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Review

Behavioral evolution by diverging cell type composition

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Recent advances in single-cell genomics are propelling a flurry of discoveries about the cellular composition of the brain and other organs across species. These discoveries, coupled with experimental manipulations, have begun to reveal how variation between species in the proportion of cell types, including the outright disappearance of some cell types and the emergence of new ones, contributes to the evolution of behavior. This review highlights these emerging findings in the context of more traditional approaches to study the evolution of behavior and discusses important outstanding questions in this field.

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How does behavior evolve?

Behavior can vary dramatically even among closely related species: Some birds are attracted to sweet nectar, whereas others are insensitive to sugar; some fish see and others are blind, some build nests for their young, whereas others incubate the fertilized eggs in their mouth; some mice are expert climbers and others dig deep burrows; some mammals are promiscuous, while others are monogamous. The search for mechanisms by which species diverge in their behavior has long fascinated biologists. Part of this interest stems from behavior being orchestrated by the brain, a remarkably complex organ whose workings are still so poorly understood. Therefore, studying the evolution of behavior is particularly challenging because it requires figuring out how interspecies differences in enigmatic nervous systems affect behavior. But these challenges of studying behavior from an evolutionary approach can come with rewards and complement the more traditional neuroscience methods, which tend to focus on single species. Indeed, this evolutionary approach has led to important neuroscience discoveries of how behavior is generated within individuals and of how it evolves between species. For example, molecular and biophysical comparisons between snakes with a pit organ (such as rattlesnakes) and without (such as Texas rat snakes) were used to discover the mechanisms for infrared sensation in snakes [1]. This led to the discovery that the transient receptor potential A1 channel evolved high sensitivity to heat to function as an infrared sensor in rattlesnakes and gained expression in the trigeminal sensory neurons that innervate the pit organ [1]. Similarly, behavioral and neurobiological comparisons between closely related species of *Drosophila* flies were also used to discover new features of the neurobiology of mate choice, from pheromonal communication, through sensory neurons, brain interneurons, to motor outputs [2,3]. This *Drosophila* work is a beautiful example of virtuous cycles that emerge when comparative neurobiology builds on solid scientific foundations from single-species studies, furthering our mechanistic understanding of behavior within species and its evolution across species.

Behavior can evolve through multiple nonmutually exclusive mechanisms [4,5]. These include (1) 'simple' changes to sensory receptors, such as missense or loss-of-function mutations of olfactory, gustatory, and thermal receptors that shape habitat and dietary preferences [6–10]; (2) sensory-circuit reconfigurations that alter, for example, thermal, host, and mate choices [3,9–11]; (3) changes in descending and motor circuits that can modify motor program orchestration such as gait or song [12,13]; (4) variation affecting neurotransmitters and hormones or their receptors [14–22]; and (5) more complex changes to central neuronal circuits, such as changes to excitatory–inhibitory balance that affect mate choice decisions [2]. Examples of these evolutionary mechanisms include mutations in gustatory receptors that conferred passerine birds the ability to taste sweet, an ability absent in most birds [8]. Additional prominent examples come from studies across *Drosophila* species. *D. mojavensis*, which prefers warmer North American desert temperatures compared to the

lower temperatures preferred by other *Drosophila* species, has evolved changes in the temperature-sensing Gr28b.d receptors and changes in the projections of those sensory neurons in the brain [11]. On the other side of the world, in the Seychelles, *D. sechellia* has evolved protein-coding changes to its olfactory receptors and anatomical changes to the projections of the olfactory circuits that contribute to its attraction to noni fruit [6,9,10,23,24]. *Drosophila* species have also diverged their peripheral responses to pheromones and the central processing of such pheromones, leading to the evolution of species recognition and preference during mate choice [2,3]. In mammals and birds, variation in the production of androgens and other steroid hormones and in the expression of estrogen receptors in the brain shapes social behaviors, including aggression and parental care [14,15,19]; variation in the vasopressin and oxytocin neuropeptide systems also modulate monogamy-related behaviors [16–18,20].

Genetic, anatomical, and physiological approaches to study the evolution of behavior have been possible for many years now. However, the recent blooming of a technical and analytical ecosystem for single-cell genomics provided a great leap forward [25]. These new technologies have allowed an unprecedented view into the variation in cellular composition of the brain and other organs, opening the door to characterizing how cellular diversification contributes to the evolution of behavior. This review provides a framework of the multiple approaches that have traditionally been taken to study the evolution of behavior and of how recent analyses of cellular composition are providing novel insights into fundamental questions. It concludes by presenting outstanding questions in this emerging field.

Traditional ways to study the biological bases of behavioral evolution

There are three main approaches to study the evolution of behavior: (1) a 'forward' genetics approach to map the genetic basis of a behavioral trait, typically by testing genes throughout the genome; (2) a study of specific genes or gene classes (e.g. taste receptors [7,8] or clock genes [21]) hypothesized to mediate differences in behavior between species or individuals; and (3) a comparative approach focusing on biological mechanisms of an evolving trait rather than starting at the level of genetic variation — for example, comparing neuronal circuit anatomy and function or hormonal biology and its behavioral effects. Intersections of these methodological approaches provide a more comprehensive understanding of how behavior evolves, helping answer both the 'proximate' (developmental and mechanistic) and 'ultimate' (evolutionary) levels proposed by Tinbergen [26].

Forward genetic approaches take three main strategies: (1) molecular evolution analyses to identify genes or classes of genes evolving exceptionally fast in species sharing a particular behavioral trait, such as echolocation in bats [27]; (2) linkage-based quantitative trait locus (QTL) mapping using laboratory-generated crosses between species or lineages with behavioral differences, such as studies of communication and mate choice in crickets and butterflies, and of social, foraging and other behaviors in flies, worms, and rodents [18,28–35]. For instance, a study starting with QTL mapping between promiscuous deer mice (*Peromyscus maniculatus*), in which only mothers provide parental care, and monogamous oldfield mice (*P. polionotus*), in which both parents raise their young, found that oldfield mice build more elaborate nests for their young partly because they have fewer vasopressin-producing hypothalamic neurons and each of those neurons produces less vasopressin than in deer mice [18,36]; and (3) population genetic approaches, including scans of selection or genetic differentiation and admixture mapping and genome-wide association studies in species hybrids, have been used to study bird migration patterns and butterfly mate choice, among other behaviors [37–39].

Studies of specific candidate genes have also been insightful. This approach has been particularly successful in the study of sensory receptors — for temperature or chemicals — which evolve particularly fast [40–42]. Because sensory receptors typically have very specific functions, studying their functional variation is more tractable than that of genes with more pleiotropic effects on nervous systems.

Comparative neurobiology and endocrinology methods characterize how differences between species in and outside the brain contribute to species-typical behaviors. For example, comparative connectomics led to the discovery that *Drosophila santomea* larvae are more likely to roll in response to noxious stimuli than *D. melanogaster* because *D. santomea* mdIV nociceptive sensory neurons synapse with additional downstream interneurons [43]. Similarly, transcriptomic and physiological comparisons between hibernating rodents (thirteen-lined ground squirrels [*Ictidomys tridecemlineatus*]) and nonhibernating rodents (house mice [*Mus musculus*]) led to the discovery that the cyclic nucleotide-gated ion channel CNGA3 is a cold sensor in the hypothalamus [44]. As a further example, hormonal and genetic comparisons have been performed among ruff sandpiper reproductive morphs, in which a supergene causes dramatic differences in morphology and aggressive behavior. These comparisons pointed to variation in 17-beta hydroxysteroid dehydrogenase 2 (HSD17B2) inside the supergene as a cause of large differences in circulating androgens, which likely contribute to the differences in aggressive

behaviors among the morphs [15]. Altogether, forward genetics, studies of specific genes, and comparative approaches have revealed genetic and mechanistic bases of the evolution of behavior.

Evolution of behavior through changes in cell type composition

Santiago Ramón y Cajal was the first to note that homologous neuronal cell types — based on morphology and location — were present in the brains of many species [45]. Whereas many cell types are evolutionarily conserved, there are also many differences in the types of neurons that compose nervous systems across species. These cell-type similarities and differences across species have been described for many years [46], yet the development of high-throughput single-cell RNA sequencing has enabled an unprecedented characterization of the cellular composition of the brain and other tissues in many species. Using these new technologies, the similarities and differences in neuronal composition across animals are now being thoroughly studied.

In humans, there are 461 to ~3300 brain cell types depending on how they are classified [47]. Notably, all major classes of excitatory and inhibitory neurons in the cortex are conserved between mice and humans [48]. However, there are major differences in the proportions of some types of neurons between the species. Particularly prominent is the variation in the ratio of excitatory to inhibitory neurons in primary motor cortex: 2:1 in humans, 3:1 in marmosets, and 5:1 in mice [49]. There are also large differences in the proportion of more specific types of neurons. For example, cortical neurons that project outside the telencephalon (also known as pyramidal tract neurons) constitute only 1% of layer 5 excitatory neurons in humans but 20% in mice [48] and a class of interneurons that express *LAMP5* and *LHX6* are eightfold more abundant in humans and other primates than in mice [50]. An even more extreme case is that of interneurons that express *VIP* and *TAC3* in the striatum. This cell type constitutes ~30% of the striatum of humans and marmosets but is absent from the striatum of mice [50]. Thus, the neuronal composition of the primate brain differs significantly from that of mice, and there are also important differences between humans and other primates.

The cell type composition of nonprimate brains has also been studied. Among closely related species, single-cell transcriptomic characterization of the whole brains of three *Drosophila* species (*D. melanogaster*, *D. simulans*, and *D. sechellia*) found that there were no cell types that were unique to any of these species but did find differences in the abundance of two neuronal and one nonneuronal (the perineurial glial cells that form the blood–brain barrier) cell types in *D. sechellia* [51]. Two distantly related teleost fishes (zebrafish and Mexican tetra) share over 75% of cell types in the hypothalamus [52]. More distant taxonomic comparisons of neuronal cell types among mammals, amphibians, and sauropsids, including lizards, turtles, and birds, have shown that inhibitory GABAergic neurons are more conserved than excitatory cells and have revealed lineage-specific cell type innovations [53–57]. It is currently unknown whether these cell type differences across species contribute to species-typical behaviors, but their identification now provides molecular handles for future study.

Consequences of evolutionary changes to cell type composition

Part of the difficulty in characterizing how variation in cell type composition among vertebrate brains affects behavior is that many of the compared species diverged from each other millions of years ago [49,50,58–61]. Thus, in addition to differences in cell type composition, homologous cell types often differ greatly in their gene expression, even in the very marker genes that distinguish them from other cell types of their own species [61]. Furthermore, it is challenging to manipulate the abundance of specific cell types to measure effects on behavior. Finally, the tetrapod studies above focused on the pallium (largely corresponding to the cortex in mammals) and striatum, where neurons rarely specialize in single behaviors such as eating, fighting, or escaping — as is more commonly the case for neurons in the hypothalamus and midbrain — making it difficult to even hypothesize the consequences of the evolution of cell type composition.

A clear example of how evolutionary changes to the abundance of cells with defined neurobiological functions can lead to clear interpretations comes from a comparative study of the retina of nocturnal house mice and diurnal four-striped mice (*Rhabdomys pumilio*) [62]. In house mice, only 3% of photoreceptors are cones, whereas in striped mice, it is over 65%. This, coupled with 18× more rod bipolar cells, more OFF-bipolar cells and more OFF and ON-OFF retinal ganglion cells in striped mice likely contributes to their higher spatiotemporal visual resolution, which benefits them under daylight.

As with photoreceptors, other sensory neurons are also specialized to respond to specific physicochemical stimuli. They are often connected to the brain via 'labeled lines' that trigger particular innate behaviors, such as attraction or avoidance. Therefore, changes to sensory neurons among individuals and species can have specific effects on behavior and hence fewer detrimental pleiotropic effects. Indeed, in addition to genetic variation that retunes sensory receptors or alters their expression levels (reviewed in Refs. [4,63]), evolutionary changes in

the number of specific sensory neurons have also been documented. For example, ducks that forage guided by touch using their bill have more sensory neurons expressing the mechanically gated channel Piezo2 innervating the bill compared to chickens, which forage guided by vision [64].

Ducks and chickens are evolutionarily quite distant, yet the rather defined function of the mechanosensitive neurons in their bill makes for a plausible contribution to their distinct tactile foraging behaviors. Still, studying the evolution of cell types across species that are more closely related facilitates functional and genetic interrogation. For example, *Prionotus carolinus* sea robins use their 'legs' (modified pectoral fins) to dig in the sand and to taste while foraging for prey [65]. Leg chemosensation is mediated by specialized sensory papillae that express T1r2 and T1r3 taste receptors. Notably, the closely related sea robin *P. evolans* does not dig in the sand to find food and lacks sensory papillae and taste receptor expression in its legs. A powerful comparative system has been the study of closely related *Drosophila* species. *Drosophila sechellia* flies, which specialize in noni fruit, have ~2.5× more olfactory sensory neurons specialized in detecting noni fruit odors — those expressing the olfactory receptors Or22a, Or85b, and Ir75b — than *D. simulans*, from which they diverged only ~150 000 years ago [6,9,66] (Figure 1). The functional significance of the increase in Or85b and Ir75b has not yet been tested, but the extra Or22a sensory neurons contribute to noni odor tracking by *D. sechellia* [23].

Two recent studies comparing closely related species documented the evolutionary loss and gain of specific cell types and the behavioral consequences. *Drosophila yakuba* lack ventral nerve cord TN1A neurons, which in *Drosophila melanogaster* trigger sine song during male courtship of females (Figure 1). Accordingly, *D. yakuba* does not produce sine song [67]. In mammals, monogamous and biparental oldfield mice (*Peromyscus polionotus*) have recently evolved a new cell type — the *zona inaudita* — in their adrenal gland. The *zona inaudita* is not present in house mice, humans, or even other *Peromyscus* species, including in closely related cloudland deer mice (*P. maniculatus nubiterrae*), from which oldfield mice diverged only around 20 000 years ago [19] (Figure 1). This new cell type produces the steroid hormone 20α-hydroxyprogesterone, which contributes to the elevated parental behavior of this species [19]. Thus, both losses of existing cell types and gains of new ones shape the evolution of behavior. The case of the *zona inaudita* shows that to comprehensively investigate the evolution of behavior, characterizing cell type composition throughout the body — beyond brain neurons — will be important.

Outstanding questions about how the evolution of cellular composition shapes behavior

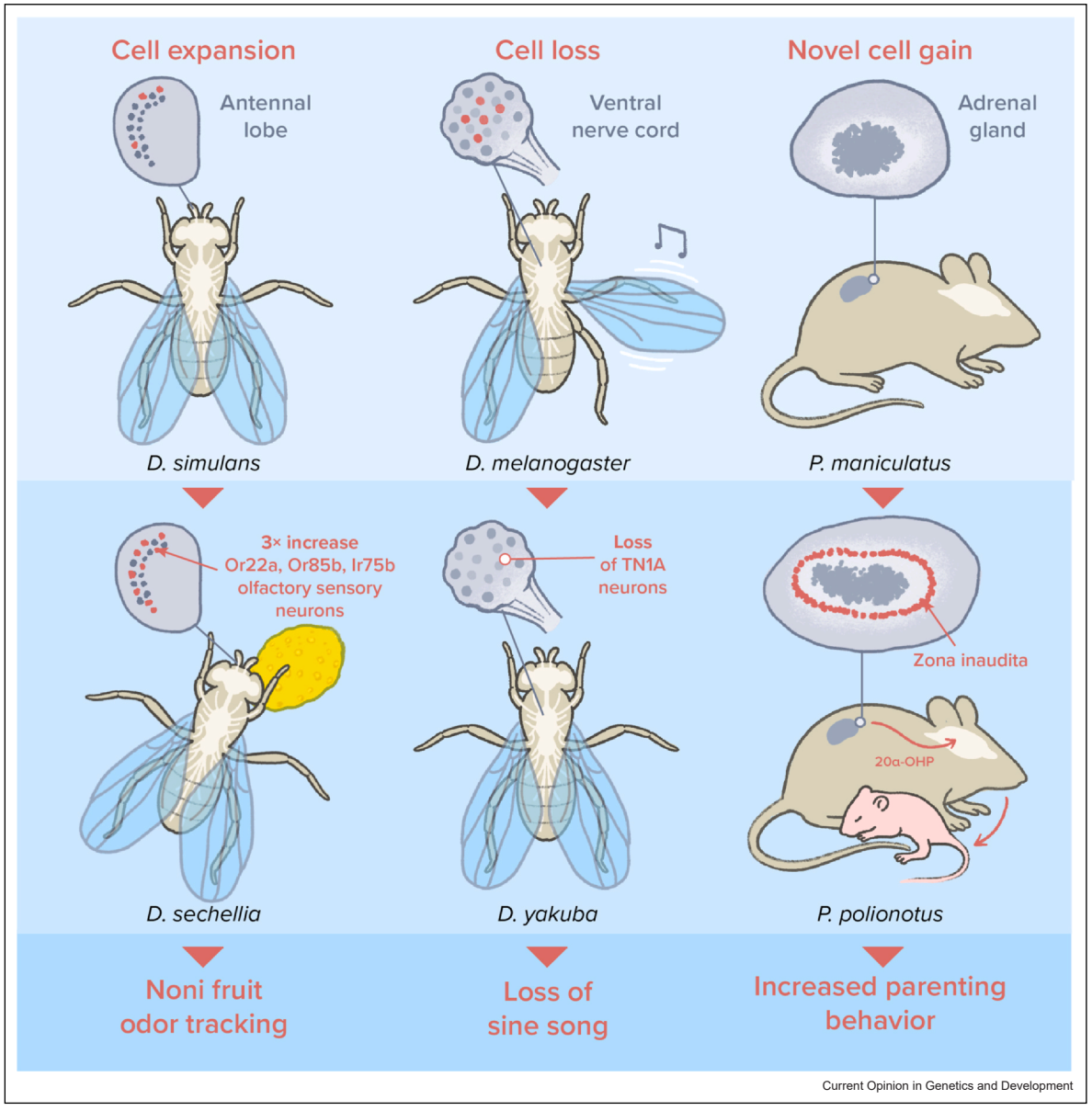
We are only beginning to understand how evolution of cell type composition in and outside the brain affects behavior and many questions remain. What is the genetic architecture that shapes cellular composition and which are the causal genes? The expansion of Or22a olfactory sensory neurons in *D. sechellia* involved many genetic changes that remain to be clearly mapped [23]. A QTL-mapping study of the emergence of the *zona inaudita* cell of the oldfield mouse adrenal revealed that multiple genetic changes contributed to the evolution of this cell type. The most prominent genetic changes corresponded to *cis*-regulatory elements affecting Gadd45a (encoding a nuclear protein) and Tenascin N (encoding an extracellular matrix protein) acting cell autonomously in the cells that become the *zona inaudita* [19]. The biochemistry and cell biology by which the gain of expression of Gadd45a and Tenascin N facilitate the ontogenesis of the *zona inaudita* remains to be determined. In *D. yakuba*, loss of the TN1A neurons is caused by developmental apoptosis mediated by *doublesex* [67]. The molecular mechanisms by which *doublesex* causes apoptosis of these neurons specifically in *D. yakuba* are still unknown, as are the causal genetic changes.

Powerful genetic tools in *Drosophila* have enabled testing how cellular composition affects behavior. However, these experiments are still challenging even in *Drosophila* but especially in vertebrates. New tools, possibly empowered by a thorough understanding of developmental programs, would facilitate answering questions such as how evolutionary changes in the proportion of pallial excitatory and inhibitory neurons and other brain neurons shape neuronal computations and behavior.

Whereas comprehensively characterizing neurons that are abundant in a tissue through single-cell technologies is now routinely possible, it is still challenging to include rare cell types. Considering that the human brain has $\sim 10^{11}$ cells and that the high cost of single-cell RNA sequencing typically allows for sampling a very small fraction of those cells, rare cell types tend to be underrepresented. Thus, more complete catalogs of cell types in and outside the brain across species might require a combination of large investments in deep sampling, spatial transcriptomics, and targeted approaches for low-abundance cell types.

Another outstanding challenge is the computational comparison of single-cell genomic data across species. Multiple algorithms have been developed for this

Figure 1



Changes in cell type composition, including cell expansions, cell losses, and novel cell gains, are associated with the evolution of specific behaviors. Figure credit: Matteo Farinella.

purpose [53,68,69], including reciprocal principal component analysis [70], which appears robust in some studies [19,51]. However, the performance of these methods for cross-species integration could be more thoroughly evaluated. Part of the integration challenge is compounded by the difficulty of defining cell types. Using single-cell RNA sequencing methods, cell types are often defined as cells that cluster together in multidimensional gene-expression space. Yet, these clusters can sometimes reflect transient cell states instead of more stable cell types. Moreover, the number of clusters is sensitive to computational parameters. These two issues lead to ambiguity in the classification of cell types, their accurate quantification, and their integration between species. Furthermore, the transcriptomic definition of cell types may not match a functional definition. For example, cells that differ in biochemical or physiological function due to variation in a small number of key functional genes might not differ in enough genes to cluster separately. It is therefore useful to consider both genomic and functional methods to study cell type evolution.

The thorough characterization of cell type composition enabled by single-cell genomics has added a new dimension to the study of the evolution of behavior. This, coupled with emerging tools to manipulate the abundance and function of cell types that differ between species, promises to give us a richer understanding of how behavior evolves.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

None.

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