TechSights

Of Molecules and Mechanisms

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Without question, molecular biology drives modern neuroscience. The past 50 years has been nothing short of revolutionary as key findings have moved the field from correlation toward causation. Most obvious are the discoveries and strategies that have been used to build tools for visualizing circuits, measuring activity, and regulating behavior. Less flashy, but arguably as important are the myriad investigations uncovering the actions of single molecules, macromolecular structures, and integrated machines that serve as the basis for constructing cellular and signaling pathways identified in wide-scale gene or RNA studies and for feeding data into informational networks used in systems biology. This review follows the pathways that were opened in neuroscience by major discoveries and set the stage for the next 50 years.

Key words: **PCR; RNA; transfection and transduction; SNARE and synapse composition; nanoscale imaging; CRISPR-Cas9**

Introduction

Modern neuroscience rests on the back of molecular biology. Without question, the tools and tactics that have emerged from molecular biology coupled with what has been learned about individual molecules and molecular machines have changed fundamentally the nature of the questions that neuroscientists ask and the perspective from which data are interpreted. Major discoveries in molecular biology have blazed a trail toward advances in neuroscience.

They have generated tools that are used to probe pathways, to identify and classify cell types and states of differentiation, and to illuminate intracellular signaling pathways running from synapses to the nucleus and back. More importantly, a full understanding of the biological function of individual molecules and their role within macromolecular networks has provided the field with essential building blocks of information needed to comprehend the normal function of cells and circuits, as well as to understand how circuits develop, how they are shaped by use or by insult, how they have evolved, and how they behave in the context of disease-causing gene mutations. The function of a surprisingly large body of biologically active gene products in the nervous system is not yet known, but it is significant that use of the word "mechanism" in the literature shows a strong correlation with the appearance of the word "molecule" or "molecular"; $r = 0.96$, *Journal of Neuroscience* PubMed data from 1981 to 2019.

The past 50 years has been something of a golden age for molecular biology (Fig. 1), that was initiated by two DNA-related discoveries. The first is the development of recombinant DNA technology (Jackson et al., 1972; Cohen et al., 1973), in which DNA sequences from two different species are ligated and expressed in a host cell. The second is Sanger DNA sequencing, a method that coupled the incorporation of radiolabeled nucleotides to random chain termination so that after size separation on a gel, the nucleotide code could be read on film. Together, the two techniques enabled molecular cloning, sequencing, and expression of a broad array of novel coding sequences. These discoveries led to the large-scale production of insulin (Goeddel et al., 1979a), somatostatin (Itakura et al., 1977), and human growth hormone (Goeddel et al., 1979b) in bacteria, and coupled with the development of techniques to transfect DNA into eukaryotic cells (Wigler et al., 1977), provided the foundation for the modern biotech industry. It was also appreciated at the time that along with extraordinary opportunity comes the possibility of unknown and unintended consequences, and the discovery of recombinant DNA was also soon followed by the 1975 Asilomar Conference in which researchers, the press, and the public debated and adopted stringent guidelines addressing the manipulation of DNA from different species (Berg et al., 1975). This largely successful effort at self-regulation by scientists has been codified in regulations set out by a variety of international agencies, that along with subsequent guideline-writing conferences (Baltimore et al., 2015), have proven mostly, but not entirely (Wang et al., 2019), successful at setting up guardrails.

The discovery of PCR acted like an accelerant for discovery in the life sciences in the 1980s (Saiki et al., 1985; Mullis et al., 1986), and it is hard to overestimate its practical significance. Its impact on neuroscience was almost immediate because the concept—multiple rounds of DNA polymerization between two annealed primers—was simple, and its implementation, using an automated thermocycler and a heat-resistant polymerase (Taq), was inexpensive and easy. Expectations grew from a place in which the discovery and sequencing of a single expressed gene constituted an entire PhD thesis, to one in which a family of structurally or functionally related genes, splice variants, and cellular distribution patterns could be found along with a chapter using experimental manipulations. PCR revolutionized approaches for many laboratories and it changed the course of my own thesis

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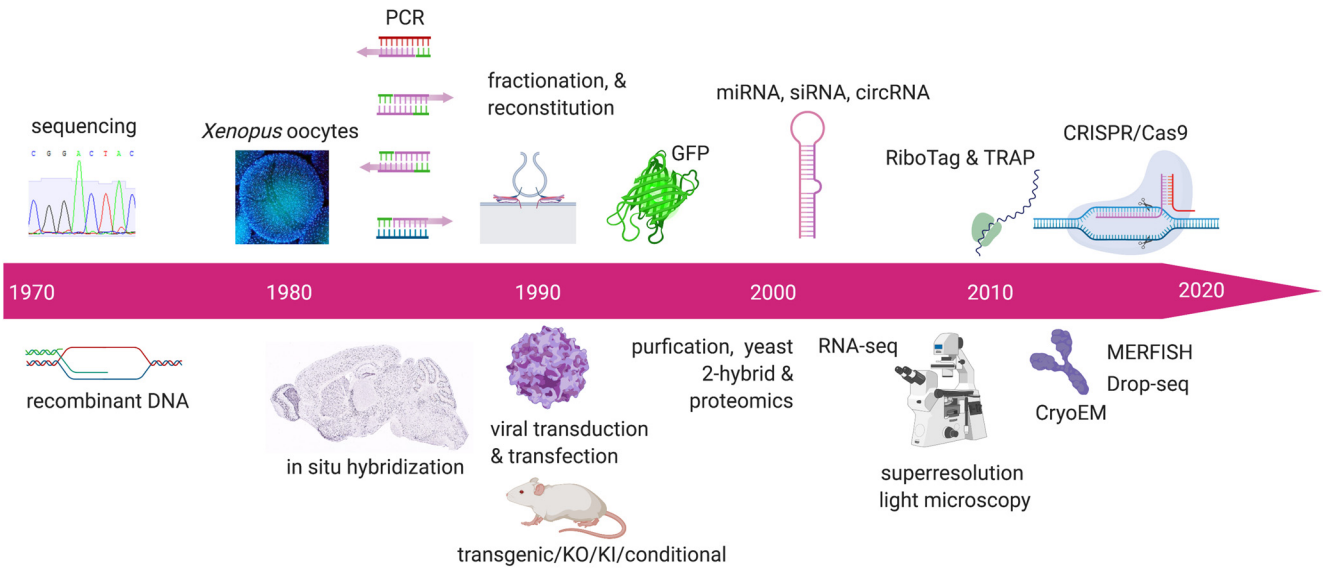

Figure 1. Timeline shows some of the major technical breakthroughs in molecular biology that have driven the field forward over the past 50 years. KO, knock-out; KI, knock-in. Hoechst-stained oocyte image from Vincent Pasque (https://creativecommons.org/licenses/by-nc-nd/2.0/uk/); sagittal section of mouse brain showing Gad1 mRNA distribution from Allen Brain Atlas (Lein et al., 2007). Figure created in BioRender.com.

work. But my experience was not unusual; according to Google Scholar, PCR was featured in 72,000 papers in its first 10 years.

The vast majority of bench-type neuroscience laboratories still use PCR regularly, most commonly for genotyping or for generating plasmid cDNA constructs, but in its first years, before the completion of the Human Genome Project, it was a tool for discovery. The 1980s and early 1990s saw a dramatic increase in the number of novel transcription factors, ion channels, and neuropeptides that were identified. Strategies to identify entirely unknown coding sequences still relied on challenging or iterative methods like chromosome walking in *Drosophila* or *Caenorhabditis elegans* (Davis and Davidson, 1984; Finney et al., 1988) or expression cloning (Lechan et al., 1986), but once a sequence was identified, standard PCR methods using degenerate oligonucleotide primers could be used to rapidly identify isoforms, family members, and even distant relatives (Keinänen et al., 1990). The rapid pace of discovery drove the development of approaches that could be used to examine the distribution and function of particular molecules. Two standouts in this arena are *in situ* hybridization and the use of heterologous cells.

In situ hybridization was first described in 1969 (Pardue and Gall, 1969), but its widespread use awaited PCR and the development of non-isotopic tags in the 1980s. In neuroscience, its use expanded exponentially with the explosion of primary sequence information. As soon as a new coding sequence or a splice variant was identified, RNA and oligonucleotide probes could be easily and cheaply synthesized, and cellular and regional distribution patterns over time or in response to a stimulus could be determined. For example, *in situ* hybridization experiments revealed that the onset and duration of c-fos, NGF, and BDNF expression following seizure showed regional and cell-type specificity (Morgan et al., 1987; Gall and Isackson, 1989; Isackson et al., 1991); and that the distribution of receptor-subunit mRNAs could be used to predict relevant subunit combinations and changes in function observed over the course of development (Monyer et al., 1994). Particularly noteworthy was the discovery that particular mRNAs were transported into dendrites and axons (Burgin et al., 1990; Benson et al., 1992; Bassell et al., 1998) where their distribution and translation could be regulated by activity, insult, or external cues (Aakalu et al., 2001; Campbell and Holt, 2001; Zheng et al., 2001). Indeed, *in situ* hybridization using probes specific for nuclear-localized primary transcripts (hnRNA) was used to study rapid regulation of gene transcription in neuroendocrine and neural cells and tissues (Fremeau et al., 1986).

The utility of *in situ* hybridization has only continued to grow. Baseline distribution patterns for most mRNAs are now publicly available for both developing and adult mouse (GenePaint, Visel et al., 2004; Allen Brain Atlas, Lein et al., 2007), human (Miller et al., 2014), and marmoset brain (Brain/MINDS; Woodward et al., 2018). The Allen Mouse Brain Atlas alone provides data for the localization of >20,000 genes and has been cited >3000 times. Recent advances have also fueled an expansion of scale. In particular, multiplexing and iterative strategies that use a rainbow of color tags and spectral separation allow examination of the location of several mRNAs in tissue or even within whole animals (Lovett-Barron et al., 2017; Meissner et al., 2019). MERFISH (multiplexed error-robust fluorescence *in situ* hybridization) is an outstanding example of this strategy, in that it uses a single-molecule imaging approach to identify thousands of mRNAs in single cells in intact tissue (K. H. Chen et al., 2015; Moffitt et al., 2016). Such *in situ* studies have also established the framework for current efforts aiming to identify the entire transcriptome of single cells (Macosko et al., 2015).

Knowing what a molecule does is even more important than where it acts, and the creative use of heterologous, reduced preparations has played a significant role in assessing function. For signaling molecules that are broadly expressed, predictions about what they do can be based on known actions in cells outside the nervous system, at least as a starting point. But for the many molecules unique to the nervous system, heterologous preparations have proven to be extremely valuable for identifying and measuring activity. In the wake of PCR, longstanding efforts to identify and characterize the primary structure of ion channels like nicotinic acetylcholine receptors (Giraudat et al., 1982; Noda et al., 1982; Patrick et al., 1983) or sodium channels (Hartshorne and Catterall, 1984; Noda et al., 1986), were overtaken by the need to assign functional attributes and rules of assembly to the ion channel subunits encoded by a growing number of sequences.

In 1982, Ricardo Miledi and Katumi Sumikawa showed that exogenous mRNAs encoding individual ion channel subunits, when introduced into *Xenopus* oocytes, were translated and assembled into functional channels, the properties of which could be recorded electrophysiologically (Miledi et al., 1982). The method was simple and was widely adopted to assess basic functional properties, as well as to determine subunit identity and stoichiometry, to identify protein interacting sequences and ligand binding sites, and to evaluate the impact of signaling partners. Two decades later, a different heterologous strategy was taken to screen and evaluate properties of cell adhesion proteins capable of promoting the generation of synapses. In its first iteration, Scheiffele et al. (2000) tested whether synapse-like junctions would form between presynaptic axons growing from a pontine explant across HEK cells expressing candidate postsynaptic synaptogenic proteins. This basic assay established canonical properties of molecules promoting synaptogenesis and it and its variants have helped to identify most of the adhesion proteins known to occupy the synaptic cleft (Biederer et al., 2002; Seabold et al., 2008; Woo et al., 2009; Siddiqui et al., 2010). These are just two examples among many that demonstrate the usefulness of heterologous preparations for establishing the nature and limits of molecular function.

Heterologous preparations are not an end. They are ultimately and obviously limited because they may lack a fully relevant signaling environment or express modifying factors that would never be found in homologous cells. However, until the 1990s, it was virtually impossible to introduce nucleotides into neurons outside of whole-animal transgenics, a situation that promoted the successful use of genetically accessible organisms like *Drosophila* and *C. elegans* to test molecular function (Doe et al., 1988; Way and Chalfie, 1988) and spurred the development of knockout, knock-in, and conditional approaches that could be used in mice (Doetschman et al., 1987; Thomas and Capecchi, 1987; Tsien et al., 1996). Introducing nucleotides into postmitotic cells remains an ongoing challenge, but several independent strategies emerged over a 10 year period that are still in use. The methods can be categorized into chemical (lipofection: Felgner et al., 1987; calcium phosphate: Xia et al., 1996), physical (injection: Gagliardini et al., 1994; electroporation: Muramatsu et al., 1997; biolistics: Lo, 2001), and viral-mediated transduction (HSV: Geller and Freese, 1990; AAV: Kaplitt et al., 1994; lentivirus: Naldini et al., 1996; Sindbis: Altman-Hamamdzic et al., 1997). All have pros and cons that can be exploited toward particular experimental ends, but AAV, although limited to sequences smaller than 5 kb, has become the gold standard. It works very well *in vivo*, does not compromise cell health or integrate (usually) into the host genome, and depending on serotype, displays specificity for particular neuron subtypes (Choi et al., 2005; Chan et al., 2017), oligodendrocytes (Powell et al., 2016), or microglia (Cucchiarini et al., 2003; Rosario et al., 2016). Some of the first studies to take advantage of transfection were protein domain swapping experiments that identified protein signatures and pathways used by neurons to target membrane proteins selectively to axons, dendrites, or synapses (Jareb and Banker, 1998; Stowell and Craig, 1999). Such studies typically combined transfection, transduction, or genetic engineering with immunolabeling for a unique exogenous tag, a strategy that was limited mostly to fixed preparations.

The need for better tags was fulfilled by the discovery of green fluorescent protein (GFP). The Chalfie laboratory first illustrated the experimental utility of GFP shortly after its sequencing by expressing it downstream of a β-tubulin promoter in a small population of sensory neurons in *C. elegans*. Their data showed that GFP could be used as a reporter for transcriptional activity and also filled the branches of a developing neuron, highlighting its potential for live cell imaging (Chalfie et al., 1994). Within 2 years, GFP was being used as a fusion tag to track protein localization in fixed and living neurons (Moriyoshi et al., 1996), to measure vesicle trafficking and release in living cells, and to report changes in voltage (Siegel and Isacoff, 1997) and signaling activity (Tannahill et al., 1995). The advent of multiple color, unstable, pH-sensitive, Ca^{2+}-sensitive, and photo-switchable variants of GFP and the discovery of related fluorescent proteins (Rodriguez et al., 2017) has provided the field with a series of tools that permit measurements within cells. GFP has pushed the development of optical tools like GCaMP (Nakai et al., 2001), which reports changes in calcium levels as a proxy for activity in coordinated neural networks of awake, behaving animals (Tian et al., 2009), and of FRET-based tools that can detect nanoscale protein–protein interactions (Heim and Tsien, 1996; Mitra et al., 1996). Although most GFP-based reporters are independent markers or fusion proteins expressed episomally or transgenically, recent advances have made possible the generation of knock-in tags in postmitotic cells to track localization of endogenous protein (Nishiyama et al., 2017).

Concurrent with the emergence of advances in protein tagging and expression, some of the most significant research in neuroscience in the 1980s and 1990s emerged from studies using sophisticated biochemical techniques that took advantage of properties unique to synapses. The best example of this is the SNARE hypothesis, which grew out of sophisticated fractionation, isolation, affinity purification, and reconstitution strategies that converged with efforts to dissect the secretory pathway in yeast using genetics. The identification of NSF (N-ethylmaleimide-sensitive fusion protein; Malhotra et al., 1988), VAMP/synaptobrevin (Trimble et al., 1988; Südhof et al., 1989), SNAPs (NSF attachment proteins; Clary et al., 1990), synaptotagmin (Perin et al., 1991; Brose et al., 1992), and Munc18 (Hata et al., 1993), and their relevance to exocytosis interdigitated with work delineating the role of *SEC* genes in the secretory pathway in yeast (Kaiser and Schekman, 1990). The mechanism unfolded like a story published in installments from the laboratories of James Rothman, Thomas Südhof, Reinhard Jahn, Richard Scheller, and Randy Schekman. Their findings were joined by the observations that toxins blocking transmitter release (tetanus and botulinum) cleaved VAMP/synaptobrevin, SNAP25, and Syntaxin (Schiavo et al., 1992; Blasi et al., 1993a,b), and together the data provide an elegant demonstration of the importance of identifying the molecules that compose a function, characterizing the individual components, and reconstituting the function *in vitro*.

The ability to purify biochemically the mostly insoluble material that constitutes glutamatergic postsynaptic densities has also greatly advanced our understanding of the molecular organization of postsynapses. In separate efforts in the late 1990s, Mary Kennedy and Craig Garner identified and characterized PSD95/SAP90 using purified postsynaptic densities as a starting material (Cho et al., 1992; Kistner et al., 1993). Both laboratories noted three repeating ~90 aa protein domains, which based on similarity to domains in *Drosophila* discs large and Zonula occludens, were dubbed PDZ domains (Kennedy, 1995). The discovery of PDZ domains snowballed into an elementary understanding of synaptic scaffolds and the rules underlying their organization. They bind to short sequences that are usually at the C-terminus of proteins like Shaker K^+ channels and GluN2 receptors (Niethammer et al., 1996; Songyang et al., 1997); and in PSD95/SAP90 and the hundreds of other proteins that have been

identified carrying PDZ domains since, they serve to appropriately localize proteins, to bring signaling partners into close proximity, and as a means to generate an organized synaptic scaffold (Garner et al., 2000; Valtschanoff and Weinberg, 2001). Yeast-2-hybrid screening for cytoplasmic protein–protein interactions proved to be an outstanding tool for identifying additional synaptic scaffolding proteins and their interacting partners (Garner et al., 2000; Kim and Sheng, 2004). Advances in liquid chromatography coupled to tandem mass spectrometry supported a series of successful efforts in the early 2000s to identify a generic, glutamatergic PSD proteome (Jordan et al., 2004; Peng et al., 2004; Yoshimura et al., 2004; Phillips et al., 2005). Although PSD function is less well understood than vesicle fusion, recent work using newly developed biochemical approaches based on proximity tagging, are contributing to a new wave of discovery, mapping interprotein relationships and identifying subcellular proteomes (Loh et al., 2016; Spence et al., 2019).

Recent advances in microscopy are also steadily chipping away at the gap in knowledge that lies between the attributes of individual molecules and their actions in cells. Single particle cryo-electron microscopy (cryo-EM), which makes visible molecules and macromolecular structures at subnanometer resolution, has been carving out new territory in ion channel biology (Liao et al., 2013; Fan et al., 2018; Shen et al., 2018; Laverty et al., 2019). For example, a recent analysis of native AMPA receptors isolated from rat brain showed that GluA subunit positioning and overall receptor architecture differed from predictions based on recombinant receptors and further revealed that ~one-third of the receptors were triheteromers, a composition that is novel (Zhao et al., 2019). Examination of the transsynaptic structure generated by presynaptic β-neurexin1, extracellular cerebellin1, and postsynaptic GluAD at parallel fiber–Purkinje cell synapses not only illustrated the molecular composition and interactions that promote assembly of this synapse, but also revealed the structural alterations in GluAD that are likely to support long-term depression (Elegheert et al., 2016). Such single-particle data are being extended by advances in cryo-electron tomography (cryo-ET), where multiple EM images of unfixed, rapidly frozen cells taken at different angles are assembled into tomograms of a 3D cellular landscape at a resolution of ~4 nm. The molecular complexes observed in cryo-ET can be difficult to attribute, but an expanding library of single-particle EM data, correlative light EM (CLEM) techniques, and targeted experimental manipulations are permitting the identification of structures as well as the roles played by particular molecules in forming or regulating macromolecular assemblies (Fernández-Busnadiego et al., 2010; Perez de Arce et al., 2015; Vargas et al., 2017; Tao et al., 2018). These EM strategies have been joined by the development of super-resolution light microscopy, which permits the assessment of single, labeled molecules and relationships between molecules within cells, even living cells (Chamma et al., 2016), at resolutions that can approach 10 nm. High-resolution examination of synaptic proteins is revealing a transsynaptic, modular organization (Dani et al., 2010; Perez de Arce et al., 2015; Tang et al., 2016; Biederer et al., 2017; Hruska et al., 2018) that is evident in cryo-ET (Perez de Arce et al., 2015). Expansion microscopy is also proving to be valuable for showing relationships between proteins, or between proteins and RNA (F. Chen et al., 2015; Chozinski et al., 2016; Hafner et al., 2019). Most exciting, though, is the capacity for these high-resolution strategies to reveal novel molecular structures and interactions. A beautiful illustration of this is the actin-rich rings in axons discovered in Xiowei Zhuang's laboratory using a single-molecule, stochastic-labeling approach called STORM. The regularly spaced actin bands are separated from one another by spectrin tetramers (Xu et al., 2013). They are most prominent in axons, evident in some dendrites, and observed in neurons across species from *C. elegans* to human (He et al., 2016). The existence of this entirely novel scaffold has been verified by other techniques in several laboratories (D'Este et al., 2015; Ganguly et al., 2015) and provides an excellent example of a molecular observation prompting functional questions: What do the rings do? How are they modified to permit the emergence of protrusions or synaptic boutons? Can they counter axonal degeneration?

The early 2000s were led by the discovery that RNA can regulate genes. When Andrew Fire and Craig Mello showed that exogenously introduced double-stranded RNAs produced a dramatic and selective inhibition of protein translation in *C. elegans*, the impact reverberated across all of biology (Fire et al., 1998). A series of rapid-fire investigations uncovered the endogenous cellular strategies used to generate short interfering RNAs (siRNAs) that destroy mRNA, block translation, or inhibit transcription (Novina and Sharp, 2004). Because siRNAs could be introduced exogenously into identified cells at particular times, siRNA-based experimental approaches fully enabled fast and inexpensive ways to conduct loss-of-function studies that were temporally and spatially controlled. They were used widely and aggressively to identify and dissect the contributions of single molecules and protein motifs to a variety of signaling and cellular pathways in neurons (Bai et al., 2003; Pekarik et al., 2003; Hamilton et al., 2005). However, in mammalian cells, the delivery of siRNAs also provokes cellular defense mechanisms against viruses and activates the interferon pathway (Gantier and Williams, 2007). This and other off-target effects have diminished enthusiasm for its wide-scale use. However, largely in parallel to the discovery of siRNA, it was discovered that microRNAs (miRNAs) are liberally sprinkled throughout the genome of most organisms (Lee et al., 1993). miRNAs share processing steps with siRNA (Kim and Rossi, 2007), but unlike siRNA, a single miRNA regulates a multitude of mRNAs through complete or partial complementarity to sequences contained mostly within 3′ untranslated regions. Ablation of Dicer, the enzyme that cleaves the hairpin loop from a microRNA in a rate-limiting processing step, revealed that an astounding array of miRNA-dependent events control nervous system development, including stem-cell proliferation, cell-type specification, and neural differentiation (Davis et al., 2008; De Pietri Tonelli et al., 2008). And single miRNAs, like miR-124 (Smirnova et al., 2005; Conaco et al., 2006; Visvanathan et al., 2007) or miR-9 (Shibata et al., 2011; Clovis et al., 2012; Dajas-Bailador et al., 2012) display different functions in different cellular contexts. Massive, parallel RNA sequencing (RNA-Seq) has more recently confirmed that siRNAs can be found naturally within the genome and has also helped to identify additional noncoding regulatory RNAs like piRNAs and circular RNAs generated from introns and exons (Chen and Schuman, 2016). This rich network of naturally occurring RNA elements outlines a highly nuanced picture of transcriptional and translational control. Significantly, many noncoding RNAs are more enriched in brain, contained within genes encoding synapse proteins, and are regulated by synapse activity (Cohen et al., 2011; Fiore et al., 2011). Coupled with data showing that nearly half of all long, noncoding (lnc) RNAs are expressed in brain (Mercer et al., 2008) and that miRNA and lncRNA diversity and number appear to have increased dramatically during the evolution of primate brains (Heimberg et al., 2008; Kosik and Nowakowski, 2018), it is clear that it will be of critical importance to understand the mo-

lecular mechanisms controlling their collective actions (Weiss et al., 2015; Kosik and Nowakowski, 2018).

Current molecular approaches are dominated by unbiased strategies that serve to outline the limits of possibility. A regional or even cell-type-specific transcriptome can be used to identify regulatory RNA elements and the set of mRNAs that have the potential to be translated, and a connectome typically uses molecular tools to pinpoint brain areas or individual neurons that are interconnected. Cell-type-specific expression of ribosome tags (RiboTag and TRAP) can be used to further reduce complexity by permitting the identification of mRNAs that are loaded onto ribosomes and thus are most likely to be translated (Heiman et al., 2008; Sanz et al., 2013). However, all of these strategies stop short of indicating what particular molecules do or the sites within cells in which they act. Recent advances coupling fractionation (Zivraj et al., 2010; Poulopoulos et al., 2019) or the use of intact sections with RNA-Seq (Rodriques et al., 2019) are starting to bridge this information gap.

Single-molecule manipulations have received a major boost by the discovery of CRISPR-Cas9. Like PCR was to the mass adaptation of sequence identification, CRISPR-Cas9 is to sequence manipulation *in vivo*. Discovered as a viral defense mechanism (Jinek et al., 2012), Cas9 (or its relatives) can be targeted with a single guide RNA in eukaryotes to generate site-specific DNA breaks (Mali et al., 2013; Hsu et al., 2014). Although still in its infancy, the ease of its application has already enabled phenotype-based screens in human cells (Koike-Yusa et al., 2014; Wang et al., 2014; Zhou et al., 2014); and targeted manipulations in terminally differentiated cells (Platt et al., 2014). Coupled with cell-type-specific inducible promoters, the strategy enables experiments *in vivo* that used to be practical only *ex vivo*. Like siRNA, widespread use is revealing some unwanted consequences (Zhang et al., 2015), but the use of CRISPR-based strategies as a molecular tool cannot be understated. As it brings within reach the possibility of corrective therapy for certain genetic diseases, its potential for healing is tantalizing, but the possibility of irreparable damage and unknown negative effects will hopefully keep this application at arm's length for the near future.

Currently, investigations of molecular function and molecular assemblies are on the upswing. Enormous progress has been made over the last decade identifying neural circuits driving particular behaviors. Such studies have often been merged with gene and protein expression data to reveal collections of molecules that are coordinately regulated in identified cells and networks in the context of a behavior or in response to pathology. However, many of the molecules identified have unknown or poorly characterized functions. Recent technical advances in labeling strategies, microscopy, and gene manipulation have provided the field with unparalleled opportunities to identify and characterize the function of key molecules and molecular machines, to determine when and where they act, and to assess their actions *in vivo*. These data are essential for moving the field forward, for understanding how the actions of molecules drive and change behavior, as well as for providing a substrate for understanding the consequences of gene modifications associated with human disease.

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