Behavior-Related Gene Regulatory Networks: A New Level of Organization in the Brain

Saurabh Sinha[1,2,†] , Beryl M. Jones[2,3*], Ian M. Traniello[2,3,*], Syed A. Bukhari[2,5], Marc S. Halfon[6], Hans A. Hofmann[7], Sui Huang[8], Paul S. Katz[9], Jason Keagy[10], Vincent J. Lynch[11], Marla B. Sokolowski[12,13], Lisa J. Stubbs[2,14], Shayan Tabe-Bordbar[1], Mariana F. Wolfner[15], Gene E. Robinson[2,3,4,16†]

[*] These authors contributed equally

[†] Corresponding authors

[1]Department of Computer Science, University of Illinois, Urbana-Champaign, IL, USA. [2]Carl R. Woese Institute for Genomic Biology, University of Illinois, Urbana-Champaign, IL, USA. [3]Department of Ecology and Evolutionary Biology, Princeton University, Princeton, NJ, USA. [4]Neuroscience Program, University of Illinois, Urbana-Champaign, IL, USA. [5]Informatics Program, University of Illinois, Urbana-Champaign, IL, USA. [6]Department of Biochemistry, University at Buffalo-State University of New York, NY, USA. [7]The University of Texas at Austin, Department of Integrative Biology and Institute for Cellular and Molecular Biology, Center for Computational Biology & Bioinformatics, 2415 Speedway C-0990, Austin, TX 78712, USA. [8]Institute for Systems Biology, Seattle, WA, USA. [9]Department of Biology, University of Massachusetts Amherst, MA, USA. [10]Department of Evolution, Ecology, and Behavior, School of Integrative Biology, University of Illinois, Urbana-Champaign, IL, USA. [11]Department of Biological Sciences, University at Buffalo-State University of New York, NY, USA. [12]Department of Ecology and Evolutionary Biology, University of Toronto, Toronto, ON M5S 3B2, Canada. [13]Program in Child and Brain Development, CIFAR, MaRS Centre, Toronto, ON, M5G 1M1, Canada. [14]Department of Cell and Developmental Biology, University of Illinois, Urbana-Champaign, IL, USA. [15]Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY, USA. [16]Department of Entomology, University, University of Illinois, Urbana-Champaign, IL, USA.

Abstract

Neuronal networks are the standard heuristic model today for describing brain activity associated with animal behavior. Recent studies have revealed an extensive role for a completely distinct layer of networked activities in the brain – the gene regulatory network (GRN) – that orchestrates expression levels of hundreds to thousands of genes in a behavior-related manner. We examine emerging insights into the relationships between these two types of networks and discuss their interplay in spatial as well as temporal dimensions, across multiple scales of organization. We discuss properties expected of behavior-related GRNs by drawing inspiration from the rich literature on GRNs related to animal development, comparing and contrasting these two broad classes of GRNs as they relate to their respective phenotypic manifestations. Developmental GRNs also represent a third layer of network biology, playing out over a third time scale, which is believed to play a crucial mediatory role between neuronal networks and behavioral GRNs. We end with a special emphasis on social behavior, discuss whether unique GRN organization and cis-regulatory architecture underlies this special class of behavior, and review literature that suggests an affirmative answer.

Significance Statement

Behavior is controlled by neural and molecular systems in the brain. Models of neuronal networks (NNs) have proven to be useful to describe the electrochemical activities of brain cells, and recently models of behaviorally related gene regulatory networks (bGRNs) have been developed to describe the activities of genes inside these brain cells. This effort has been spurred by a growing literature demonstrating strong and specific changes in brain gene expression associated with specific behavioral responses. We compare and contrast NNs and bGRNs, aided by the wealth of knowledge that exists for developmentally related GRNs (dGRNs). Considering these three layers of network biology, operating over three time scales, provides intriguing new insights into brain and behavior.

Animal behavior arises in large part from the coordinated activities of cells in the nervous system. It is common to model this activity with neuronal networks (NNs) (1-4), which seek to describe how circuits of neurons transmitting electrochemical signals from one neuron to the next control sensory, integrative, and motor functions of an organism (5). NNs provide quantitative representations of the signal processing activities that integrate perceptions of environmental stimuli with internal physiological states to produce the neuronal signals that orchestrate adaptive behavior (6).

A rich body of genetic and, more recently, genomic, studies have revealed that behavior is also associated with the coordinated activities of genes that operate in brain cells. Many studies have found significant, predictable, and specific changes in brain gene expression profiles associated with behavioral responses to particular environmental stimuli (7-14). These findings suggest that a second layer of network biology – that of gene regulatory networks (GRNs) – also underlies behavior. Expression of thousands of genes in the genome must be coordinated in order to generate the gene expression profiles that establish cell types, states and functions, and such coordination is also necessary to induce the characteristic expression changes associated with behavior. Orchestration of gene expression within a cell is achieved by regulatory interactions through which genes influence one another's activity, often in response to extracellular signals, jointly establishing the GRN. A GRN is a collection of regulatory relationships among genes that helps us understand how "input" signals and cellular context map to "output" gene expression levels. Applied to the brain, a GRN is thus a natural construct to help explain the observed behavior-associated changes in gene expression profiles in mechanistic terms. It is worth noting that the GRN in our discussion refers to the regulatory relationships and interactions operative within the cell, and not to the statistical relationships determined as part of GRN reconstruction efforts (8).

Which gene regulatory interactions most impact the expression changes associated with a particular behavior? These interactions comprise a sub-network of the genome-wide GRN that we will refer to as a "behavior-associated GRN" ("bGRN"). Models of bGRNs have only recently been developed (8), and despite their usefulness, many questions remain unanswered regarding their composition and structural/functional characteristics, as well as their relationship to NNs.

Here, we highlight emerging concepts and open questions related to bGRNs. We argue that integrating both networks – NNs and bGRNs – holds great potential for a better understanding of

how neurons and the genes expressed within them together regulate organismal behavior and channel its evolution (6, 15). While bGRNs are intracellular networks whose direct "outputs" are changes in gene expression, these intracellular changes are influenced by behavioral context and in turn feed back into NNs, with functional consequences in behavior. We also outline some paths for future research, with a special focus on social behavior, a particularly active area of research on bGRNs.

To guide our exploration of bGRNs we draw inspiration from the field of developmental biology, because metazoan gene regulatory networks are perhaps best understood in the context of development (16, 17). Developmental biology has already produced mature descriptions and theories of developmental GRNs ("dGRNs") and also have inspired other researchers studying brain and behavior (18, 19). Moreover, there are deep connections between development and behavior, as we discuss below, and this also provides a strong framework for comparative analysis. We thus use dGRNs as a point of comparison and contrast for bGRNs. Even if we find, upon pushing the comparison further, that dGRNs are in fact a poor model for understanding behavioral regulation, the rich literature on dGRNs will have allowed us to frame baseline expectations about bGRN characteristics, and identifying departures from this baseline will help us appreciate unique aspects of behavioral gene regulation. In referring to developmental studies, we focus on the development of new cell types from precursor cells (17, 20), rather than organ development and other processes involving groups of cells. We do not claim to describe or compare all the many ways in which behavior and development have been studied; rather, we comment on salient properties vis-à-vis their associated networks and control mechanisms.

Gene regulatory networks in development and behavior

Cellular states in development and behavior

An important concept for mechanistic studies of development is the "cellular state" (21, 22). Development of the various cell types in the metazoan body can be seen as a temporal succession of cellular state transitions, operating in parallel on multiple cell lineages in the organism. Gene expression profiles (measured as genome-wide transcript profiles or "transcriptomes") have emerged as a convenient yet powerful surrogate for cell states. GRNs, which control these profiles, are the underlying systems that drive cell state transitions (23). For instance, if a set of genes change expression as a cell transitions from one state to another, the GRN may explain those changes as the effects of one or more transcription factors (TFs) that were activated or deactivated in the transition (17). In fact, GRNs not only explain state transitions,

they also underlie the very existence of stable transcriptomic profiles representing cell states (24, 25) (**Figure 1A**).

Organisms also show transitions from one distinct behavior to another. In some cases, each behavior is performed briefly, while in other cases each behavior is performed for a relatively long period time, giving rise to "behavioral states" [7]. In species living in complex societies with division of labor, social dominance hierarchies, alternative reproductive tactics, and other forms of behavioral plasticity, some individuals perform the same set of behaviors repeatedly, sometimes for days or longer, thus exhibiting extreme behavioral states (26). Gene expression profiles in specific brain regions or even whole brains have proven useful as surrogates for behavioral states (**Figure 1A**); in some cases the correspondence between brain gene expression profile and behavior is strong enough to use the former to predict the latter (7, 27). This is similar to how gene expression profiles serve as reliable signatures of developmental stages and corresponding cellular states.

There are similarities and differences in the delicate balance between the stability and flexibility (openness to transitions) of states in both development and behavior. Transcriptomic studies have revealed not only that behavioral stimuli change brain gene expression profiles in brain tissues in a predictable and reproducible manner, but also that the new expression profile is stable, commensurate with the stability of the behavioral state. In other words, the brain or brain region being profiled transitions from one stable molecular state to another in response to the stimulus, and a sufficient number of cells apparently must undergo the same or similar cell state transitions so that their measured aggregate expression profiles at the tissue level still reflect this transition. This speculation suggests that individual cells in the brain switch states in a coordinated manner – akin to cellular state transitions during development. However, behaviors are in general more ephemeral than typical cellular states in development, so we would expect that the transcriptomic correlates of behavior are correspondingly more fluid (28) than those of cells in a developmental context. Both dGRNs and bGRNs govern cell state dynamics, but it is plausible that the dynamical features of bGRNs are more skewed toward flexibility.

A second point of comparison between developmental and behavioral systems with implications for underlying GRNs lies in the multi-scale organization of states and their coordinated transitions. Development is the determination and differentiation of many different cell types through time. Each cell type emerges by a transition from a precursor cell type, with multiple transitions

occurring in parallel across space that are coordinated by local as well as longer-range signaling. Likewise, transcriptomic state transitions associated with behavior are manifested in multiple brain regions simultaneously (29), presumably coordinated by neuronal connections as well as humoral cell-cell communication involving hormones and neuromodulators. We therefore expect to see common themes shared between development and behavior regarding how GRNs operate in different spatial locations coordinate their activities.

Gene expression changes during development and behavior

Changes in brain gene expression associated with behavioral changes are generally of modest magnitudes, with studies reporting statistically significant changes to be two-fold or less (29). As a point of contrast, more dramatic expression changes are seen in early development (16, 17, 30), where transcriptomes first establish cell lineages that will give rise to a vast diversity of tissues of the body. A simple explanation of this contrast may be differences in cellular heterogeneity between tissues analyzed – the early embryo will give rise to tissues ranging from gonads to brain, whereas in behavioral studies cells of a smaller range of similar lineages are being studied. Additionally, most behavioral transcriptomics studies have so far relied on "bulk" (whole brain region or even whole brain) rather than single-cell expression measurements, and the relatively modest expression changes noted may be the consequence of only a subset of cells in the bulk sample participating in the change. On the other hand, developmental studies often make use of single cell sequencing (31) and/or spatial expression profiles such as those based on *in situ* hybridization (32), allowing construction of higher resolution transcriptomic maps that resolve cellular heterogeneity.

However, there may be a biological reason for observed differences in the magnitude of gene expression differences between early development and behavior, related to differences in the persistence of behavioral and developmental states. Behavioral changes, especially when associated with an active stimulus, are generally more plastic than development; animals can rapidly transition from one behavior to a variety of other behaviors, depending on the social and ecological context. This stands in stark contrast to the typically unidirectional nature of developmental progression, which ultimately establishes different cell types with distinct expression profiles. Different behaviors are seen to induce different directions of change in brain gene expression profiles (10), and by extrapolation we expect that the number of distinct behaviorally related transcriptomic changes exceeds the diversity of paths normally taken from any given developmental state. It is reasonable to speculate that the less pronounced

transcriptomic changes seen in behavioral contexts (compared to those noted in early development) are related to this greater plasticity. If true, these points of contrast between developmental and behavioral changes in expression would suggest the existence of corresponding differences in regulatory mechanisms at multiple levels. These include transcriptional gene regulation in the GRNs (trans- and cis-elements), the architecture of gene regulatory circuits (feedback loops) within the bGRNs or dGRNS, and the control of GRN dynamics exerted by cell-cell interactions in the respective cell communities.

Differences between bGRNs and dGRNs

The above analyses of similarities and differences between development and behavior in terms of cellular states and gene expression set the stage to compare their underlying GRNs directly. One possible difference between the two types of GRNs is that dGRNs have a greater connectivity (frequency of regulatory edges) among TFs than do bGRNs. To understand this, let us consider the space of all possible transcriptomic states achievable by a system (the cell), with transitions among states (gene expression profiles) being determined by the GRN. Most of these states are unstable because they violate gene regulatory interactions. However, a distinct subset of them satisfy all regulatory interactions, are stable (robust to molecular noise), and can perform important biological roles such as maintaining cell type identity. Such stable transcriptomic states are called the “attractors” of the space (24, 25). The term “attractor,” borrowed from dynamical systems theory, refers to a state toward which a system tends to evolve and revert to if perturbed, e.g., due to fluctuations arising from gene expression noise. Attractors may be conceptualized as valleys in a landscape that depicts the stability of all possible transcriptomic states (**Figure 1B**). A GRN with many TF-TF regulatory interactions is likely to have feedback loops, which are known to result in a transcriptomic landscape characterized by many “deep” attractors from which there is no escape other than experimentally induced cell type reprogramming (33). By contrast, a paucity of TF-TF regulatory interactions and feedback loops in a GRN is expected to result in more malleable gene expression profiles that can reversibly transition into each other, depicted by shallow valleys in the transcriptomic landscape (**Figure 1C**). Characteristics of gene expression changes associated with the greater plasticity of behavior, discussed above, thus suggest that bGRNs should have fewer TF-TF regulatory interactions than dGRNs. We expect that the continuing efforts at reconstructing genome-wide GRNs through identification of trans- and cis-regulatory connections between all gene loci will help to test this prediction.

Figure 1D illustrates one way to test the above prediction, by comparing a bGRN reconstructed from transcriptomic profiles of behavioral states in mouse (29) and a dGRN reconstructed from transcriptomic profiles associated with eye development in *Drosophila* (34). This comparison, which provides support for the prediction, is merely one suggestive example, guided largely by the limited availability of GRNs at scale. Future tests will need to account for the fact that GRN characteristics can differ depending on the specific behavior and developmental process under study.

We noted above that a transcriptomic landscape with shallow attractors enables frequent transitions between cellular states. Shallow attractors are also associated with greater fluctuations in gene expression, which translates to the prediction of more stochastic gene expression in transcriptomic states associated with a behavior. Similarly, experimental studies using single-cell transcriptomics have revealed a greater dispersion in expression during differentiation events (35, 36), when deep attractors representing precursor cell types are destabilized and rendered shallower to facilitate state transition (25). Future work utilizing single-cell transcriptomics to analyze cells from the brain will help us test this hypothesis regarding behavior-associated cell states.

bGRNs and dGRNs in evolution

In addition to the above mechanistic comparisons, an important insight into the parallels between behavioral and developmental gene regulation comes from evolutionary analysis. The rich literature on evolutionary developmental biology ("evo-devo") (16, 37) has revealed genetic "toolkits" that have been deployed repeatedly in the independent evolutions of sometimes-parallel features of animal morphology, and these toolkits have been traced to the level of GRNs (17). Recent behavioral studies have undertaken increasingly comprehensive cross-species comparisons at the transcriptomic level and also have reported the existence of toolkits of genes and gene modules underlying parallel behaviors (9, 38), loosely analogous to developmental toolkits (16). dGRNs have provided a systems-level construct at which similarities of developmental regulation emerge across great evolutionary spans despite extensive sequence-level divergence (39, 40). Similarly, bGRNs and associated co-expression modules provide glimpses of shared mechanisms of behavior in different species even if such evolutionary toolkits are not apparent at the individual gene level (9, 41). In addition, such comparisons can also give insights into how entirely new behaviors might evolve. For example, analogous to the redeployment (and sometimes tweaking) of toolkits or dGRNs in the evolution of morphological

novelties (42-44), a new behavior's appearance might be facilitated (in an appropriate selective situation) by redeploying all or part of an existing bGRN in a new time, neural context, or in response to a different stimulus (19). Concepts and approaches developed in evo-devo for cross-species comparisons of dGRNs are already proving useful for similar cross-species comparisons of bGRNs (19).

Integrating gene regulatory networks and neuronal networks: multi-scale dynamics

The recognition of the bGRN as an important molecular substrate of behavior raises exciting possibilities to consider the interplay of the bGRN with the network most directly related to behavior – the neuronal network (NN). Such interactions would integrate across two distinct levels of biological networks, resulting in increased complexity of network dynamics compared to either network alone. The NN is based on physical connections among neurons, and the messages transmitted through it may interface with the bGRN (45). For instance, the bGRN operating within a neuron may respond to the synaptic activity among the neurons in the NN (46), as well as hormones and other secreted mediators that bind to its receptors, resulting in changes in gene regulatory activity. In one study, the temporal kinetics of neuronal firing was found to be intimately linked to GRN activity in dorsal root ganglia neurons, suggesting that the patterning of neuronal activity is interpreted by the GRN (47). Similarly, in the mouse cortex, expression levels of a transcriptional switch, the TF Er81, are directly correlated with firing properties in a subtype of interneuron, and activation of these interneurons in the context of learning modulates Er81 expression (48). Conversely, the bGRN indirectly controls NN activity via setting the production levels of neurotransmitter receptors, ion channels, axon outgrowths and dendritic arborizations, and other physico-chemical components of the NN (49-52). A case in point is the highly conserved TF Foxp2, a component of bGRNs in the basal ganglia song nucleus, Area X, that is associated with avian song learning (53). Knock-down of FoxP2 is known to impact vocal imitation and song variability. Mechanistic studies have shown Foxp2 to regulate genes that contribute to neurite outgrowth and NN formation (51), and to influence dopamine-modulated cortical circuits (54) in the mouse brain. Similarly, in the Drosophila brain, complex regulatory cascades of gene expression establish specific features of Tv1 neurons such as neurite morphology or neurotransmitter identity (55). GRNs have also been shown to constrain variability in neuron identity and function among similar neurons despite substantial variation in the expression of specific genes (56). bGRNs thus have the ability to directly influence the architecture and activity of NNs by modulating neuronal excitability and connectivity (57). Despite a fundamental difference between the two networks – the NN being an intercellular network and the GRN being an

intracellular network (with signal transduction crossing between GRNs in different cells) – they clearly influence each other's activities, presenting an exciting frontier of future research. Moreover, we also suggest that the wiring of NNs in the brain imparts a qualitatively different characteristic to the coordination of bGRNs across different spatial locations.

Spatiotemporal dimensions of bGRN-NN interplay

The interactions between NNs and bGRNs play out at multiple spatial and temporal scales. In the spatial dimension (**Figure 2**), the activities of bGRNs differ across brain regions and cell types; each location may thus exhibit distinct gene expression changes during a specific behavior (29). bGRN activities at different locations also influence each other, e.g., via the NN and neuroendocrine signaling (58). Likewise, the NN is meshed across the entire nervous system, with even single neurons known to link distant regions (59). Thus, with both networks exhibiting spatial patterns of activity, their interplay will assume a level of complexity above and beyond that of either network alone. This may lead to an increased number of stable transcriptional states (attractors), as has been shown in computer simulations that connect each cell's GRNs to a cell-cell interaction network (60). Such higher-level interactions can also influence the stability of, and transitions between, attractors. This results in more dynamic gene expression profiles, an important anticipated feature of bGRNs, as noted above. A key direction for future efforts must be the coupling of real-time neural activity measurements (61) with high resolution single-cell transcriptomics (62), in specific behavioral contexts.

There also are differences between the GRN and NN in temporal dimensions (**Figure 2**). The NN operates on the millisecond to second scales (for neuronal firing) and may induce the rapid activation of immediate early genes (IEGs) associated with behavior (57). By contrast, expression and epigenetic changes controlled by the GRN usually happen over a scale of minutes to hours or even days (63, 64). Aforementioned feedback from the GRN into the NN, such as modulation of neuronal connections via changes of receptor and transmitter levels, can take place over even longer time scales (65), and the GRN may serve the role of a temporal "integrator" of organismal experiences over such time scales. Back-and-forth interactions between bGRNs and NNs may prove to be an important mechanism for learning and memory and for past experiences to influence future behavior, possibly even across generations (66). In short, how this two-layered network architecture of the brain orchestrates behavioral responses almost certainly involves rich multi-scale spatiotemporal patterns and intricate phenomena that fall outside the realm of current knowledge.

Developmental mechanisms of bGRN-NN interplay

The dGRN is important in the understanding of the molecular basis of behavior in its own right, and not just in comparison to the bGRN. Cross-talk between bGRNs and NNs can be mediated by developmental processes, thus bringing dGRNs into the fold and suggesting an intermeshing of three different networks with functional consequences for behavior. For instance, transcriptomic changes associated with behavior – the consequence of bGRN activity – often include genes involved in nervous system development (67). This suggests that developmental changes, which in the postnatal periods pertain to the phenomenon of brain plasticity, can be caused by bGRN activity. Also, it is known that hormonal signals operating at various time scales can reorganize brain morphology and NN structure or function (68). These changes are driven by a variety of factors including environmentally induced changes in sex, dominance hierarchy, and predation threat, and may span across generations. Developmental processes thus triggered by bGRNs may result in NN rewiring and growth (69) or changes of cell type proportions in the brain (70), serving as a major mechanism for feedback from bGRN to NN, and thus to future behavior.

There are different ways to consider the cycle of relationships from behavior to GRNs to development and back to NN and behavior (**Figure 2**). The possibility of three-way interactions between NNs, bGRNs and dGRNs is strong for developmental processes that are regulated in an experience-dependent manner. Notably, when the feedback from the bGRN to NN involves developmental processes (controlled by the dGRN), it is expected to have greater longevity than feedback mediated by changes in neurotransmitter levels. In addition, mechanisms that give rise to individual differences in behavior can be mapped to aspects of early brain or synapse structure that are set up during development. Drawing on the relatively mature concepts and tools of developmental biology, especially brain development (19), should therefore be very useful for future work that aims to elucidate the three-way network interactions that underlie behavior.

Environmental influences mediated by bGRNs and NNs

The brain not only orchestrates behavior, it predicts what behavioral response would be most suited to environmental conditions and as mentioned above, the reciprocal interactions between bGRNs and NNs also mediate the influence of the environment on behavior. Developmental processes invoked by bGRNs provide a way for environmental changes to impact brain morphology and neuronal networks (71), which in turn bear upon future behavior. For some behaviors there are “critical” periods in behavioral development during which individuals are more

receptive to environmental influence (72), and such periods may coincide with critical periods in morphological development such as expansion of particular brain regions. Recent work has identified some of the GRN components activated in these periods (73, 74). GRNs also have an intimate theoretical connection to critical periods: bifurcations of developmental trajectories (cell fate decisions) can be attributed to non-linear gene-gene interactions in the GRN, and a critical developmental period is the period just upstream of a bifurcation point, when an irreversible binary decision is made, mediated by the GRN and potentially influenced by environmental inputs. This concept has been demonstrated for cell fate decisions by cytokines in cell differentiation (25, 75) and we suggest that analogous dynamics also may play a role in brain development.

The role of experiences and environmental inputs during critical periods may thus result in the "fine-tuning" of behavioral development via NN- and GRN-mediated mechanisms. The relative extent to which the two types of networks are engaged in environmentally influenced modulation of behavior likely depends on the nature of the environmental input. For instance, an acute change in environment may act directly on the NN (76) or trigger specialized signal transduction pathways that modulate bGRN dynamics resulting in temporary modulation of the NN (77), and hence of behavior. By contrast, more permanent responses to chronic environmental change may be mediated by developmental processes and/or epigenetic mechanisms. For instance, early adverse experiences have been shown to prime the genome, via DNA methylation at specific loci related to stress-response pathways, so that the individual responds differently to future stressful events (78-80). Generally speaking, such responses may be thought to involve drastic changes in individual regulatory interactions of developmental genes so as to distort the topography of the transcriptional landscape, opening access to maladaptive developmental trajectories. Such "decanalization" of development results in lasting developmental anomalies with all the behavioral consequences of an improperly wired NN (81-83). This reciprocity between genes and neurons also depends on individual differences in temperament due to genotype and experience, and is the foundation for the brain's ability to predict the future (84).

GRNs in social behavior

A special focus of behavioral transcriptomics during its first two decades has been social behavior, from both mechanistic and evolutionary perspectives (85-87). Should we expect fundamental differences in bGRNs related to social behavior relative to those associated with other types of behaviors? Treating bGRNs as a mapping of inputs (cell communication signals and cellular context) to outputs (gene expression levels), a reasonable null hypothesis is that it should not

matter whether the inputs were triggered by a social or non-social stimulus. According to this logic there is nothing special about social bGRNs relative to other types of bGRNs for behaviors that do not involve social interactions among conspecifics, such as food acquisition or nest construction in some species. On the other hand, there are also good reasons for anticipating differences between social bGRNs and other bGRNs. Social behavior involves repeated interactions between individuals, an iterative exchange of stimulus and response that is fundamentally different from a unidirectional intake of stimuli from abiotic sources. This adds yet another network layer – the social network – to the information-processing system, potentially leading to specialized patterns and dynamics in bGRN and NN activity, and hence to special structural properties of these networks.

The need for balance of stability and flexibility is ostensibly more acute in social behavior compared to non-social behaviors. This is because social behavior involves responding repeatedly to a greater variety of environmental (social) cues and must be adaptive yet stable within a range of variation of signals. Animals with busy social lives have to respond to all the same environmental stimuli as do less social animals (abiotic as well as biotic, such as predator-prey interactions) and in some cases also have to maintain a set of individual relationships with conspecifics. An alternative viewpoint is that animals living in social groups inhabit a less challenging world, as social groups might buffer against environmental noise and reduce pressures such as predation or lead to niche construction. Per this view, whether social behavior results in a more or less complex bGRN (e.g., by the above-mentioned aspects of GRN complexity) will depend on the stimuli that are encountered and how being in a social group impacts those stimuli and the potential behavioral responses to them. In light of the above considerations, the nature of social bGRNs and their special properties compared to bGRNs in general poses an intriguing open problem.

Evidence for a cis-regulatory code for social behavior: evolutionary perspectives

One finding that supports the possibility that bGRNs for social behavior have distinct features relative to other bGRNs comes from a comparative genomics analysis of the genomes of ten species of bees exhibiting different levels of social organization. Kapheim et al. (88) bioinformatically detected greater TF binding site presence (reflecting stronger binding of TFs) in gene regulatory regions from social bees compared to orthologs from solitary bee species. This result suggested that gene regulation in social bees has increased capacity and complexity relative to non-social bees, encoded in the DNA. These finding and those in refs. (89-91)support

403 the prediction that changes in gene regulation are key features of the evolutionary transition from
404 solitary to social life, at least in the social insects. Perhaps this is related to the appearance of
405 extreme behavioral states in species of social insects with division of labor and the performance
406 of the same set of behaviors by individuals for an extended period of time.
407
408 The result from Kapheim et al. (88) gives the first glimpse of a special signature tied to GRNs for
409 social behavior, but this is intriguingly reminiscent of a cis-regulatory signature seen in
410 developmental studies in *Drosophila*. Li et al. (92) reported greater homotypic TF binding site
411 clustering in blastoderm-stage (early) enhancers than in those for other developmental programs,
412 possibly reflecting the greater complexity of cell fate decisions driven by positional information in
413 the early *Drosophila* embryo. We speculate that just as the greater complexity of expression
414 patterns achieved in the syncitial embryo is reflected in the complexity of associated enhancers,
415 perhaps the increased phenotypic complexity of social behavior is achieved, in part, by increases
416 in the complexity of cis-regulatory architectures and GRNs. The cis-regulatory basis of
417 evolutionary changes in social behavior was also investigated by York et al. (93), who studied
418 divergence in bower-building behavior among Lake Malawi cichlid fishes. They identified
419 behavior-associated genetic variants and reported allele-specific brain gene expression that
420 depended on behavioral context. Their study provides a concrete example of the connection
421 between cis-regulatory evolution and diversity of social behavior.
422
423 How might GRNs become more complex? With respect to cis-regulatory organization this could
424 involve greater numbers of enhancers (94) or greater numbers of TFs regulating each enhancer
425 (95). In the case of early embryonic developmental enhancers it is the latter, but it is not yet known
426 which scenario accounts for the increased TF binding site presence observed for social bees.
427 Improved methods for enhancer discovery, e.g., chromatin accessibility profiling via ATAC-seq,
428 massively parallel activity assays such as STARR-seq, or effective insect-specific computational
429 approaches should help to address this question (39, 96, 97).
430
431 For species with an extensive repertoire of social behavior, experience and exposure to specific
432 social stimuli can be recorded quantitatively as changes in the gene expression profile (98), much
433 like an odometer records distance. Principles of cis-regulatory organization associated with such
434 a quantitative recording of temporal information may thus have similarities to those related to the
435 precise spatial readout in early embryo body-plan development, offering another perspective on
436 the observation by Kapheim et al. It is still too early to know conclusively whether social behavior

involves unique features of GRNs and cis-regulatory sequences, but emerging evidence seems to point to an affirmative answer. The correlation of cis-regulatory potential and social complexity is remarkable given the large gap it bridges from genotype to behavioral phenotype and needs rigorous confirmation in the future.

The question of unique features of bGRNs for social behavior may also relate to unique aspects of the evolutionary dynamics of social behavior. The multi-layered network architecture underlying social behavior, including the social network layer, with spatially diverse and temporally dynamic cross-talk between layers, is likely to impose a range of evolutionary constraints, with parallels in the co-evolutionary dynamics of multiple signal transduction pathways that exhibit cross-talk (99). An interesting evolutionary perspective into social behavior also arises from the fact that the unit of selection lies, at least in cases of extreme sociality, above the individual and at the societal level (100). This special evolutionary status of certain social behaviors may be reflected in the molecular mechanisms evolved to implement them. A recent study has also examined the provocative idea that social organization can drive the evolution of GRNs by affecting genome structure (101).

Future directions

This is a particularly exciting time for molecular explorations of behavior. Gene regulatory networks are a unifying construct today for scientists embarking on such explorations along diverse routes. Detailed analyses of bGRNs will not only break new ground in our understanding of behavior (8), but also provide broader insights into gene regulation, complementary to those obtained from developmental studies.

A number of emerging technologies will play key roles in future research on bGRNs. Perhaps leading this pack is the rapidly evolving technology of single-cell RNA sequencing (scRNA-seq) (35, 36), which allows transcriptomic profiling at cellular resolution, as well as single-cell epigenomic profiling (102). These new developments will help us bridge the existing gap between the true bGRNs operational within different cell types and the approximate reconstruction afforded by traditional “bulk” assays. They will also help solve a major mystery about bGRNs: that transcriptomic profiles of brain regions or even whole brains often show a striking correspondence with behaviors even though the GRNs underlying these profiles are properties of individual cells. That this relates to the fact that behavior is an emergent property of many cells seems intuitive, but the precise mechanisms of integration are currently unknown (103). By contrast,

developmental states do not present this mismatch of scales, since developmental phenotypes, whether at the cellular or tissue level, as well as the associated GRNs, are cellular properties, even if they are influenced by inter-cellular communication. Do bGRNs have a level of organization that transcends the cell, as theories of coupled GRNs have suggested (60)? The ability to tease apart transcriptomic profiles and GRNs at the individual cell level, especially in the face of extreme spatial diversity and cell type heterogeneity in the brain, will play a crucial role in finding answers to this and other pressing questions. Emerging technologies for "spatial transcriptomics" (104) and their combination with scRNA-seq will prove to be particularly noteworthy in this regard, and also allow the above cellular insights to begin to be connected to neural circuitry.

While our discussion focuses on mRNA levels as representing the regulatory processes in play, this is a simplification motivated by the current sparsity of data on other levels such as non-coding RNA (e.g., microRNA and lncRNA), exosomes, epigenetics, peptides, proteins, lipids, carbohydrates, and metabolites, all of which are part of what define a cell state and have been reported as being important in behavior (105-107). Various "omics" technologies are already available and will soon become well-established approaches to better inform these complementary views of molecular processes. Powerful new techniques to control and manipulate neural and gene activity, such as directed cell CRISPR (108) and optogenetics (109), as well as approaches such as 3D brain organoids (110) that facilitate controlled sample generation, are likely to be crucial in teasing apart cell type- and region- specific activities of bGRNs. In addition to more accurate reconstructions of GRNs, future investigations will have to map out the cross-talk between bGRNs, dGRNs and NNs in various behavioral contexts, and large-scale efforts in connectomics, such as the Human Connectome Project (4), and Brainbow (111), will provide a solid foundation for such studies. Information from all of these sources should enable the development of a comprehensive theory of behavior in molecular terms.

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Figure Captions

Figure 1. Transcriptomic states, stability and relation to Gene Regulatory Network connectivity. (A) Gene Regulatory Network (GRN) along with cellular environment determines the gene expression profiles (transcriptomic states) of cells, which in turn are surrogates of cellular states. Cellular states that are stable but flexible transitions between states are also possible, also under the influence of GRN. Cellular states in the brain have been found to be strongly predictive of behavioral states. **(B)** Landscape depicting stability of transcriptomic states, with the x-y axes representing all possible states (state space) and the z-axis representing their stability. Valleys in this transcriptomic landscape represent regions of stability, or attractors. Stable states correspond to attractors of the landscape. **(C)** GRN connection patterns shape stability in the transcriptomic landscape. GRNs with more edges between transcription factors (TFs) and feedback loops exhibit deep valleys (more stable attractors) in the landscape, while fewer TF-TF edges in a GRN are associated with shallow valleys. **(D)** Comparison of TF-TF connectivity between a behavioral GRN (bGRN) in mouse (Saul et al. 2017) and a developmental GRN (dGRN) in fruit fly (Potier et al. 2014). The two GRNs were reconstructed from genome-wide expression data in the respective studies and consist of TF-gene edges. For each TF, we counted the number of its target genes that encode TFs and calculated the ratio of this count and the total number of target genes of that TF (since the GRN was constructed separately in each species, with different criteria for defining edges). We normalized this ratio further by the overall TF-to-gene count ratio in the species. Shown is the histogram of (normalized) TF-TF edge frequencies in each species, revealing that a TF typically had more TF targets in the dGRN relative to the bGRN.

Figure 2: Neuronal Network-Gene Regulatory Network interactions. Spatial dimensions (bottom): Different cells (neurons), connected by the neuronal network (NN), may exhibit different Gene Regulatory Network (GRN) activities, even though the GRN itself is unchanged. GRN includes activating (green arrow) and repressive (red hammer) relationships between genes (circles). Gene expression is indicated by black or grey border, representing high and low expression, respectively. Signals carried by NN may influence gene expression in a cell (arrow labeled “neural signaling”) and activity of a GRN in one cell may influence gene expression in

another cell, for instance via neuroendocrine signaling. **Temporal dimensions** (top right, thicker arrows indicate faster interactions)**:** Fast (millisecond – second scale) message transmission by the NN ("neural firing") can induce, via neural signaling, the activity of immediate early genes (IEGs) associated with behavior, setting off a cascade of slower transcriptional and epigenetic changes mediated by a behavioral GRN (bGRN) on the scale of seconds to days. These changes may feed back to the NN if levels of neuroreceptors or neurotransmitters are affected. In some cases, bGRN-mediated changes can lead to developmental changes, mediated by developmental GRNs, on a slow time scale of days, months or even across generations. These slow developmental changes may affect brain morphology and cause neuronal growth or rewiring, thus feeding back into the NN.