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Evolution of Animal Neural Systems

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Abstract

Nervous systems are among the most spectacular products of evolution. Their provenance and evolution have been of interest and often the subjects of intense debate since the late nineteenth century. The genomics era has provided researchers with a new set of tools with which to study the early evolution of neurons, and recent progress on the molecular evolution of the first neurons has been both exciting and frustrating. It has become increasingly obvious that genomic data are often insufficient to reconstruct complex phenotypes in deep evolutionary time because too little is known about how gene function evolves over deep time. Therefore, additional functional data across the animal tree are a prerequisite to a fuller understanding of cell evolution. To this end, we review the functional modules of neurons and the evolution of their molecular components, and we introduce the idea of hierarchical molecular evolution.

INTRODUCTION

When Anton van Leeuwenhoek looked through his microscope, saw swimming microorganisms, and called them "animalcules" due to their rapid motions, he was the first witness to the remarkable similarities between the behavior of microbes and animals (van Leewenhoeck 1677). Behavior is widespread among unicellular life forms, but among multicellular organisms, animals are preeminent in the rapidity and diversity of their behavioral repertoire. This preeminence is due to key differences in the ways that animals have achieved a multicellular lifestyle as compared with plants, fungi, and red and brown algae, the other large multicellular eukaryotic lineages that sacrificed motility for stability in the transition to multicellularity. The development of novel pathways for cell adhesion (Abedin & King 2010) and intercellular signaling (Babonis & Martindale 2017) laid the groundwork, but the advent of neurons and muscles were the key adaptations that allowed animals to maintain complex motility while evolving large multicellular bodies.

With the availability of genome sequences from diverse animal phyla (Moroz et al. 2014; Ryan et al. 2013; Srivastava et al. 2008, 2010), a lively debate has sprung up over the early evolution of neurons and, more broadly, over how to interpret genomic data in a way that best enlightens the deep origins of complex tissue types (Achim & Arendt 2014, Hejnol & Lowe 2015, Jekely et al. 2015, Moroz 2009, Moroz et al. 2014, Ryan 2014). During 2015–2017 alone, there were five journal issues dedicated completely or in part to the early evolution of nervous systems. This trend is a continuation of perennial debates over the nature and evolutionary history of the first neurons (Bishop 1956, Mackie 1990): When did the first neurons evolve? What kinds of cells did they evolve from? What were their early functions?

For the first time, these debates are centered on the interpretation of molecular and genomic, rather than phenotypic or physiological, data. Yet the new genomic data have not been able resolve the old debates. Why? Neurons, and especially neural systems, are not monolithic entities. They consist of numerous molecular machines and their constituent proteins, each carrying out the processes we identify with neural function. These constituents may have evolutionary histories that differ both from one another and from the phenotypes they mediate, making it difficult to distinguish among scenarios of phenotypic evolution using genomic data alone (Hejnol & Lowe 2015, Liebeskind et al. 2016). Knowledge of how proteins are used in different cell types across taxa can help by contextualizing genomics data and guiding inquiry toward relevant gene families. Here, we review the key functional modules of neurons, detail what is known about the diverse evolutionary histories of the molecular constituents of these modules, and discuss pitfalls of comparative genomics. We end with an example of how genomic data can be contextualized and interpreted in a hierarchical fashion by exploring the surprisingly plastic evolution of the neuromuscular junction (NMJ).

PHYLOGENETIC CONTEXT OF EARLY NEURAL EVOLUTION

When did the first neurons arise, and how? Although these questions are still hotly debated, the phylogenetic context for early neural evolution is being resolved in conjunction with the growing acceptance of a revised animal tree of life (Dunn et al. 2014). Perhaps the most radical change to the animal tree of life has been a growing consensus that ctenophores, which have a complex neural system and behavior, branched off first from the remaining animal lineages (**Figure 1**). Sponges had previously been considered to be the earliest branching lineage, and their lack of neurons and sedentary lifestyle as adults were thought to be the ancestral condition of animals. Although it is still possible that the placement of ctenophores results from problems in phylogenetic inference (Pisani et al. 2015, Simion et al. 2017), the early splitting of ctenophores is now supported by

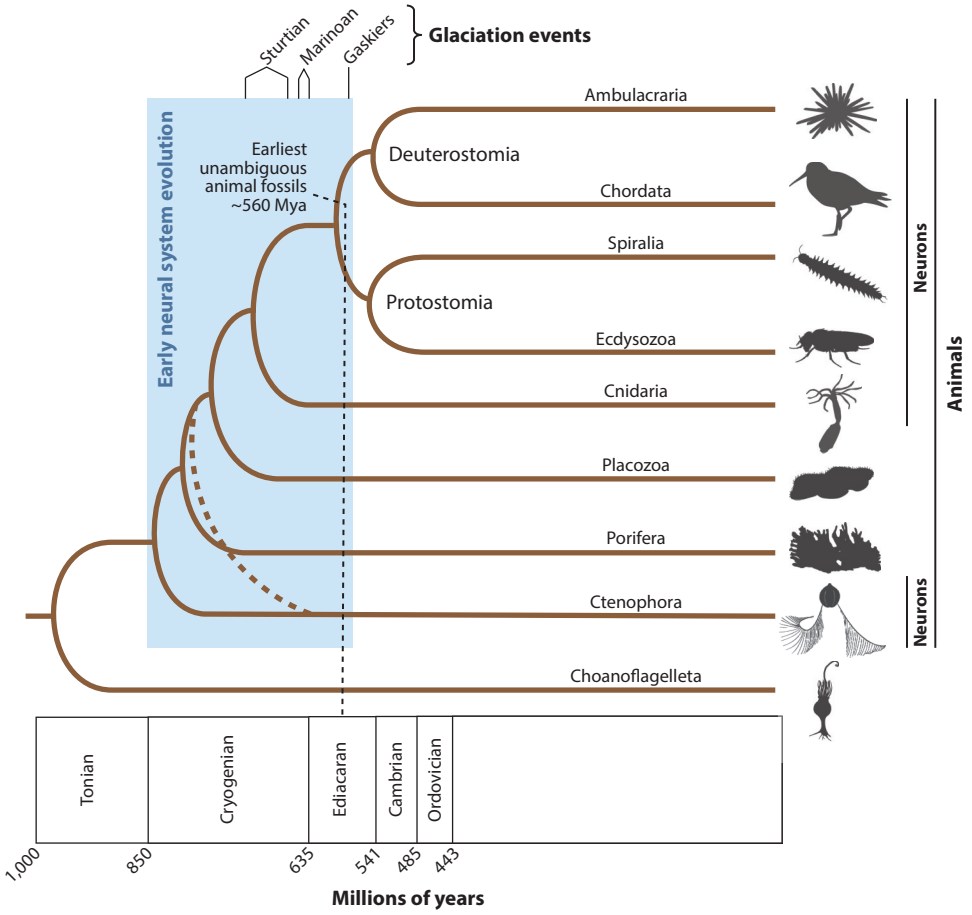


Figure 1

Time tree of early animal divergence. The deepest divergences predate unambiguous animal fossils by hundreds of millions of years, even when taking plausible confidence intervals into account (dos Reis et al. 2015). The most likely explanation for this discrepancy is that the first animals were too small and simple to fossilize as recognizable metazoans. Early neurons arose during this period, so their origins must be reconstructed primarily from extant animal lineages. Silhouettes here and throughout are from PhyloPic (**http://phylopic.org**). Abbreviation: Mya, million years ago.

a variety of careful studies (Arcila et al. 2017; Dunn et al. 2008, 2014; Moroz et al. 2014; Ryan et al. 2013; Whelan et al. 2015). This placement of ctenophores raises the question of whether neurons arose once in animal evolution and were lost in sponges and placozoans (the single origin hypothesis) or arose independently in ctenophores and Planulozoa (cnidarians plus bilaterians) (the multiple origins hypothesis) (**Figures 1** and **2**) (Dunn et al. 2008, Moroz et al. 2014).

The placement of sponges and ctenophores is important but not decisive for reconstructing the origin(s) of neurons (Liebeskind et al. 2016). The debate has often operated on the assumption that the two topologies suggest either a ctenophore-like or sponge-like animal ancestor, but neither is likely to be the case. The deepest divergences of extant animal lineages stretch back to the Cryogenian (720–635 million years ago), long before the appearance of large animal fossils (**Figure 1**) (Cunningham et al. 2017, dos Reis et al. 2015). Thus, it is entirely plausible that animals

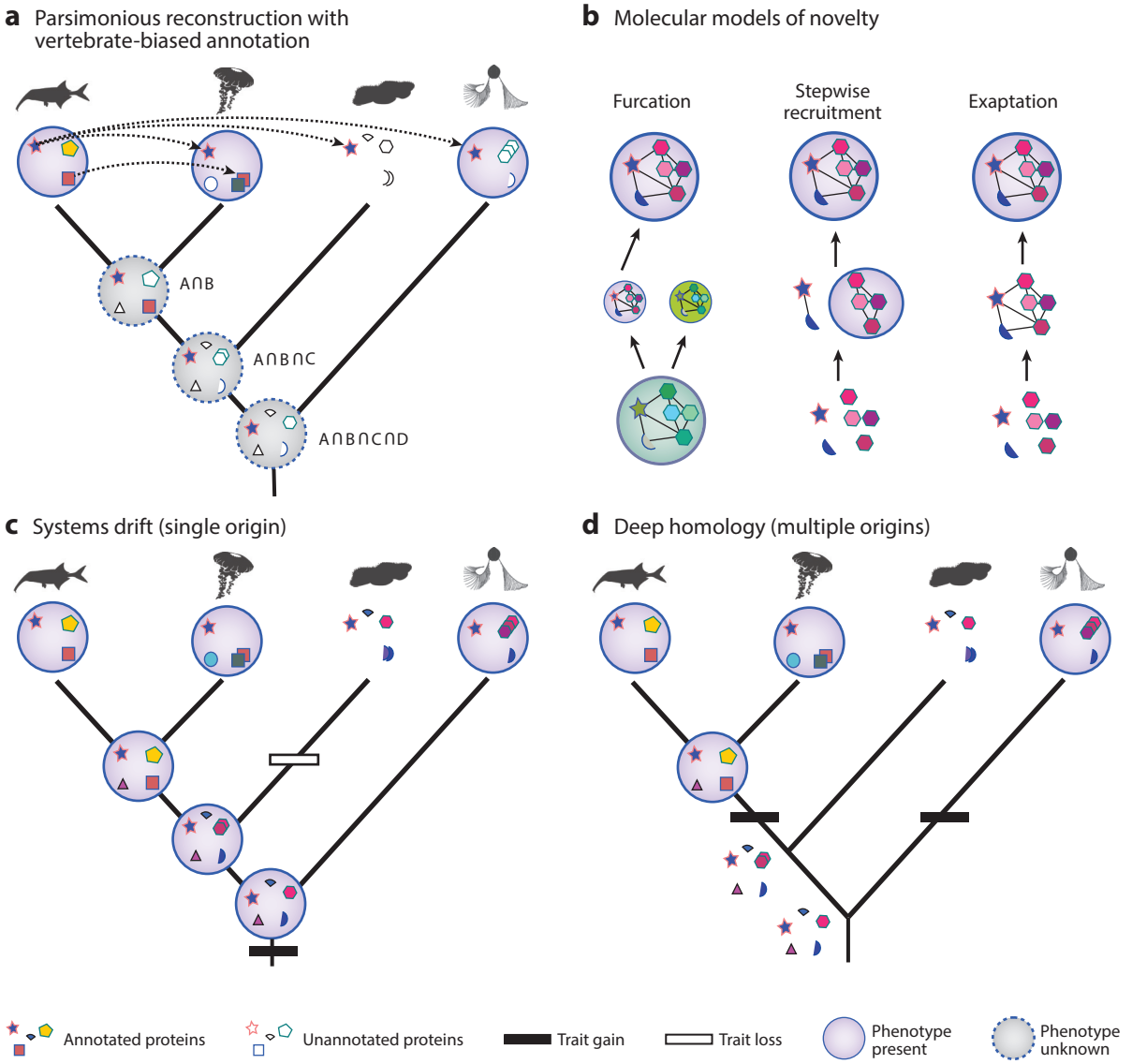


Figure 2

Practical and theoretical difficulties in the inference of phenotypic evolution from genomic data. (*a*) Biased gene annotation obscures the complexity of nonfocal organisms and internal nodes. (*b*) Three models of trait origins at the molecular level. Furcation (sensu Oakley et al. 2007) is the simple splitting of cell lineages or other phenotypes. Stepwise recruitment takes into account the fact that many mechanisms predate the phenotypes they currently mediate (Achim & Arendt 2014). Exaptation (sensu Gould & Vrba 1982) is the wholesale reappropriation of molecular modules into a novel phenotype. These models are not mutually exclusive and could all be relevant for different molecular components of a phenotype. (*c*,*d*) Identical taxonomic distributions of phenotypes and molecular components can be used to support a single origin or multiple origins of neural systems or any other phenotype, depending on whether one assumes (*c*) systems drift or (*d*) deep homology.

began diversifying before becoming more complex, but these early animals were too small and delicate to fossilize or to be recognizable as animals. Importantly, no extant lineage is frozen in time, so resolution of the tree is necessary but not sufficient for ancestral character reconstruction. As the early evolution of animal neural systems occurred during this dark period in the Cryogenian (**Figure 1**), we must use extant molecular and physiological data to ask whether neurons evolved once or repeatedly and what the first neurons were like.

THE PROMISE AND PERIL OF GENOMIC DATA

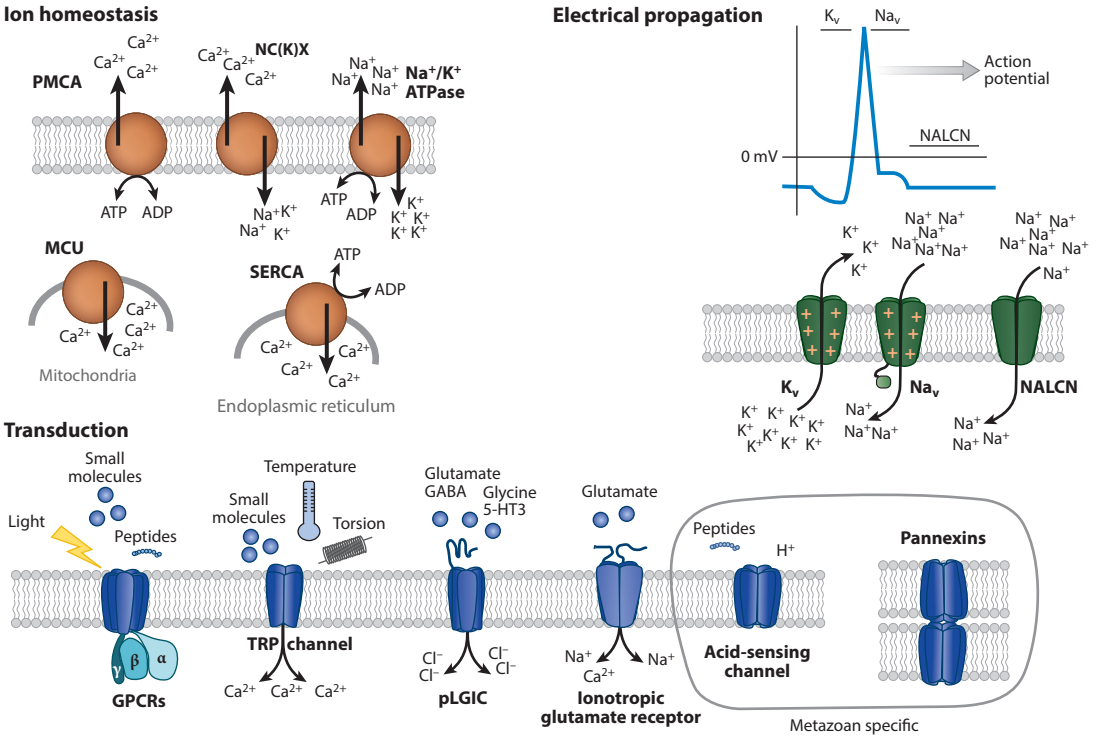
Despite the new genomic data from diverse animal phyla and improved resolution of the animal tree of life, the field has failed to reach agreement on the nature and timing of the early evolution of neurons. There are several reasons for this. Predicting ancient phenotypes from genomic data requires us to (*a*) reconstruct ancestral genotypes and then (*b*) predict phenotypes from these data by identifying how the proteins and phenotypes came to be associated. The best way to infer ancestral genotypes is a topic beyond the scope of this review, but we note one common artifact: Gene annotation biased toward a particular lineage will have the effect of masking complexity in other lineages and ancestral states (**Figure 2*a***) (Dunn et al. 2015), giving the impression of an upward march of complexity toward the focal lineage.

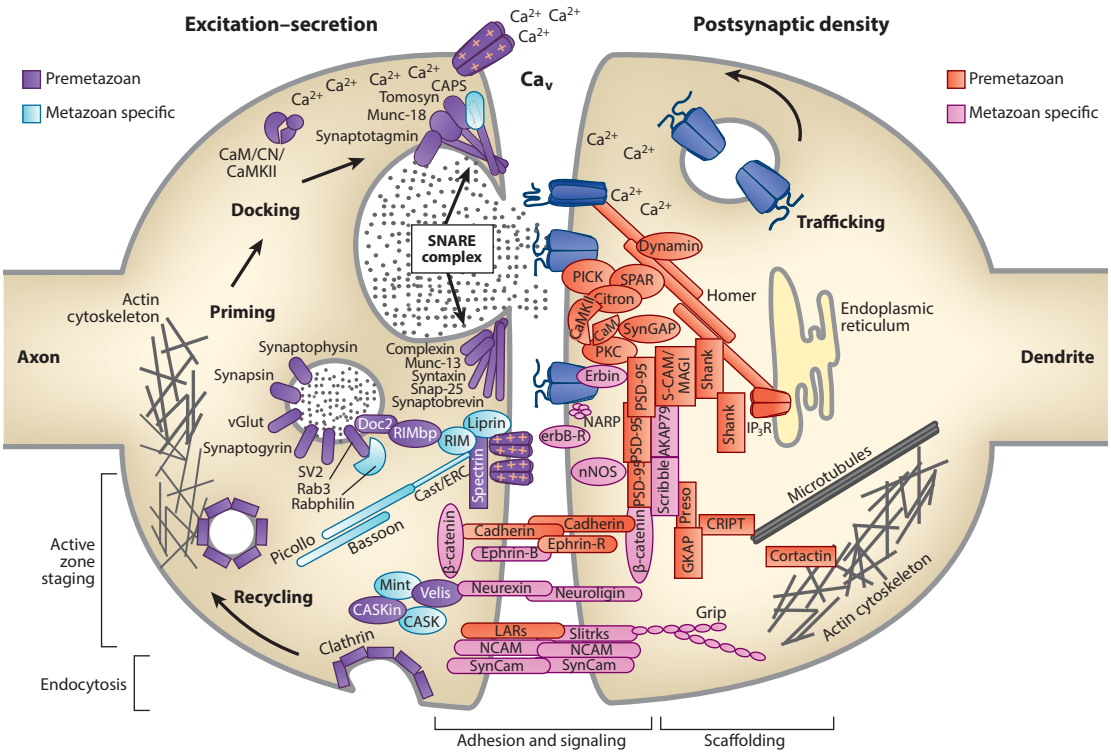
The prediction of phenotypes from data (step *b* above) requires us to use one or several of the models of novel trait origins to predict phenotypes from the reconstructed genotypes (**Figure 2*b***). These models include furcation (sensu Oakley et al. 2007), or lineage splitting, of, for example, a cell type and the proteins expressed there, followed by modification; stepwise recruitment of already-existing proteins into novel interaction networks within a new cell type (Achim & Arendt 2014, Arendt et al. 2016); and the wholesale recruitment or exaptation (Gould & Vrba 1982) of preexisting functional networks into a novel phenotype. These models are simplifications; several may apply to the various mechanistic modules within complex cell types, making it difficult to come to conclusions about phenotypes. Furthermore, the structure of proteins and molecular systems is not uniquely determined by the phenotypes they encode, and vice versa. For instance, homologous phenotypes can diverge in molecular composition (known as systems drift; True & Haag 2001), and homoplastic phenotypes can independently recruit homologous molecular modules (known as deep homology; Shubin et al. 1997) (**Figure 2*c*,*d***). Therefore, the identical reconstructions of ancestral genomes can be used to support opposite phenotypic conclusions (**Figure 2*b–d***), depending only on a priori assumptions.

It seems clear, then, that we cannot always predict ancient phenotypes from genomic data alone. A robust determination of homology must rely on a better understanding of how molecular modules evolve within cell-level phenotypes. The next challenge is to start putting the genomic data back into its physiological context, and this will involve developing comparative methods that explicitly take into account not just genes but also whole physiological systems. Only then will we be able to distinguish between the models in **Figure 2*b***. Because neurons are among the most complex and best-studied cell types, comparative neuroscience offers an ideal system within which to employ such hierarchical comparative methods to reach a better understanding of cell-type evolution. To this end, we next review the evolutionary dynamics of neural molecular systems in their physiological context and then give an example of how comparative approaches can, and must, go beyond the level of genes.

WHAT IS A NERVOUS SYSTEM?

By nervous system we typically mean the network of neurons that underlies animal behavior. It has long been appreciated that the term nervous system is imprecise (Bishop 1956). Many other





cell types beside neurons are nervous—that is, electrically excitable—and exist in systems, such as pancreatic or muscle cells. Plants and unicellular organisms also make use of electrical excitability to mediate behavior, but are not said to have nervous systems. It is the presence of neurons that really distinguishes animal nervous systems, so we prefer the term neural systems. A neuron is typically defined morphologically and physiologically as an elongated and excitable cell that synapses onto another cell. To this admittedly vague definition (glial cells can also be excitable and make synapses), we add the functional qualification that neurons are usually said to encode information. That is, their particular activity is an arbitrary symbol or code of downstream activity, ultimately of animal behavior. A neural system encodes information in two ways, which we use to structure our discussion below: first, in an electrical code within neurons and, second, in the wiring code or connectome between neurons. Plasticity in these signaling modes is responsible for learning, memory, development, and behavioral complexity.

ELECTRICAL CODE

Electrical excitability is a defining feature of neurons. Most neurons propagate regenerative, all-or-none electrical disturbances called action potentials. These spikes can be sent over meters-long axons and arrive at the nerve terminal with near-perfect fidelity; thus, they constitute a digital signal. Like fidelity, conduction speed is a major adaptive feature of neurons, allowing organisms to move rapidly. Thus, giant axons, which reduce internal resistance, have evolved multiple times as part of escape behavior circuits, most famously in crayfish, squid, and teleost fish, but also in cnidarians and ctenophores (Edwards et al. 1999, Mackie 1989, Mackie et al. 1992). Myelination, which insulates axons, was previously thought to be a vertebrate-specific innovation but, in fact, has evolved several times in protostomes as well (Castelfranco & Hartline 2016). The creation of the neural electrical code relies on three modules of proteins: one that creates the potential energy for the action potential (ion homeostasis), one that transduces sensory and intercellular signals into the electrical code (transduction), and one that propagates the electrical signals along neurons (propagation) (**Figure 3**).

Ion Homeostasis

The flow of ions that creates an action potential is powered by actively maintained electrochemical gradients. The maintenance of these gradients is a common feature of all cells, but in neurons the energetic cost is much steeper. In humans, the proteins that pump ions in and out of neurons use up to 10% of the body's energy in the brain alone (Laughlin et al. 1998). The key tasks of neuronal ion homeostasis are maintaining a low cytoplasmic calcium level to protect the fidelity of calcium signaling and maintaining a negative voltage across the membrane to power action potentials.

The primary means of maintaining membrane potential is active pumping via ATPase (adenosine triphosphatase) pumps (**Figure 3**). Proteins from the same family as neuronal ATPases, called

Figure 3

Molecular details of four molecular modules of neurons for which extensive evolutionary information is available. Most channels, pumps, and exchangers are evolutionarily ancient, whereas certain submodules in synapses are more recent innovations. Abbreviations: ADP, adenosine diphosphate; ATPase, adenosine triphosphatase; GABA, γ-aminobutyric acid; GPCR, G protein–coupled receptor; MCU, mitochondrial calcium uniporter; NALCN, sodium leak channel nonselective; pLGIC, pentameric ligand–gated ion channel; PMCA, plasma membrane calcium ATPase; SERCA, sarco/endoplasmic reticulum calcium ATPase; TRP, transient receptor potential; v, voltage gated.

P-type ATPases, can be found in all cellular life forms (Barrero-Gil et al. 2005, Thever & Saier 2009). Proteins that maintain low cytoplasmic calcium levels (**Figure 3**) are found across eukaryotes as part of a pan-eukaryotic calcium-signaling toolkit (Cai & Clapham 2012, Cai & Lytton 2004).

The origin of neurons does not seem to be correlated with an increased number of genes for these pumps and exchangers. For instance, humans have 24 P-type ATPases, whereas yeast have 14 and flies 12. The plant *Arabidopsis thaliana* has 46 (Thever & Saier 2009). Thus, the ion homeostasis module is both evolutionarily ancient and pleiotropically expressed in multiple cell types. Although there may have been protein-level specializations associated with the origin of neurons in this module, they are not currently well understood.

Transduction

The first step of neural signaling is often the transduction of an input signal, either from the environment or another cell, into an electrical signal. This occurs both at synapses between neurons and at sensory neurons, such as photoreceptors or olfactory neurons. These two distinct tasks, sensory transduction and synaptic transduction, are often treated separately, but many of the same proteins are expressed in both submodules across animals. Of the many protein families involved in transduction, only two are metazoan novelties (**Figure 3**): All others were present in the unicellular ancestors of animals (Jaiteh et al. 2016, Liebeskind et al. 2015, Ryan et al. 2013), where they were probably used for environmental sensing. The proteins involved in the transduction of extracellular signals into electrical signals in neurons are members of large gene families that have rapid turnover and many instances of convergent evolution of ligand specificity, even across nonrelated gene families. Correspondingly, these proteins are used differently by different animal lineages.

Two major families of proteins transduce environmental stimuli into electrical signals in vertebrate neurons: transient receptor potential (TRP) channels and G protein–coupled receptors (GPCRs) (**Figure 3**) (Dalton & Lomvardas 2015, Touhara & Vosshall 2009). GPCRs form the largest gene superfamily in tetrapods, with the majority being olfactory receptors (Niimura 2012). Individual GPCR genes, especially olfactory receptors, are rapidly duplicated, lost, or inactivated (Nei & Rooney 2005). GPCRs also mediate taste and photoreception. This latter sensory modality is mediated by opsins, which bind a chromophore whose response to light sets off the G protein signaling cascade. There have been independent expansions of opsins in cnidarians (Sakarya et al. 2008), notably in cubozoan jellyfish, which represent one of three lineages of animals to have evolved lens-bearing eyes (Liegertová et al. 2015). Curiously, although opsins were lost in the sponge lineage, ciliated sponge larvae are phototactic. This behavior is mediated by cryptochromes instead of opsins (Rivera et al. 2012), probably a case of systems drift.

Whereas mammalian odorant receptors are all GPCRs, hexapod odorant receptors are all ion channels: a remarkable case of evolutionary flexibility that is not yet fully understood. Some hexapod channels belong to the same class of seven transmembrane segment proteins as do GPCRs (odorant receptors and gustatory receptors; Silbering & Benton 2010, Touhara & Vosshall 2009), and others are part of the ionotropic glutamate receptor (iGluR) family (ionotropic receptors; Croset et al. 2010), which in vertebrates are solely expressed in synapses. Nematodes have a large number of GPCRs, some of which play a part in chemoreception, as well as orthologs of ionotropic receptors whose function is unclear (Silbering & Benton 2010). Cnidarians have homologs of hexapod gustatory receptors that, curiously, have a role in development, with no indication of a role in chemosensation (Saina et al. 2015). Thus, it is not clear whether the bilaterian ancestor primarily used GPCRs for chemoreception, ion channels, or both.

TRP channel families are not nearly as large as those of GPCRs, but are molecularly diverse. They underlie mechanosensation, thermosensation, and certain types of gustatory compounds, such as capsaicin and menthol (Julius & Nathans 2012). The ASC (acid-sensing channel) family has a role in both sensation (salt taste) and synaptic transmission (Bianchi & Driscoll 2002), with a larger role in synaptic transmission in cnidarians (Assmann et al. 2014).

Like sensory receptors, synaptic channels and receptors exist in large gene families that have undergone substantial independent expansions in a number of animal lineages, and this may have played an important part in the convergent origins of synaptic complexity (Liebeskind et al. 2015). In vertebrates, the most important synaptic receptors are either GPCRs or ion channels of the iGluRs or pentameric ligand–gated ion channel families (pLGICs, also called Cys-loop receptors). Nearly all the major neurotransmitters target both ionotropic and metabotropic receptors, and there are key differences between nonvertebrate and vertebrate receptors that suggest widespread convergent evolution for ligand specificity, just as in olfactory sensation. For instance, ionotropic glutamate receptors in vertebrates are all part of the ion channel superfamily iGluR, an ancient group of tetrameric channels distantly related to voltage-gated potassium channels (Chiu et al. 1999). These channels create an excitatory response in downstream neurons. In protostomes, such as *Drosophila* and *Caenorhabditis elegans*, however, some ionotropic glutamate receptors belong to the pLGIC channel family and create an inhibitory response. Moreover, these protostome glutamate receptors have evolved several times independently (Kehoe et al. 2009, Lynagh et al. 2015). Ctenophores have lost pLGICs altogether, the only lineage with a neural system known to have done so, and may rely on other channel families for the same function, such as iGluRs and ASCs (Liebeskind et al. 2015, Moroz et al. 2014, Ryan et al. 2013). Consistent with this hypothesis, it has recently been shown that the radiation of the iGluR family in ctenophores has resulted in the evolution of both glutamate- and glycine-sensitive isoforms convergently with bilaterians (Alberstein et al. 2015).

Taken together, these considerations show how flexible sensory and chemical transduction systems can be in terms of their constituent proteins. Many different proteins can have the same role within a neuron.

Electrical Propagation

Local electrical signals are transient and will attenuate over space. To propagate signals over large distances, neurons use ion channels that respond to the voltage itself, called voltage-gated ion channels. Voltage-gated ion channels and the regenerative potentials they create are found across the tree of life. For instance, plants with rapid behaviors, such as the Venus flytrap (*Dionaea muscipula*) and the sensitive plant (*Mimosa pudica*), trigger these behaviors with ionic action potentials conducted through the phloem (Simons 1981). Protists also use action potentials to trigger bioluminescence (Oami et al. 1995), to coordinate cell deformations (Febvre-Chevalier et al. 1986), and to control ciliary beating (Saimi & Kung 1987). Bacterial biofilms also use regenerative potential changes to coordinate colony growth (Prindle et al. 2015). In animals, neurons are not the only cell types to make use of electrical excitability: Insulin release from the β-cells of the pancreas, muscle contraction, and chemotaxis in sperm are all mediated by electrical signaling (Hille 2001, Lishko et al. 2012).

In plants, protists, and fungi, the change in electrical potential is often a by-product of the main task of the impulse: the delivery of an ion species that effects a change in cell biology. Typically, this ion is calcium, which triggers a variety of cellular processes. In plants, the action potential creates an osmotic potential, and the resultant turgor pressure is the plant equivalent of muscle contraction. However, in animal axons, the action potential is a signal, and it does not

substantially alter the cell biology of an axon until it arrives at the terminus. How did neurons elaborate a primarily intracellular signaling pathway into a complex electrical code, often expressed as dynamic bursts of action potentials?

The key change that allowed this development was the evolution of voltage-gated sodium channels (Na_v) from preexisting voltage-gated calcium channels (Ca_v), as Na^+ does not broadly trigger cellular signaling pathways as Ca^{2+} does. This allowed animal axons to generate action potentials at a high rate without poisoning the cell with Ca^{2+} (Hille 2001). Bona fide Na^+-selective channels arose twice, once in cnidarians, and once in the bilaterian ancestor (Gur Barzilai et al. 2012, Liebeskind et al. 2011), suggesting convergence toward a complex neural code in these lineages. Another member of the voltage-gated family has lost its sensitivity to voltage, and has been called sodium leak channel nonselective (NALCN). This channel family keeps neurons near the threshold for Na_v activation and is crucial for rhythmically firing neurons that mediate, for example, breathing in mice and snails (Ren 2011). NALCN channels have also likely converged toward Na^+ permeability via similar mutations in their ion selectivity filter (Liebeskind et al. 2012, Senatore et al. 2013).

After a positive displacement by Na_v channels, action potentials are then repolarized by K^+ efflux through voltage-gated potassium channels (K_v). Therefore, K_v channels have the central role in shaping the neural electric code, and they are a large and diverse family. Although there are typically fewer than 5 Na_v genes in animal genomes (despite having radiated to 8 in fish and 10 in tetrapods), there are typically 10 times as many K_v genes (Hille 2001). In animals, the K_v gene family has radiated independently in a number of lineages (Li et al. 2015, Liebeskind et al. 2015), presumably for the regulation of an independently expanding electrical code. In a remarkable case of convergence, Erg-type K_v channels have radiated independently in cnidarians and bilaterians, and in both radiations they converged toward two distinct types of pore closure (Martinson et al. 2014).

Therefore, the broad similarities between electrical signaling in cnidarians and bilaterians are largely a result of convergent evolution. Almost nothing is known about the physiology of ctenophore neurons, although their muscles make mostly calcium-based action potentials (Bilbaut et al. 1989). Glass sponges can propagate electrical impulses through their syncytial tissue (Leys et al. 1999), but demosponges (and perhaps other sponge lineages) have lost all voltage-gated-channel genes (Liebeskind et al. 2015, Srivastava et al. 2008). Much remains to be discovered about the early evolution of electrical signaling.

WIRING CODE

Besides the electrical code within neurons, animal behavior is also encoded in specific wiring diagrams (or connectomes) between neurons. The connections between neurons, and between neurons and downstream effector cells, occur at specialized cell junctions called synapses. Synapses can occur by direct electrical coupling between two cells, but chemical synapses, in which communication is via the release of a neurotransmitter, are more common and are involved in more complex information processing, so we focus our attention on them. The proteins involved in chemical synaptic transmission are much more numerous and diverse than those involved in electrical conduction, and they function in medium-to-large protein complexes whose structure and function are not yet fully understood (**Figure 3**). In vertebrates, synaptic transmission usually travels in one direction (as shown in **Figure 3**), but ctenophore and cnidarian synapses are often bidirectional (Anderson 1985, Hernandez-Nicaise 1973). Little is known about the proteins involved in cnidarian and ctenophore synapses, and there is probably much undiscovered complexity there. We divide the synapse into (*a*) a presynaptic module, in which calcium signals are transduced into

chemical secretions (known as excitation–secretion coupling); (*b*) a postsynaptic module (postsynaptic density), which comprises the proteins that support the specialized postsynaptic membrane and the signaling that goes on there; and (*c*) a module that determines the specific wiring diagram of neurons during development (axonogenesis) (**Figure 3**).

Excitation–Secretion Coupling

Chemical synapses transduce a neuron's electrical code into an excreted chemical signal. This happens when the sodium-based action potentials of the axon reach the axon terminus and trigger the opening of Ca_v channels (**Figure 3**). The influx of calcium triggers neurotransmitter-filled vesicles to release from their priming area and fuse with the presynaptic membrane. After release, the membrane is then recycled into new vesicles (**Figure 3**). Despite its apparent specialization for neuronal signaling, the excitation–secretion system in neurons comprises many ancient gene families. However, like the transduction module, these gene families are often used differently in the various animal lineages.

The proteins involved in docking and in recycling are, for the most part, conserved across eukaryotes (Burkhardt et al. 2014, Emes & Grant 2012). The well-known SNARE complex is found across eukaryotes, where it serves a similar function in endomembrane trafficking and exocytosis (Kloepper et al. 2007). Interestingly, SNARE proteins have expanded independently in the major multicellular lineages, suggesting a key role in cell-type differentiation (Richter & King 2013). Endocytosis mediated by clathrin proteins (**Figure 3**) is also found in all eukaryotes (Becker & Melkonian 1996). However, many presynaptic proteins involved in scaffolding and in vesicle priming arose only within Metazoa (**Figure 3**) (Burkhardt et al. 2014, Emes & Grant 2012).

A nearly complete set of excitation–secretion-related proteins are present in choanoflagellates (Burkhardt et al. 2011), and both sponges and placozoans have a distinct flask-shaped cell type that is enriched for vesicles, synaptic genes, and Ca_v channels, as well as possessing a nonmotile cilium, consistent with a role in environmental sensing (Sakarya et al. 2007; Smith et al. 2014, 2017). In the demosponge *Amphimedon queenslandica*, the flask cells have been shown to transduce sensory cues into induction of settlement and metamorphosis in a calcium-dependent manner (Nakanishi et al. 2015). Thus, these flask cells are probably the best candidates for neuron-like cells in sponges and placozoans; although without detailed knowledge of ancestral animal cells, we cannot say whether they are descendants of protoneurons, simplified neurons, or have some other complex history.

Several types of molecules are used as neurotransmitters; their evolutionary deployment in different synapse types across animals is fascinating and still poorly understood. Many are used widely in eukaryotes for intercellular communication, but some of the biogenic amines may be present in animals as a result of the late horizontal transfer of synthesis enzymes from bacteria (Iyer et al. 2004). For instance, epinephrine and norepinephrine are important neurotransmitters in vertebrates but not in protostomes (but see Bauknecht & Jékely 2017), whereas the opposite is true of octopamine and tyramine (**Figure 4**). Cnidarians make a set of neurotransmitters similar to those in vertebrates (Kass-Simon & Pierobon 2007), but *Nematostella* expresses most nonpeptide types in the endoderm near the pharynx and testes—only peptide transmitters are found in neurons (Oren et al. 2014).

Intriguingly, ctenophores seem to use a much more restricted set, as glutamate is the only well-validated neurotransmitter (Moroz et al. 2014). This is consistent with the theory that neurons arose independently in ctenophores and planulozoans because vertebrates and most protostomes use acetylcholine at the NMJ. However, arthropods use glutamate at the NMJ, just as ctenophores do (Jan & Jan 1976), and cnidarians probably use neuropeptides (Oren et al. 2014). Although

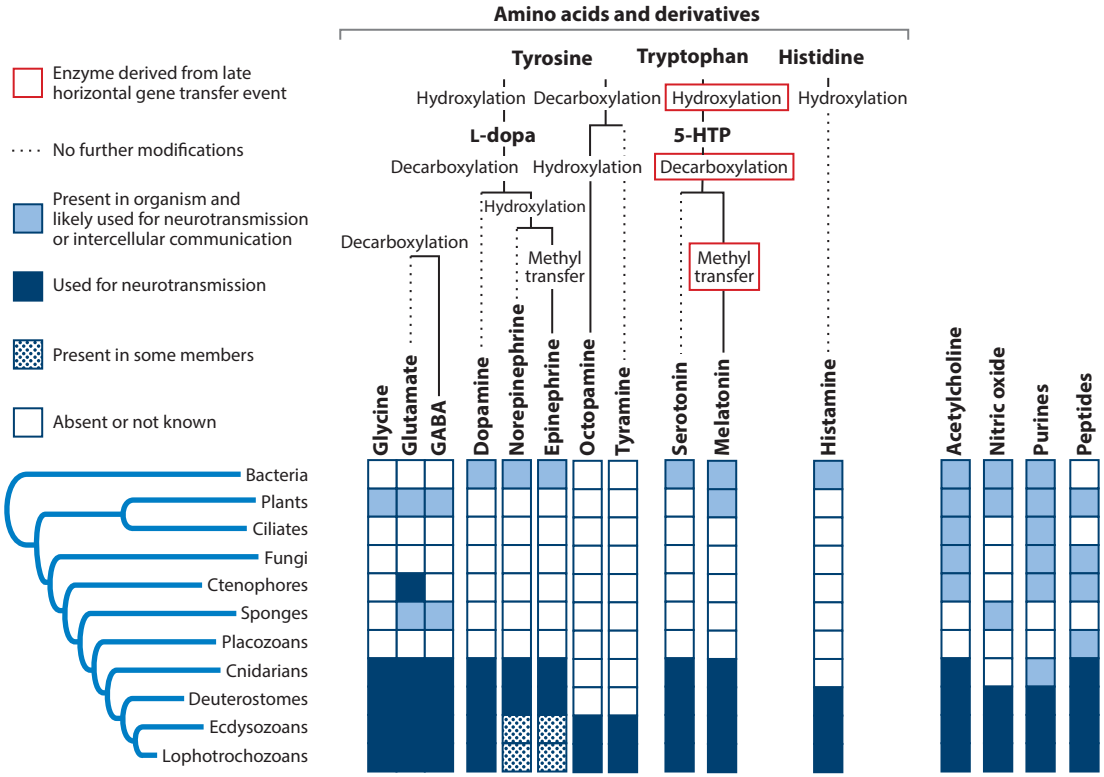


Figure 4

Evolution of neurotransmitter types. The derivatives of tyrosine, tryptophan, and histidine have a restricted taxon distribution, and they may be present in planulozoans (bilaterians plus cnidarians) as a result of the late horizontal gene transfer of key enzymes from bacteria (Iyer et al. 2004). Other neurotransmitter types are used for intercellular signaling across a wider range of eukaryotes. Presence and absence data are derived from Elliott & Leys (2010), Kass-Simon & Pierobon (2007), Roshchina (2010), and Ruggieri et al. (2004). Abbreviations: 5-HTP, 5-hydroxytryptophan; GABA: γ-aminobutyric acid; L-dopa: L-3,4-dihydroxyphenylalanine.

sponges do not have true synapses, they use γ-aminobutyric acid (GABA), glutamate, and nitric oxide to coordinate contractions (Elliott & Leys 2010). *Trichoplax* individuals also lack synapses, but their secretory flask cells label for FMRFamide, suggesting a conserved role in transmission for this peptide class (Smith et al. 2014).

Postsynaptic Density

One of the defining features of synapses is an electron-dense, disc-shaped structure on the postsynaptic side, called the postsynaptic density (**Figure 3**). In addition to the ion channels that transduce the neurotransmitter signal into postsynaptic potentials, this density includes a large number of scaffolding and signaling proteins that modulate synaptic strength. The proteins of the postsynaptic density can be roughly classified into adhesion and signaling molecules that exist near the postsynaptic membrane, and scaffolding proteins (Burkhardt et al. 2014). Many of the adhesion and signaling molecules are newer innovations, particularly those that interact specifically with certain classes of postsynaptic ion channels (Emes & Grant 2012), although the pattern of a progressive advance in complexity toward mammals described by these authors probably comes

from mammalian-biased sampling of synaptic proteomes (Liebeskind et al. 2016). Scaffolding proteins, in contrast, tend to be found outside of Metazoa as well (Burkhardt et al. 2014, Sakarya et al. 2007). One protein, Homer (**Figure 3**), has been shown to be ancestrally expressed at the nuclear envelope, based on its expression in choanoflagellates and in mammalian glia (Burkhardt et al. 2014), highlighting the pleiotropic nature of many of the genes in this module.

A nearly complete set of postsynaptic density proteins are found in sponges and in *Trichoplax*, despite the lack of obvious synapses in these organisms (Sakarya et al. 2007). Intriguingly, disc-shaped, electron-dense junctions are found in syncytial cell types of hexactinellid sponges (Leys 2003), *Trichoplax* (Smith et al. 2014), and colonial choanoflagellates (Dayel et al. 2011). It is not known which proteins are expressed at these structures, however, and postsynaptic density genes do not appear to be coexpressed in the sponge *A. queenslandica* (Conaco et al. 2012). The flask cells of sponges, which express presynaptic genes and have vesicles, also express postsynaptic scaffolding and signaling proteins (Sakarya et al. 2007).

Axonogenesis

Neurons develop from ectoderm in most bilaterians, cnidarians, and ctenophores (Hartenstein & Stollewerk 2015, Martindale & Henry 2015). However, they can also derive from mesoderm in planarians (Rink 2013), endoderm in sea urchins (Wei et al. 2011), and interstitial stem cells in hydrozoan cnidarians (Bode 1996). The gene repertoire involved in early neuron specification is largely conserved in bilaterians and cnidarians, but ctenophores are missing some of this core set of neurodevelopment genes (Martindale & Henry 2015, Ryan et al. 2013). However, they do have genes in the Lim-homeobox family, which may have a role in neuron development as they do in bilaterians (Simmons et al. 2012). They also express the Wnt pathway, which is important for body patterning and neurogenesis in cnidarians and bilaterians, but only in adults and only in a localized area near the mouth (Jager et al. 2011). In general, the developmental gene content of ctenophores more closely resembles that of sponges, but in the neuronless *Trichoplax*, it largely resembles that of cnidarians and bilaterians (Ryan et al. 2013). The well-known ability of developmental genes to be repurposed (Shubin et al. 1997) makes it difficult to infer the presence of a phenotype from the presence of a morphogenetic gene family (Hejnol & Lowe 2015).

During development, and after injury, axons must grow and find their correct synaptic targets. The proteins responsible for this targeting include secreted and membrane-bound signals and receptors that have not been studied in an evolutionary framework as well as the other modules. We plotted the evolutionary distribution of some of the best-known families in **Figure 5**. Most families have clustered, shared-domain repeats (de Wit & Ghosh 2016) and evolve by domain swapping (Stolzer et al. 2015), a reticulating evolutionary process. All of the domains shown in **Figure 5** are found outside of Metazoa, except for the Semaphorin domain. Proteins with Immunoglobulin (Ig) family domains are well represented, suggesting a close functional and evolutionary relationship between immunity and axon guidance. Many of these cell adhesion proteins are also found in epithelial junctions, such as the pleated septate junction (nonvertebrates) and tight junctions (vertebrates), and in intermodal septate junctions between myelinating glia and axons. Therefore, it has been suggested that some synapse types evolved from epithelial precursors (Harden et al. 2016). This suggestion is intriguing, as both sponges and placozoans have recently been shown to have occluding adherens junctions (Adams et al. 2010, Smith & Reese 2016). In fact, both choanoflagellates and the slime mold *Dictyostelium* make electron-dense junctions, the latter of which are known to involve β-catenin (Dayel et al. 2011, Grimson et al. 2000).

How is the final wiring diagram in the brain specified? In *Drosophila*, the *DSCAM* (Down syndrome cell adhesion molecule) gene has clustered series of exons that are alternately spliced to

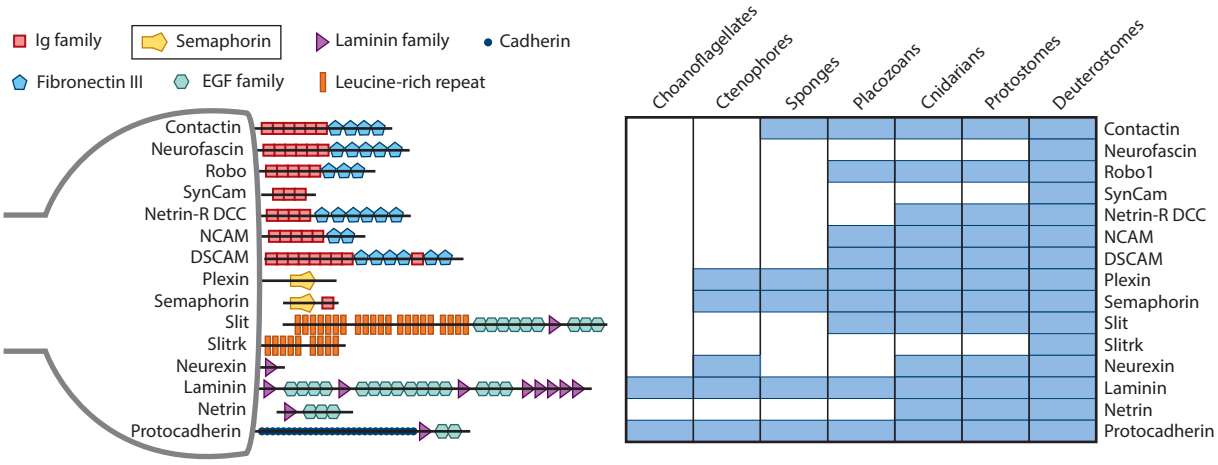


Figure 5

Surface and secreted proteins involved in axonogenesis and synaptic targeting, arranged by protein domain composition. Most are made up of multiple domains and have probably evolved by domain shuffling (Stolzer et al. 2015). Many are expressed in other cell types, especially epithelial and immune cells. All domains are found outside of metazoans, with the exception of the Semaphorin domain (*boxed*). Abbreviations: DCC, deleted in colorectal cancer; DSCAM, Down syndrome cell adhesion molecule; EGF, epidermal growth factor; Ig, immunoglobulin; NCAM, neural cell adhesion molecule; SynCAM, synaptic cell adhesion molecule.

produce an enormous variety of mature protein isoforms. It is thought that this diversity creates a combinatorial code determining the wiring diagram of the fly neural system (Schmucker et al. 2000). In vertebrates, however, *DSCAM* is not extensively spliced. Instead, vertebrate genomes have clustered arrays of protocadherins, which play a part in dendritic self-avoidance and synaptic pruning (Kostadinov & Sanes 2015). Little is known about these mechanisms outside of current model systems, but intriguingly, protocadherins have convergently radiated in octopods, where they are also found in clustered genomic repeats and are heavily expressed in the brain (Albertin et al. 2015)—a convergent feature of large brains in cephalopods and vertebrates.

HIERARCHICAL MOLECULAR EVOLUTION

Currently, the evidence presented for the homology or nonhomology of neurons in ctenophores and other animals relies chiefly on comparative genomic data, usually presented as lists of genes or pathways present or not present in the common animal ancestor (Moroz & Kohn 2016, Ryan 2014). However, genes cannot be interpreted apart from their functional context. Proteins function in the hierarchy of molecular processes that make up complex cell types: They form protein complexes; protein complexes function in pathways and subcellular structures; and these structures come together to mediate cell-level phenotypes. It is a nontrivial task to reconstruct the evolutionary dynamics of the higher level phenotypes from lower level mechanisms. Doing so becomes more difficult as less is known about intermediate molecular functions, as fewer taxa are sampled across the phylogeny, and as the interior branches of the tree become deeper. Deep neural system evolution exists in the worst part of this space. There is little we can do about the interior branch lengths of the animal tree, but more functional data across more taxa are within our reach.

To this end, we have described above how neural modules have undergone different rates and modes of molecular evolution, and how this might have affected neural system evolution. For instance, functional changes in ion channels, such as those in the propagation or transduction

modules, are typically characterized by a few, often convergent, amino acid changes at key sites (Liebeskind et al. 2011, Lynagh et al. 2015, Martinson et al. 2014). This is consistent with the furcation model of novelty (**Figure 2*b***). Adhesion and scaffolding proteins expressed in the axonogenesis and postsynaptic modules are pleiotropic and may have been repurposed from epithelial tissue with little change to their biophysical function (Harden et al. 2016, Schmitz et al. 2000). This is more consistent with the stepwise recruitment or exaptation models (**Figure 2*b***). It is possible that whole pathways for neurotransmitter synthesis may have been transferred from bacteria into animals (Iyer et al. 2004). Can this be considered an extreme version of repurposing? The fact that gene families come to mediate phenotypes by distinct processes and evolve at different rates and with different modes suggests that no single model of trait origins (**Figure 2*b***) is sufficient for all genes. What is needed, then, is a better understanding of neuronal protein function in a broadly sampled comparative context.

One system that might best yield to this sort of analysis is the NMJ. Moroz and colleagues (2014) found that the ctenophore NMJ was glutamatergic (glutamate is the neurotransmitter). Interestingly, this has been used as evidence both for and against the multiple origins hypothesis (Moroz & Kohn 2016, Ryan 2014). On one hand, vertebrate NMJs are cholinergic (using acetylcholine), and ctenophores have many independently expanded the gene families associated with glutamate signaling (although we note that many of the so-called glutamate receptor genes are actually glycine receptors, and the role of glycine signaling in ctenophores is unknown) (Alberstein et al. 2015). On the other hand, glutamatergic synapses and the protein families involved are found across animals. Too little is known to make a determination of homology, but the case of the NMJ shows how the study of hierarchical molecular evolution is the necessary next step in reconstructing ancient cell types.

The NMJ is a model synapse whose evolution seems likely to reflect that of neurons as a whole; muscles and neurons are always found together in animals, with the sole exception of the parasitic myxozoans, which have lost neurons (Gruhl & Okamura 2015). Yet as one looks closer at the molecular composition of NMJs, it becomes apparent that there is considerable evolutionary plasticity. Vertebrates, arthropods, cnidarians, and ctenophores all use different neurotransmitter types at the NMJ, whereas nematodes use the same neurotransmitter as vertebrates (acetylcholine), despite their closer relationship to arthropods (**Figure 6**). NMJs can also vary considerably in ultrastructure, even among vertebrates (Ackermann et al. 2015). Thus, the fact that ctenophore synapses have another distinct morphology (Hernandez-Nicaise 1973, Moroz & Kohn 2016) is not strong evidence of an independent origin. And yet, the distinct presynaptic cytomatrix structures in *Drosophila* and *Xenopus* are made up of largely homologous proteins. Some of these homologous proteins (Bruchpilot and ELKS, CAST, and ERC) have different compositions of the homologous domains and long nonhomologous stretches (**Figure 6**).

Finally, it has been shown that *Drosophila* NMJs use the same neurotransmitter (glutamate) as vertebrate excitatory synapses in the brain, and also have an overlapping set of cell-adhesion proteins (Harden et al. 2016). Inversely, *Drosophila* central excitatory synapses use the same neurotransmitter as vertebrate NMJs (acetylcholine) (**Figure 6**). Perhaps the last common ancestor of bilaterians had NMJs with multiple transmitter types, as glutamate receptors are expressed at cholinergic NMJs in mammals (Mays et al. 2009).

Synapses have often been treated as monolithic entities in the evolutionary literature, but much of what is known about the molecular composition of synapses comes from just two synapse types: the excitatory synapses of vertebrates and the *Drosophila* NMJ (Husi et al. 2000). The plasticity we describe above suggests that synapses in nontraditional model organisms may be just as complex, but mediate their functions with different molecular components. Furthermore, it makes clear that evolution at each hierarchical level—the protein domain, the gene, the pathway,

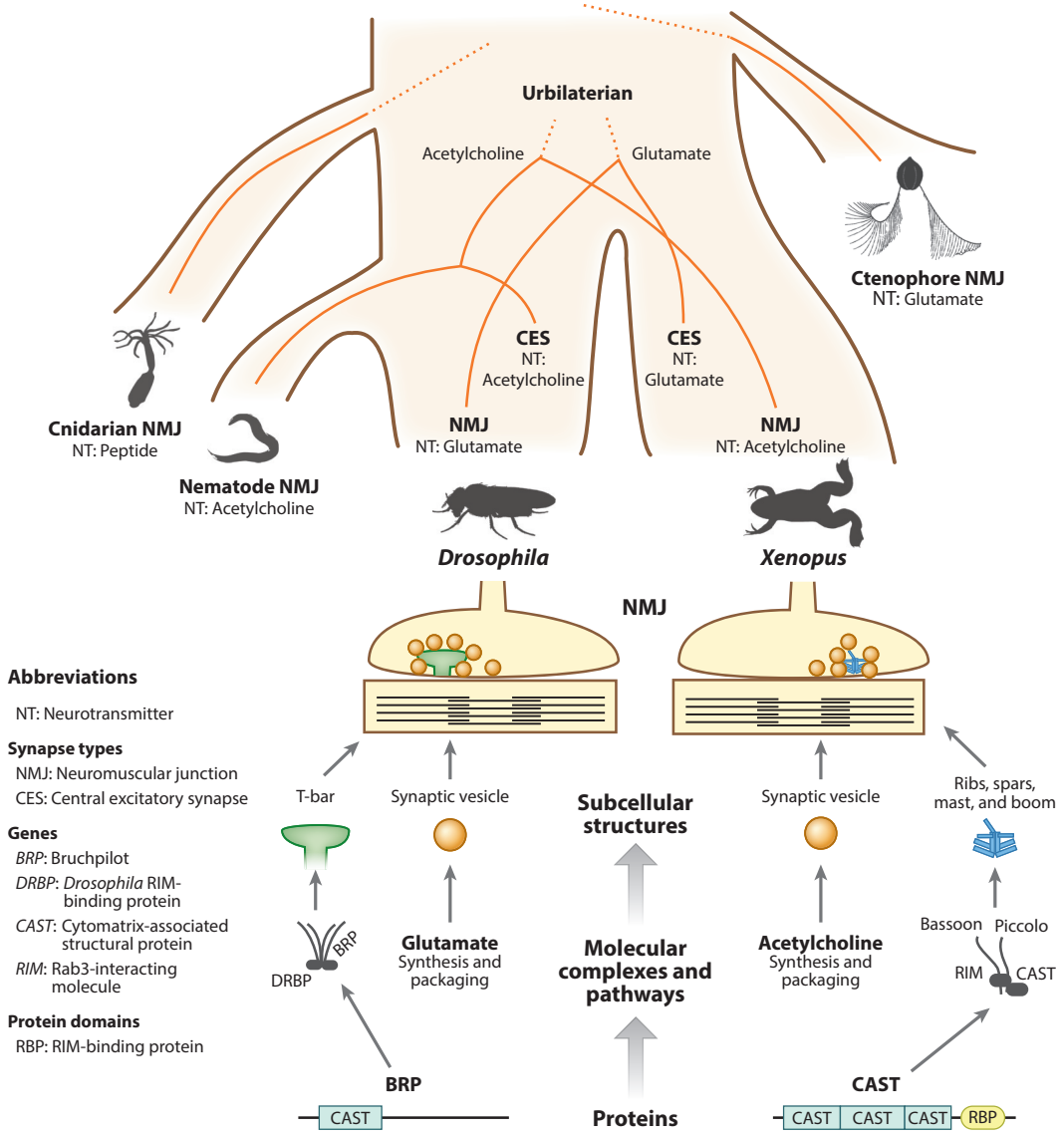


Figure 6

Evolution of the neuromuscular junction (NMJ). There are interesting differences and similarities between the two model NMJs in *Drosophila* and *Xenopus* when one looks across the hierarchy of molecular organization. Molecular components of *Drosophila* NMJs may be more closely related to excitatory synapses in the brain in vertebrates, and vice versa, *Drosophila* central excitatory synapses use the same neurotransmitter as vertebrate NMJs. Not enough is known about the molecular constituents of ctenophore and cnidarian NMJs to determine their homology with bilaterian cells.

and the subcellular structure—can evolve semi-independently because the state at each level is not uniquely determined by the state at the others. Therefore, a better understanding of molecular details across species is key to reconstructing the deep evolution of cell-level phenotypes. It also seems clear that if cell-type evolution is to be understood in a rigorous, probabilistic fashion, comparative phylogenetic methods must incorporate function across these hierarchical levels.

Such models are scant, but there are examples that can guide future research (Lartillot & Poujol 2011). However, we are far from making a determination about the homology of synapse types, much less neurons as a whole, across cnidarians, ctenophores, and bilaterians.

CONCLUSIONS

All complex cell types comprise a hierarchy of molecular components with diverse histories and are, therefore, likely to face problems similar to those we have discussed for neurons and NMJs. It should be acknowledged that all possible patterns of coevolution between proteins and the phenotypes they encode (**Figure 2*c*,*d***) are not only possible but plausible over even modest evolutionary divergences. Examples of convergence and systems drift over relatively short time frames are abundant in the evolutionary neuroscience literature (Nishikawa 2002). These include the convergence of electric organs within teleost fish (Thompson et al. 2014) and myelination within closely related copepods (Castelfranco & Hartline 2016), and systems drift in brain circuitry for swimming in nudibranchs (Katz 2016) and social behavior within vertebrates (O'Connell & Hofmann 2011). This plasticity over even short time frames shows the barriers to reconstruction in deeper time. The most promising ways to deal with this complexity are (*a*) to sample comparative molecular data that report on function, and (*b*) to develop comparative phylogenetic methods that incorporate sufficient functional detail.

Neural systems are spectacularly complex phenotypes. The primer on the molecular evolution of neurons we have provided makes it clear that the history of neural systems is more complex than often imagined. Comparative genomics has opened the door to evolutionary neuroscience across the most divergent animal taxa, but more detail about neural systems in nonmodel organisms is necessary before we can elucidate the origins of neurons.

DISCLOSURE STATEMENT

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