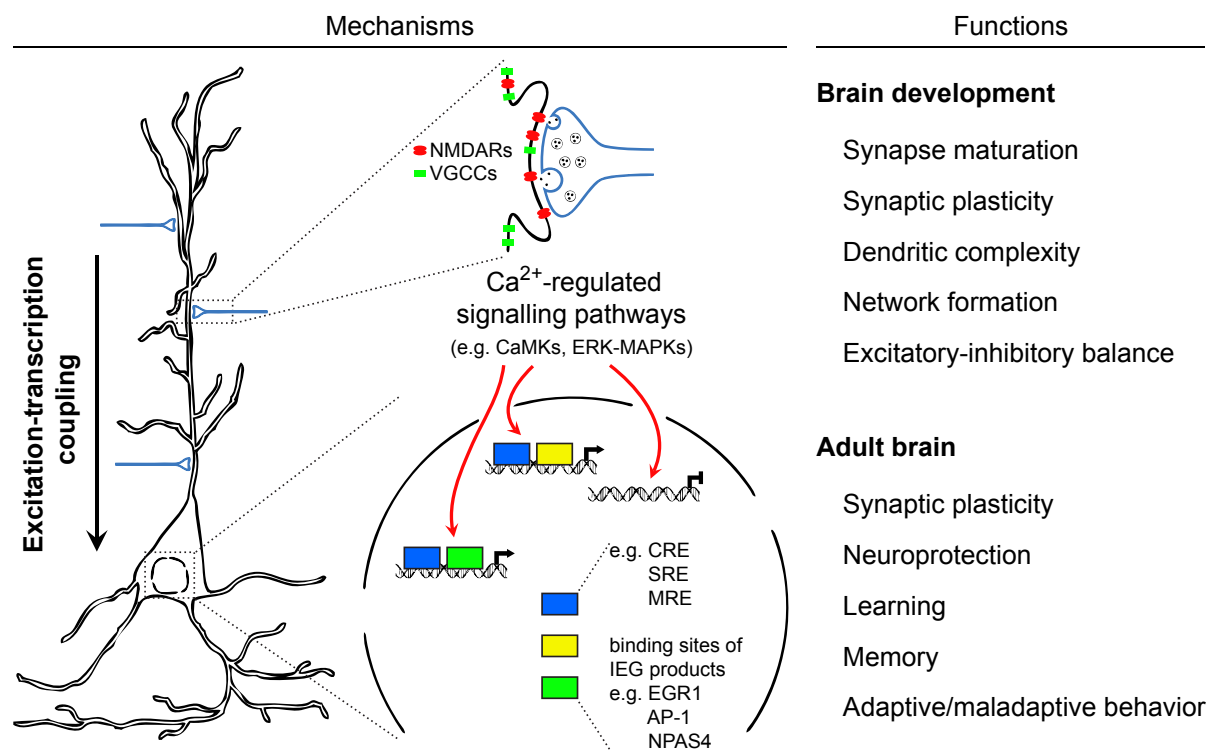
Transcriptional responses to electrical activity were first described in the rat pheochromocytoma cell line, PC12, where membrane depolarization and Ca^{2+} influx evoked by elevated extracellular K^+ concentration trigger induction of the *Fos* gene^{66,67}. Experiments with networks of rat primary hippocampal neurons identified Ca^{2+} as the principal second messenger that operates via distinct synapse-to-nucleus signaling pathways and links neuronal excitation to transcriptional regulation¹⁶. The type of transcriptional response — that is, the nature of the target genes and the temporal profile of their induction — is influenced by the pattern of electrical activity, the spatial properties of the Ca^{2+} signal, the degree of Ca^{2+} release from internal stores, the route of Ca^{2+} entry and even the oscillatory frequency of the Ca^{2+} signals^{23,68-70}.

The evolutionary conservation of the mechanisms underlying activity-regulated transcription (see the figure) was already apparent in early experiments¹⁶, in which the human *FOS* gene and mutated versions of its promoter were transfected into rat primary hippocampal neurons in order to map the activity-controlled DNA motifs that are targeted by Ca^{2+} -regulated signaling pathways. This showed that the initiators of activity-dependent transcription are synaptic N-methyl-D-aspartate (NMDA) receptors (NMDARs) and voltage-gated Ca^{2+} channels (VGCCs)^{22,70}. Synapse-to-nucleus communication is mediated by Ca^{2+} itself, which can invade the cell nucleus and activate nuclear Ca^{2+} /calmodulin-dependent protein kinases (CaMKs) and phosphatases. Ca^{2+} also activates protein-based signaling pathways, including those mediated by the extracellular signal regulated kinase-mitogen activated protein kinase (ERK-MAPK) cascade, Jacob and TORC1/2^{70,71}.

Whether or not transcription-regulating Ca^{2+} signaling pathways induce expression of a gene depends on the presence of binding sites for Ca^{2+} -responsive transcription factors in its promoter or enhancers. Examples of regulatory DNA motifs that are responsive to synaptic activity are indicated in the figure by colored boxes; they include the cAMP response element (CRE), the serum response element (SRE), the myocyte enhancer factor 2 (MEF2) response element (MRE), and the AP-1 binding site. Such DNA regulatory elements are often found in the promoters of immediate early genes (IEGs), which explains the robust induction of these genes by synaptic activity. Many IEGs encode transcription factors, such as FOS, early growth response protein 1 (EGR1) and neuronal PAS domain-containing protein 4 (NPAS4), which in turn bind their cognate target DNA elements and take part in producing the late phase of the activity-induced gene expression program^{23,62,70}.

Excitation-transcription coupling is conserved from invertebrates to vertebrates and plays a key role in translating synaptic activity into physiological responses in the developing

and the adult brain⁷²⁻⁷⁵. Many cell biological processes, including dendritic outgrowth and arborization, synapse formation and maturation, network excitatory-inhibitory balance, synaptic plasticity and neuronal survival and metabolic homeostasis are controlled by activity-dependent gene expression^{23,62,76-78}. Moreover, a number of neurodevelopmental disorders, particularly autism spectrum disorders, have been associated with mutations in genes known in mouse models to affect synaptic activity-dependent transcription^{79,80}.



Box Figure.

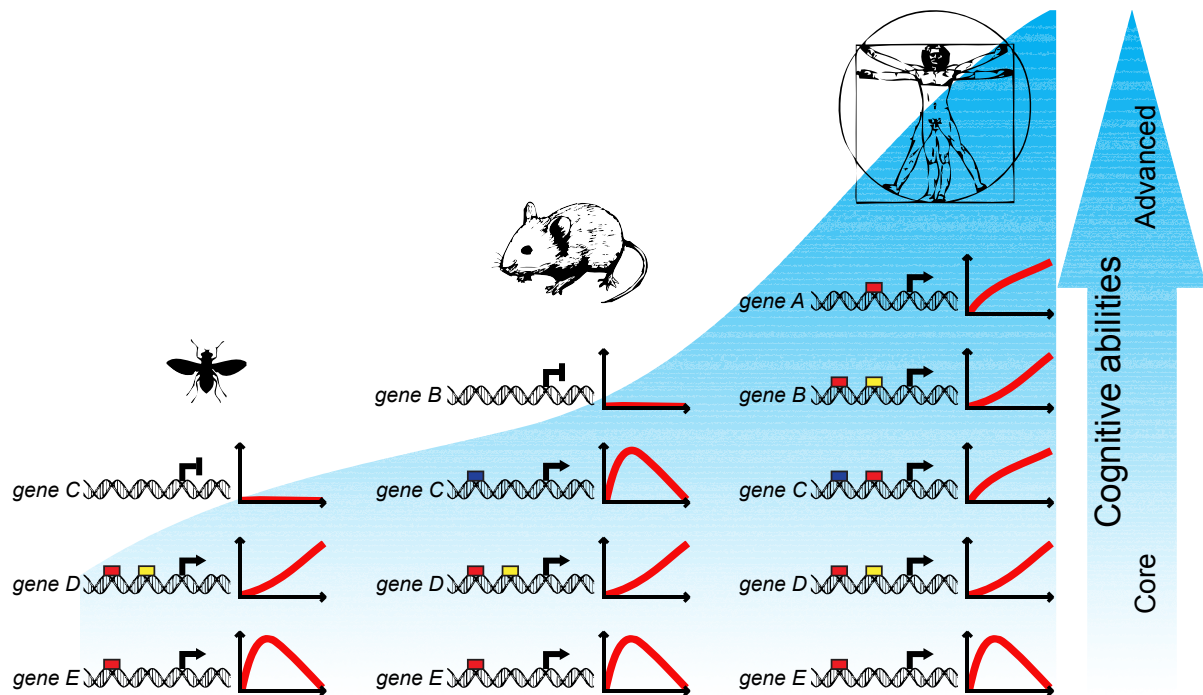


Figure 1. Linking evolutionary remodeling of promoter architectures to altered activity-dependent gene expression and cognitive abilities.

The figure shows a schematic representation of hypothetical gene orthologues present in one, two or all of the depicted species — fruit fly, mouse and human — together with graphs illustrating their transcriptional response to synaptic activity. Orthologous genes are presented in parallel and are named *gene A*, *gene B*, *gene C*, etc. According to our hypothesis, gaining activity-responsive DNA elements (depicted as colored boxes) in regulatory regions of orthologous genes in one lineage either confers inducibility (defined as a synaptic activity-regulated increase in the rate of transcription, which is indicated by arrows; blunt arrows denote that the gene is not inducible) to that gene, as shown for *gene B* (which is present in mouse and human but gains inducibility in the human lineage) or alters the kinetics and/or magnitude of its signal-responsiveness, as shown for *gene C* (which is inducible in both the mouse and human lineage, but demonstrates different response kinetics). The emergence of activity-regulated lineage-specific genes (such as *gene A*, present only in the human) increases the evolutionary divergence of activity-regulated transcriptomes between species. Combined, this may contribute to lineage-specific advancements of cognitive abilities. For simplicity, gains of activity-responsive genes are only shown for mouse and human. The fruit fly is included as an example of an invertebrate that uses excitation-transcription coupling via evolutionary conserved nuclear Ca^{2+} regulated mechanisms for core cognitive functions⁹⁹. This schematic illustration is not intended to be comprehensive and categorical and does not include all possible scenarios by which activity-responsiveness may be conferred to a gene

(for example, through the loss of a repressive DNA element) and does not imply that advancement of cognitive abilities is necessarily linear.

Table 1. Genes with divergent activity-responsiveness in human and mouse neurons

Human gene	Function and/ or relevance	Fold change in expression in response to activity		Refs
		Human neurons	Mouse neurons	
<i>ADRA1B</i>	Associated with attention deficit/hyperactivity disorder	4.3 ^c	0.9 ^c	20,22,81
<i>ATP1B3</i>	Associated with absence epilepsy	3.5 ^c	1.2 ^c	20,21,82
<i>BRE-AS1^{a,b}</i>	Not known	26.6 ^d	NA	22
<i>CAMTA1</i>	Associated with episodic memory performance and intellectual disability	1.5 ^e	0.7 ^e	20,22,32,83
<i>CCNH</i>	Associated with brain tumor risk	3.7 ^c	0.9 ^c	20,21,84
<i>CENPN</i>	Involved in regulation of chromosome segregation	2.5 ^f	0.8 ^f	20,21,85
<i>CTNNAL1</i>	Involved in modulation of Rho GTPase signaling	4.6 ^c	1.3 ^c	20,21,86
<i>DNMBP</i>	Associated with Alzheimer disease and memory performance in aged rats	2.3 ^c	0.9 ^c	20,22,87,88
<i>DUSP3</i>	Involved in modulation of ERK-MAPK signaling	2.2 ^c	1.1 ^c	20,21,89
<i>ETS2^b</i>	Implicated in apoptosis of neurons in Down syndrome	3.8 ^c	1.1 ^c	20,90
<i>GNB4</i>	Mutations cause Charcot-Marie-Tooth disease	2.2 ^f	0.9 ^f	20,21,91
<i>GREM2</i>	Associated with Warburg-Micro syndrome	8.1 ^f	1.4 ^f	20,21,92
<i>HIC1^b</i>	Involved in regulation of reelin receptor genes	6.5 ^e	1.3 ^e	22, 29
<i>LINC00473^{a,b}</i>	Involved in regulation of IEGs	48.7 ^e	NA	21,22, 93

<i>MAFG</i>	Loss of function causes neuronal degeneration in mice	2.4 ^c	1.1 ^c	20,21,94
<i>NKD2</i>	Role in negative regulation of Wnt signaling	5.4 ^c	1.1 ^c	20,22, 95
<i>OSTN^b</i>	Contributes to negative regulation of activity-dependent dendritic growth	102.9 ^f	ND	21
<i>RTL1</i>	Potential proneural functions in the developing telencephalon	4.4 ^e	1.3 ^e	20,22, 96
<i>TUNAR</i>	Involved in regulation of pluripotency and neural lineage commitment	3.8 ^c	1.0 ^c	20,22, 97
<i>ZNF331^{a,b}</i>	Tumor suppressor function	4.9 ^g	NA	21,22, 98

Table shows examples of genes that were transcriptionally induced by activity in human neurons but have mouse orthologues that displayed no induction, significantly weaker induction or different induction kinetics in mouse neurons. Because different activity-inducing stimulation paradigms and durations were used in different studies, only the highest fold change detected in human neurons (together with the corresponding change in mouse neurons) is shown. ^a, no orthologue in rodents; ^b, experimentally validated at the level of promoter activity; ^c, experiment involved 3 hr membrane depolarization; ^d, experiment involved 1 hr evoked excitatory synaptic activity; ^e, experiment involved 4 hr evoked excitatory synaptic activity; ^f, experiment involved 6 hr membrane depolarization; ^g, experiment involved 1 hr membrane depolarization; ERK-MAPK, extracellular signal regulated kinase-mitogen activated protein kinase ; IEGs, immediate early genes ;NA, not applicable; ND, not detected.