41 Neuromolecular Approaches to the Study of Language

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That language has a strong genetic basis has been clearly established in the previous chapters of this book and elsewhere. The difficult task of finding genes that underlie language skills and disorders has also been comprehensively covered in the other chapters of this part. Thus the goal of the present chapter is to discuss the ways in which we can move beyond gene identification into a deeper understanding of how these genes function, how these functions relate to normal development, and why variations or mutations in these genes lead to altered language abilities or, at the extreme, language disorders.

Communication via language is a complex multimodal task that involves a so-called *system of systems* (Levinson & Holler, 2014). To perform this task we must attend to the signal by which language is conveyed in our environment, that is, text, speech, and/or gestures, via auditory or visual perception. The information we receive must be processed via neural circuitry, and then we must plan and enact a motor program intended to produce sounds, gestures, signs, and/or writing to convey meaningful information (Fitch, Hauser, & Chomsky, 2005; Hauser, Chomsky, & Fitch, 2002; Levinson & Holler, 2014). This is a gross oversimplification of how we communicate and each of these steps can be broken down into many more intricate and interacting components that contribute to how we communicate via language. What this description is intended to do, however, is give a little insight into the complexity of the problem that we are dealing with when we talk about the genetics of language. When we say "language genetics" are we talking about genes that help us hear, speak, or read; genes that help us process linguistic information; genes that give us the ability to use grammar or learn new languages; or genes that when malfunctioning can cause language disorders? The answer is yes. We are looking for genes that do all this and more. But what we are clearly not looking for is one gene that does all these things. Rather, we are looking for many genes, some of which are likely to have very subtle influences, that together give us our unique human capacity for language.

But once we have found these genes we do not hang up our lab coats and go home. Finding the candidate genes that are involved in language phenotypes is the first step in a long and fascinating road toward understanding how these strings of letters in our DNA are able to do such an amazing task as building a "language-ready brain" (Fisher & Marcus, 2006). Once we identify a candidate language-related gene, we need to answer a range of questions to understand the function of the gene and its relationship to the capacity for language. We can ask questions such as: What sort of RNA or protein does the gene encode? When does it function—that is, is it acting developmentally to set up the biological components needed for language or is it performing some function during the very act of using language, or both? Where is it needed—that is, is it present in specific brain regions or neural circuitry subserving language? Via which molecular mechanisms does this gene produce these effects? And why does disruption of this gene cause language disorder? To answer these questions, we need a battery of approaches and model systems that we can use to explore gene function. In the following sections I will present background on the types of genetic variation that can give us clues to the genetics of language, the model systems that are widely used in a neuromolecular approach to language, as well as case studies exploring the functions of genes linked to language-related phenotypes—focusing on neuromolecular mechanisms. Finally, I will discuss approaches for finding new language-related genes, including recent advances taking a genome-wide view of language genetics.

1. Types of Genomic Variation Contributing to Language Phenotypes

The presence of genetic differences allows us to determine which genes underlie a trait in the human population by linking the presence of DNA changes to the trait under study. Although we share 99.9% of our DNA, this still leaves a lot of room for variation to occur in the approximately three billion DNA "letters" of the

human genome. Thus, each person carries approximately three million variants in their genome. Given the complexity of language, it is perhaps not surprising that many different types of genetic variation have been identified that contribute to language phenotypes. Before going into specific examples of language-related genes, I will outline some of the types of variation present in the genome that helps us find such genes. Broadly, I have classified genetic variation into classes based on size of the variation, its frequency in the population, and position in the genome.

1.1. Size of Genetic Changes The size of a genetic variant reflects how much of the genome it affects. Variation may take the form of large genomic rearrangements (copy number variants, translocations, or inversions) or small insertions or deletions (indels), or variation may change the identity of single letters in the DNA sequence. Importantly, the size of the change alone cannot be used as a proxy for the severity of the resulting phenotype, since a single base substitution in one gene could be more severe than the insertion of hundreds of bases in another part of the genome depending on whether any gene(s) are affected and what the affected genes do.

1.2. Frequency of Variation in the Population In terms of frequency, genetic variation can be broadly classed as either common—positions in the genome that are highly variable in the population, or rare—found in small numbers of people or even single individuals.

Common variation often has more subtle effects on phenotypes, as severely detrimental changes to the genome are usually not maintained in the population. The best-studied common variants are known as SNPs, which are single nucleotides in the genome that vary in large numbers of individuals, and thus can be used to track phenotypic variation in the population. The human genome has millions of these SNPs, and it is possible to look for associations between phenotypes and millions of SNPs simultaneously to determine whether one or more are linked to a trait—known as a genome-wide association study. By assaying common variation, it has been possible to identify genomic regions related to normal variation in language ability in the general population, as well as identify genetic risk factors related to language impairment or language-related disorders (Becker et al., 2016; Gialluisi et al., 2014; Newbury, Fisher, & Monaco, 2010; Newbury & Monaco, 2002, 2010; Reader, Covill, Nudel, & Newbury, 2014).

Rare changes may be inherited or de novo—that is, newly arising in the individual. These can be more severe and are often associated with disorders when they disrupt an essential gene. Rare genetic mutations can have severe consequences for language and many types of rare variation have been identified in individuals with language disorders, some examples of which will be discussed in subsequent sections (Deriziotis & Fisher, 2013; Fisher & Scharff, 2009; Kang & Drayna, 2011; Rodenas-Cuadrado, Ho, & Vernes, 2014; Vernes & Fisher, 2009, 2011).

1.3. Position of Variation in the Genome Genetic variation can be further classified into coding and non-coding variations. *Coding* DNA is the part of the genome containing genes, which encode proteins. Proteins compose much of the functional machinery of cells and therefore disruption of protein coding regions can have severe effects on phenotypes. Mutations in coding DNA might result in a change to the sequence identity of a protein, truncation of a protein (making the sequence shorter than it should be), or a complete loss of the protein.

Although crucial to phenotypes, only about 1% to 2% of the human genome codes for proteins. Much of the rest of the genome is so-called *noncoding DNA*, which has a range of functions, often having a regulatory role that controls when, where, and how much protein is produced from a gene. Although often more subtle than protein-coding variation, changes in regulatory regions of DNA can change the timing, location, or amount of gene expression and in this way have been implicated in disorders (Devanna et al., 2018; Turner et al., 2016; Vaishnavi, Manikandan, Tiwary, & Munirajan, 2013).

2. Models for Functional Testing

When attempting to understand functional links between genes and language phenotypes, there are multiple different ways to address this problem. We can ask about the general properties of the gene in its normal state. This will tell us how, when, and where the gene normally acts. We can also determine what happens when the gene is no longer present and compare this to its normal function. The effects of removing the gene from a system (via a genetic "knockout") or severely reducing its expression (via a genetic "knockdown") can tell us a lot about what it normally does. However, not all human variants or mutations completely destroy the function of a gene, so it is often of interest to also investigate the effects when a patient-identified mutation is introduced into the gene/protein.

In this section, I will briefly outline some key model systems used for neuromolecular investigations of

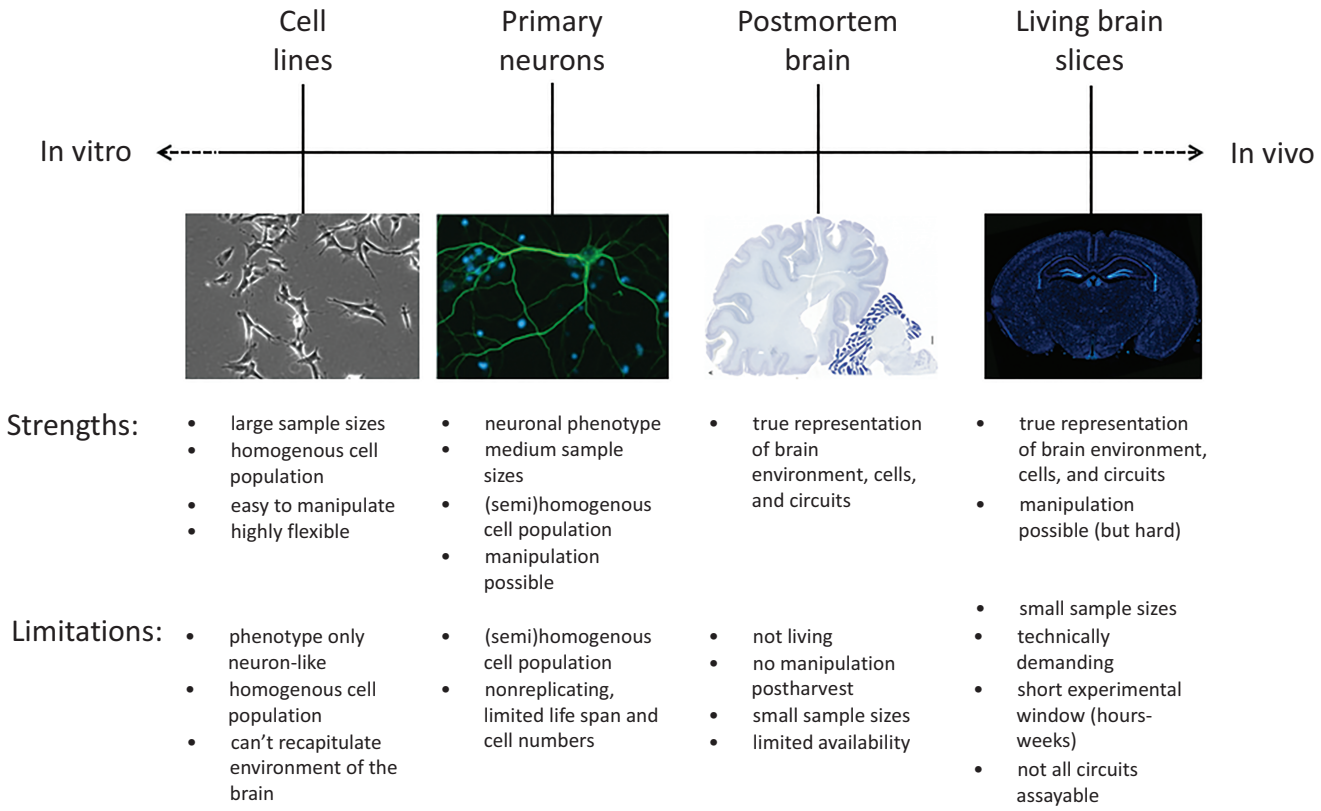

FIGURE 41.1 Summary of a selection of models used in neuromolecular approaches to studying language. Models are placed along a hypothetical scale from in vitro to more in vivo–like systems and the main strengths and limitations of each model are summarized. Postmortem human brain image adapted from Ding et al. (2016).

language-related genes. These range from in vitro systems, to models that more closely resemble the in vivo brain, and each has its strength and limitations (see figure 41.1 for summary). In sections 3–5, I will give specific examples of how these methods have been applied to understand three language-related genes.

2.1. CELL LINES One of the most simple and flexible models routinely used to explore gene function is an immortalized cell line. These cells are called *immortalized* as they can continue to divide in culture almost indefinitely and retain their properties as they divide,[1] meaning that very large quantities of highly homologous cells can be generated—a considerable advantage for experimentation at the molecular level. These cells can be frozen for long-term storage and thawed without harm. They also have the great advantage that it is relatively straightforward to manipulate their gene expression. DNA constructs that express the normal or mutant copy of a gene, or that express RNA molecules to switch off endogenous genes in the cell (known as short hairpin or small interfering RNA molecules) can be easily introduced into cell lines. Cell lines may come from almost any species (there are human, mouse, rat, bat, and fruit fly cell lines, to name just a few) and many different tissue sources (e.g., brain, skin, lung, kidney). Although these cells do not exactly match the phenotype of their source tissue, they show properties akin to cells of the original tissue including similar epigenetic marks, gene expression profiles, and morphology (Agholme, Lindstrom, Kagedal, Marcusson, & Hallbeck, 2010; Odom et al., 2007; Xie, Hu, & Li, 2010). Neurogenetics regularly makes use of human cell lines derived from neural tissue—the so-called *neuron-like cell lines* (Agholme et al., 2010; Cheung et al., 2009). An advantage of these neuron-like cells is that although they start off only moderately resembling neurons, with the addition of growth factors they can be differentiated into a state that is much more characteristic of a mature neuron and may even form active synapses and start signaling as a connected neural network (Agholme et al., 2010; Encinas et al., 2000; Jamsa, Hasslund, Cowburn, Backstrom, & Vasange, 2004; Joshi, Guleria, Pan, DiPette, & Singh, 2006). Because of these properties (easy to manipulate, able to get large quantities of homogenous cells, similarity to source tissue), these model systems facilitate a wide

range of molecular experimentation aimed at understanding the core functions of language-related genes.

Exciting advances in cellular techniques are also now making it possible to derive human neurons from non-neuronal sources such as from human embryonic stem cell lines or patient tissue biopsies (e.g., induced pluripotent stem cell lines) (Chailangkarn, Acab, & Muotri, 2012). These emerging methods direct the differentiation of cells into neurons via reprogramming of gene expression and addition of growth factors. The resulting neurons closely resemble specific in vivo phenotypes (e.g., neurons of the cortex or even complex assemblies of neuron types such as organoids) (Lancaster et al., 2013; Wapinski et al., 2013) and allow study of gene-phenotype relationships within a human neuronal system. A great benefit of this approach is the opportunity to study human neurons directly (especially neurons generated from patient samples that could carry causative genetic mutations). However, generating these models requires substantial investment as they are currently technically very demanding and time-consuming, meaning that their use is not yet routine.

2.2. Primary Neurons Another cell model that can be grown in a dish in the lab, but that is closer to the in vivo brain than to immortalized cell lines are primary neurons. Primary neurons are made by dissecting out tissue from regions of interest in the developing brain (often from mouse or rat) and treating the tissue so the cells are gently dissociated into a single cell suspension. The neurons are then maintained alive in a rich growth environment in a petri dish. Primary neurons prepared in this way start off as single cells, but after only one day the cells start extending their neurites (axons and dendrites) and after approximately two weeks they have grown long, highly branched neurites to form connected, active, neuronal networks (Falk, Zhang, Erbe, & Sherman, 2006; Geissler & Faissner, 2012; Kim & Lee, 2012). This means that the effect of gene manipulation on neurite growth, synapse formation, and network activity can be studied in real time in these living neurons in a petri dish. Primary neurons are not immortal and do not divide, thus they have a limited life span once in a petri dish, however they can survive in culture for weeks or months with the proper care. Like cell lines, primary neurons can be genetically manipulated, but they are far less receptive to the introduction of DNA constructs, thus it is often necessary to use techniques such as packaging the DNA into viruses and infecting the cells to deliver DNA to the neurons (Holehonnur, Lella, Ho, Luong, & Ploski, 2015; Manfredsson, 2016).

2.3. Postmortem Brain Tissue Directly studying postmortem brain tissue moves closer toward an environment that reflects the in vivo brain. The greatest strength of this model is that it is possible to take slices through different regions of a human or animal brain and look directly at where and how strongly a gene/protein is expressed and how brain structure might be affected by differences. However, a major limitation with this system is that because the tissue is not living it is not possible to perform any further manipulations. Thus all genetic manipulations must be done prior to harvest of the tissue. With human postmortem tissue this is ethically out of the question; however, it is possible to compare brains of people with naturally occurring variation and look for common features (although still difficult given that tissue is scarce and thus sample size is necessarily small). In animal models, particularly in mouse models, this is more straightforward, as transgenic animal lines are now routinely created in which a gene is *knocked-out*—such that it can no longer be expressed; *knocked-down*—such that its expression is reduced; or *knocked-in*—where a change, for example, a patient-identified mutation, is added to the genome. Comparison of a knockout brain versus a matched control (normal) brain is a powerful way to determine the neuronal functions of a gene.

2.4. Brain Slices Finally, it is possible to harvest brain slices from animal models and keep them alive in a dish for hours (acute slices) or weeks (organotypic slices). This can be done with transgenic animal models to look at effects of genetic manipulations, but it is also possible to infect brain slices from normal animals with viruses carrying DNA constructs to alter gene expression or apply chemicals to manipulate specific molecular pathways. In this way it is possible to directly measure the effects of genetic mutations or chemical treatments on the activity of synapses and neural circuits in real time. A major benefit of using slices is that they closely reflect many aspects of the living brain. Prior to harvest, the neurons and circuits have formed in the complex three-dimensional environment of the brain and thus slices match the in vivo brain more closely than cell models do (e.g., primary neurons). However, there are some limitations that should be considered. Working with brain slices is technically demanding and time-consuming. Often, neurons are recorded one at a time, meaning that only a handful of neurons can be measured per brain slice. Slices can also only be worked with for a limited time window. This means that, for example, genetic or chemical treatments that take days or weeks to have an effect cannot be observed in acute slices and

long-term experiments (longer than weeks) are sometimes not feasible. One way around this is to perform manipulations in the animal (by creating transgenic animals or administering chemicals to the living animal) and then harvest the brain to measure long-term effects. Another limitation is that these are, by nature, slices, thus it is not possible to measure all circuitry in the brain. Brain slices tend to be between 100 and 500 μm thick.[2] Only connections that are preserved after the slices are cut can be assayed, meaning that any connectivity in the brain extending more than a few hundred microns perpendicular to the cut will be lost.

To illustrate how the models outlined in this section can be utilized and what they have revealed about the neurogenetics of language, in the subsequent sections I will discuss two well-characterized examples and one new example of a putative language-related gene. For each gene (where the information is available), I will describe what the gene encodes; where it is expressed; what molecular, cellular, and neuronal functions it performs; and how this has informed our understanding of why mutations cause language-related disorders.

3. Function of Language-Related Genes: FOXP2

The first gene implicated in human language was *FOXP2*,[3] and it is for this gene that we have the most comprehensive investigations of how genes contribute to a language-ready brain. *FOXP2* was first identified as a monogenic cause of a rare and severe form of speech and language disorder in a large pedigree, known as the KE family (Lai, Fisher, Hurst, Vargha-Khadem, & Monaco, 2001). All affected members of this family carried a single letter DNA change in the protein coding region of one copy of this gene and that led to a single amino acid change in the protein sequence (Lai et al., 2001). Because humans have two copies of each gene in their genomes (one inherited from each parent), affected individuals in this family had one normal copy of *FOXP2* and one copy that carried the protein altering mutation—which is sufficient to cause the disorder. Since its initial identification, mutations affecting *FOXP2* (ranging from point mutations to large-scale multigene deletions) have been identified in a number of independent cases, each with a single copy of *FOXP2* disrupted and displaying similar speech and language disorders (Morgan, Fisher, Scheffer, & Hildebrand, 2017). These cases provide convincing evidence that mutation of a single copy of *FOXP2* is sufficient to cause speech/language disorder and that both copies of the *FOXP2* gene are required for normal language to develop in humans. Although it should be noted that *FOXP2* mutations are rare and can only account for the observed speech/language disorder in a small minority of cases.

3.1. When and Where Is *FOXP2* Expressed?

Understanding the location and timing of *FOXP2* expression can give clues to its role in the brain.[4] Expression of *FOXP2* has been observed throughout the developing human brain including in the developing cortex, subcortical regions (e.g., thalamus, hypothalamus, caudate nucleus), midbrain, and cerebellum (Lai, Gerrelli, Monaco, Fisher, & Copp, 2003). Little is known about *FOXP2* expression in the postnatal human brain given the difficulty in obtaining samples; however, animal models have given us insight into what adult expression pattern might look like. Human *FOXP2* and mouse *Foxp2* are highly conserved at a sequence level and the expression pattern of *Foxp2/FOXP2* was highly similar in mouse and human embryonic brains (Ferland, Cherry, Preware, Morrisey, & Walsh, 2003; Lai et al., 2003). Thus it was predicted that in the adult brain, expression patterns would also be highly conserved. In adult mice, *Foxp2* expression was maintained in the same areas as in the developing mouse and human brains. In many regions, however, expression became more restricted to subsets of neurons in these regions in adult brains. As a result, adult mice displayed *Foxp2* expression in deep layer cortical neurons, medium spiny neurons of the striatum, a subset of nuclei of the thalamus, inferior olives in the brain stem, and Purkinje cells of the cerebellum (Ferland et al., 2003; Lai et al., 2003). This evidence from human and mouse studies suggests that *FOXP2/Foxp2* is expressed across multiple structures of the developing brain, and in the adult brain is found in areas involved in higher order cognition, sensory integration, and motor control/learning. Thus a 50% reduction in functional *FOXP2*, as found in the affected KE family members, might have subtle but specific effects on development and function of these circuits.

3.2. What Does *FOXP2* Encode?

Given its links to language, it was of intense interest to discover the function of the protein encoded by the *FOXP2* gene. Analysis of the protein sequence encoded by *FOXP2* indicated that the protein contained a domain known as a forkhead-box (*FOX* for short), identifying it as part of the FOX family of genes (Katoh & Katoh, 2004; Lai et al., 2001).[5] FOX genes are known to act as transcription factors, suggesting that *FOXP2* was likely to have the same function, that is, to regulate the expression of other genes in the genome.

3.3. In Which Molecular Mechanisms Is *FOXP2* Involved? Around the same time the mutations in human *FOXP2* in the KE family were identified, researchers independently showed that the mouse Foxp2 protein was indeed acting as a transcription factor to regulate gene expression (Shu, Yang, Zhang, Lu, & Morrisey, 2001). The ability of human FOXP2 to directly regulate gene expression was also shown in human cell lines (Vernes et al., 2006), opening up the possibility to explore the genes it regulates and thus understand the downstream molecular mechanisms directed by FOXP2. Studies using human neuron-like cells, human fetal brain and developing mouse brain identified the genes that were regulated by FOXP2; its so-called *target genes* (Spiteri et al., 2007; Vernes et al., 2007; Vernes et al., 2011). These studies identified overlapping sets of genes that were involved in highly conserved molecular pathways. Across both human and mouse model systems there was a high proportion of target genes that played a role in neuronal development including such processes as neuronal migration, neurite outgrowth, and synapse function (Spiteri et al., 2007; Vernes et al., 2007; Vernes & Fisher, 2011). This gave first insight into the molecular pathways that were acting downstream of FOXP2 and furthermore suggested cellular processes and phenotypes that may be directly affected by this gene.

3.4. In Which Cellular/Neuronal Mechanisms Is *FOXP2* Involved? The molecular data described have suggested a role for FOXP2 in cellular processes including migration, neurite outgrowth, and synaptic activity—hypotheses that could be directly tested using model systems.

Precise control of neuronal migration is important to ensure that neurons reach the appropriate place in the brain at the right time during development. Neurons migrating too far, not far enough, or arriving at the right place but at the wrong time can all have important consequences for the formation of brain circuits (Marin, Valiente, Ge, & Tsai, 2010). Effects of Foxp2 on neuronal migration were first observed in the developing mouse brain, where overexpression of *Foxp2* led to reduced migration of neurons during cortical development (Clovis, Enard, Marinaro, Huttner, & De Pietri Tonelli, 2012). This study investigated the regulation of Foxp2 via microRNA molecules and its consequences for neuronal migration. However, it was also possible from this data to observe the effect of excess Foxp2 on cortical migration during development. In this study, *Foxp2* or a control DNA construct, along with a visible label, were injected into cells of the early embryonic cortex. Embryos were then left to grow and the effects were observed in the brains of postnatal animals. Mice that received excess *Foxp2*, compared to control animals, displayed more labeled cells in deep layers of the cortex, suggesting that the normal migration of neurons through the cortical layers had been disrupted.

Comparable effects were observed for human FOXP2 in a human neuron-like cell line model (Devanna, Middelbeek, & Vernes, 2014). An advantage of studying such questions in these cell lines is that is it possible to compare the behavior of pure populations of FOXP2-positive and FOXP2-negative cells in real time. Using this system to observe the migration of living cells using time-lapse photography, it was demonstrated that cells expressing FOXP2 migrated more slowly than cells lacking FOXP2 (see figure 41.2A; Devanna et al., 2014). Thus the evidence from both brain and cell models suggest that human and mouse FOXP2/Foxp2 acts to influence neuronal migration and that increasing *FOXP2/Foxp2* expression results in reduced cell migration.

Neurogenetic models were also used to investigate the role of FOXP2 in neurite outgrowth—the process by which axons and dendrites grow and branch to allow neurons to connect to each other. The developing mouse striatum is a site of high *Foxp2* expression and thus primary neurons from this region were generated to study how Foxp2 can influence the growth and development of neurites. Primary striatal neurons from mice with mutations in *Foxp2* that mirrored the mutation found in the KE family developed shorter neurites with fewer branches than did primary neurons from normal (so-called *wild-type*) animals (figure 41.2B). This showed that a normal function of Foxp2 is to promote the growth and branching of axons and dendrites in the striatum, pointing to a role for Foxp2 in establishing striatal circuitry (such as cortico-striatal-thalamic loops) in the brain (Vernes et al., 2011). Cell line models showed a comparable role for human FOXP2 in neurite outgrowth. Human neuron-like cells expressing FOXP2 grew longer and more highly branched neurites than FOXP2-negative cells did (Devanna et al., 2014), suggesting that both mouse Foxp2 and human FOXP2 normally act to promote neurite outgrowth and branching.

Once neurons have migrated to the right position in the brain and extended their neurites toward other neurons, they must make and maintain connections with other neurons via the formation of synapses. It is this signaling across synapses and the resulting strengthening or weakening of synapses (known as synaptic plasticity) that underlies the function of neural circuits. By using brain slices from wild-type or *Foxp2* mutant animals, it was possible to record directly from neurons and observe synaptic effects. Mutation of one

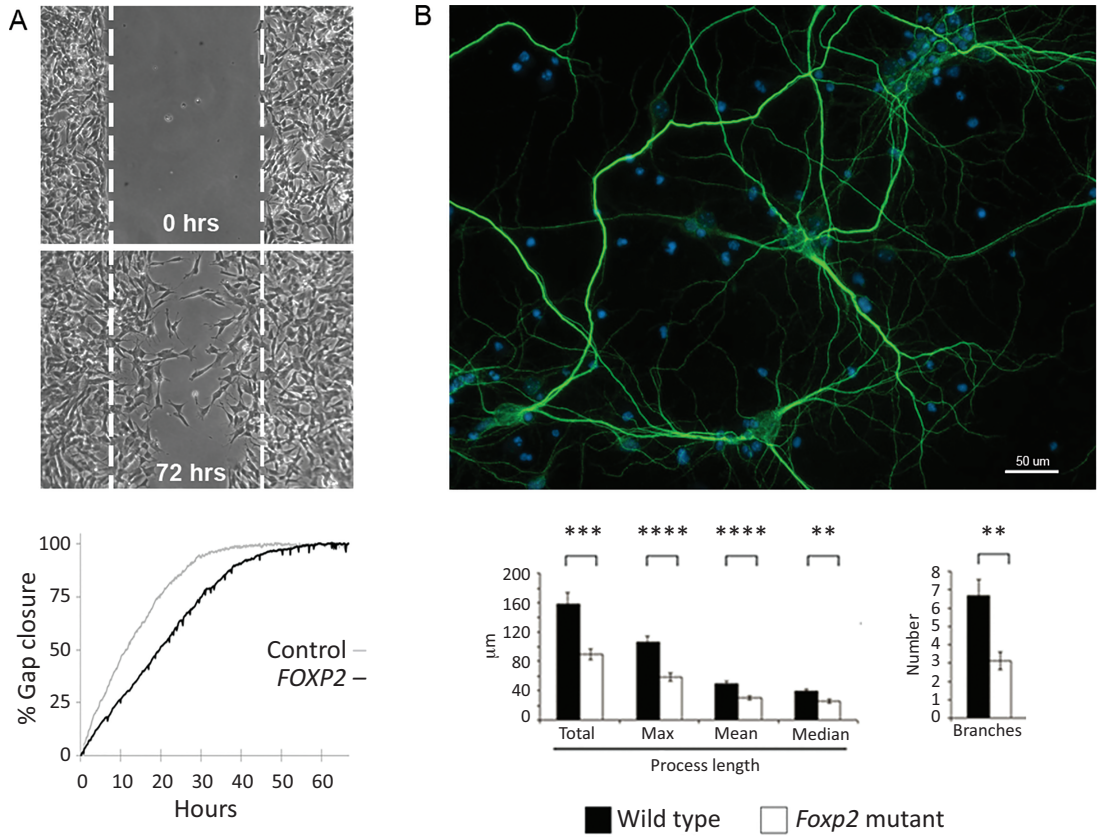

FIGURE 41.2 *FOXP2* affects neuronal migration and neurite outgrowth. (A) Observing the migration of human neuron-like cells over 72 hours showed that cells expressing *FOXP2* migrated more slowly than control cells without *FOXP2*. (B) Primary striatal neurons taken from normal wild-type mice had longer and more branched neurites than those from mice that are homozygous for mutations in *Foxp2* (*Foxp2* mutant) (**** = p=0.001, *** = p=0.001, ** = p=0.01). Figures adapted from Devanna et al. (2014) (A) and Vernes et al. (2011) (B).

copy of *Foxp2* altered synaptic plasticity in the mouse striatum, showing that *Foxp2* is involved in regulating the activity of striatal synapses and firing through striatal circuitry (Groszer et al., 2008). Recordings made by implanting electrodes in awake-behaving mice also showed aberrant striatal activity in mice with heterozygous mutations in *Foxp2* (French et al., 2012). Taken together, studies across human and mouse model systems demonstrate that *FOXP2/Foxp2* influences multiple aspects of brain development that can influence the formation of, and signaling through, specific neural circuitry.

3.5. WHY DOES MUTATION OF *FOXP2* CAUSE DISORDER? Understanding the molecular and cellular functions of FOXP2, it is possible to start to build a picture of why mutations of this gene result in language-related disorders. At the most fundamental level it was shown that introducing patient mutations (such as the mutation found in the KE family) into *FOXP2* severely affected the transcription factor function of this protein in cell lines (Vernes et al., 2006). Normally, FOXP2 is restricted to the nucleus of cells, where it binds to DNA. However, introducing the KE family mutation into FOXP2 led to increased levels of the protein outside the nucleus in human cell lines (Vernes et al., 2006). Moreover, the mutant protein was no longer able to bind to target DNA or regulate gene expression (Vernes et al., 2006). As a result, in individuals carrying these mutations, the target genes normally regulated by FOXP2 are likely to be misregulated. Since affected individuals retain one normal copy of *FOXP2*, these effects may be subtle (i.e., an altered amount of target gene being expressed rather than a complete loss of gene regulation); however, this could still lead to important changes in how the brain develops. Indeed, both structural and functional abnormalities were observed in affected members of the KE family, in regions where *FOXP2* is expressed (Belton, Salmond, Watkins, Vargha-Khadem, & Gadian, 2003; Liégeois et al., 2003; Pinel et al., 2012). For example in affected KE family members (who all have a mutation

in one copy of *FOXP2*), the caudate nucleus, putamen, and select cortical regions (including the inferior frontal gyrus) showed both structural (via voxel-based morphometry tests) and functional differences (via functional MRI) from those regions of the brains of unaffected family members (Belton et al., 2003; Liégeois et al., 2003; Pinel et al., 2012). Thus by understanding the role of FOXP2 in model systems to regulate target genes and cellular processes, together with the phenotypes observed in affected individuals, we can start to understand the mechanisms that may underlie the disorders of speech and language caused by *FOXP2* mutations.

4. Function of Language-Related Genes: CNTNAP2

Understanding FOXP2 function also led to the identification of new candidate language-related genes. Given that the function of FOXP2 is to regulate the expression of other genes, the phenotypic effects it has on cells, circuits, or behaviors must be mediated by these target genes. For this reason, it was hypothesized that the target genes acting downstream of FOXP2 would represent new candidate genes for language or language disorders. This was proven to be the case by the identification of the *CNTNAP2* gene as a target of FOXP2 (Vernes et al., 2008) and the association of common variation in this gene with disorders involving language (specific language impairment [SLI], autism spectrum disorder [ASD], dyslexia) and language-related phenotypes (early communicative behavior) (Alarcon et al., 2008; Anney et al., 2012; Arking et al., 2008; Ji et al., 2012; Newbury et al., 2011; Peter et al., 2011; Poot, 2014; Vernes et al., 2008; Whitehouse, Bishop, Ang, Pennell, & Fisher, 2011). Rare mutations of *CNTNAP2* have also been found in individuals with disorders affecting language (including language regression, ASD, and a small number of individuals with speech apraxia) and also more widespread deficits such as intellectual disability and epilepsy (Poot, 2015; Rodenas-Cuadrado et al., 2014; Rodenas-Cuadrado et al., 2016).

4.1. When and Where Is *CNTNAP2* Expressed? *CNTNAP2* expression has been well studied in mouse and human tissue and is highly expressed in a number of brain regions during development and increasingly in postnatal stages (Abrahams et al., 2007; Alarcon et al., 2008). In the human brain, expression is highest in the striatum, dorsal thalamus, and cortical areas. In the developing human cortex, expression is highest in layers II–V and enrichment in the inferior frontal gyrus and perisylvian regions has been noted (Rodenas-Cuadrado et al., 2014). The strong expression of *CNTNAP2* in brain regions involved in higher order cognitive processes, speech, and language supports a role for this gene in human language development.

4.2. What Does *CNTNAP2* Encode? The *CNTNAP2* gene encodes a protein (known as CASPR2) that is a member of the neurexin superfamily of proteins (Nakabayashi & Scherer, 2001; Rodenas-Cuadrado et al., 2014). Neurexins are well known to facilitate cell-cell interactions in the nervous system and play a role in synapse development and function, giving first hints to the likely function of CASPR2 (Dean & Dresbach, 2006; Siddiqui & Craig, 2011; Sudhof, 2008).

4.3. In Which Molecular Mechanisms Is *CNTNAP2* Involved? CASPR2 is a transmembrane protein (meaning that it sits at the cell surface) and most of the CASPR2 protein is projected outside the cell into the extracellular space (Poliak et al., 1999). Initially CASPR2 was observed at the surface of axons. The small intracellular component of CASPR2 helps to cluster ion channels together and the large extracellular component facilitates interactions with myelin (which surrounds and insulates nerve fibers), and these functions contribute to rapid conduction of nerve impulses (Horresh et al., 2008; Inda, 2006; Poliak et al., 1999; Poliak et al., 2003; Traka, 2003; Vabnick et al., 1999).

More recently, it has been shown that CASPR2 is also located at the synaptic membrane (Bakkaloglu et al., 2008; Varea et al., 2015). Like other members of the protein family to which it belongs (neurexins) it is thought to act as scaffold protein bridging the presynapse and postsynapse to facilitate synaptic stability (Bakkaloglu et al., 2008; Sudhof, 2008; Varea et al., 2015).

4.4. In Which Cellular/Neuronal Mechanisms Is *CNTNAP2* Involved? CASPR2 has been implicated in neuronal migration, network formation, and synaptic activity. Evidence for a role in neuronal migration came from patients with *CNTNAP2* mutations and a severe syndrome including epileptic seizures, autism, and language regression (Strauss et al., 2006). Following surgery aimed at controlling their epilepsy, brain biopsies were analyzed and the tissue was found to contain evidence of abnormal migration including poorly defined white and gray matter junctions, abnormal organization of neurons, and ectopic (misplaced) glia and neurons (Strauss et al., 2006). Migration defects were also observed in a mouse model of *Cntnap2*. Brain slices taken from *Cntnap2* knockout mice showed ectopic neurons in the corpus callosum and aberrant layering of neurons in the cortex, suggesting deficiencies in migration during

development (Penagarikano et al., 2011). Brain tissue from these mice also showed reduced numbers of inhibitory interneurons in the cortex, striatum, and hippocampus, which could reflect either altered neuronal migration or neurogenesis (Penagarikano et al., 2011).

In primary cortical neurons from the developing mouse brain, Caspr2 affected neuronal network formation and connectivity. Reduction in Caspr2 expression (via knockdown) resulted in reduced growth and branching of dendrites (Anderson et al., 2012). Neurons cultured from transgenic *Cntnap2* knockout mice displayed reduced density of spines—the sites where synapses may form (Varea et al., 2015). In primary neurons, Caspr2 knockdown/knockout also affected synaptic properties and transmission of signals across the synapse by altering levels of receptors at the synapse (Varea et al., 2015) and altering the amplitude of synaptic responses (Anderson et al., 2012).

4.5. Why Does Mutation of *CNTNAP2* Cause Disorder? The links between *CNTNAP2* mutations and disorder are not as clear-cut as the ones for *FOXP2*. As noted, individuals with rare *CNTNAP2* mutations display a complex profile that is not restricted to language but includes ASD, speech/language impairments, epilepsy, and intellectual disability. These phenotypes are consistently present in individuals with homozygous *CNTNAP2* mutations; however, heterozygous changes seem to have a mixed penetrance, from severe to unaffected (Murdoch et al., 2015; Rodenas-Cuadrado et al., 2014; Rodenas-Cuadrado et al., 2016). It is not clear why some heterozygous individuals have more severe phenotypes than others do. It could be due to the genetic background of the individual, making some people more vulnerable to *CNTNAP2* mutation than others are (due to other genetic variants they carry). Conversely, it may be that some mutations of *CNTNAP2* produce more severe disruptions of CASPR2 function. Functional effects of patient mutations have been directly investigated for a small number of variants in human cell lines. Some nonsynonymous point mutations caused retention of the protein in the endoplasmic reticulum of the cell (where proteins are produced, modified, and folded into their mature shape) and in some cases degradation of the mutant protein. Introducing patient mutations into cells resulted in reduced amounts of functional protein to be present in human cell lines (Falivelli et al., 2012). A homozygous frameshift mutation causing a disorder involving cortical dysplasia, focal epilepsy, intellectual disability, attention deficit/hyperactivity disorder, and ASD in an Amish population was predicted to cause a truncated CASPR2 protein (Strauss et al., 2006). This mutation was found to truncate CASPR2 prior to its transmembrane domain, meaning that instead of being lodged at the cell surface/synapses, this protein is secreted into the extracellular space and lost (Falivelli et al., 2012).

Common variation in *CNTNAP2* has been associated with language disorder phenotypes and these variants have been linked to differences in the structure and function of brain circuits related to language processing. Structural changes included effects on gray matter volume (Tan, Doke, Ashburner, Wood, & Frackowiak, 2010; Udden, Snijders, Fisher, & Hagoort, 2016); structural and functional connectivity (Scott-van Zeeland et al., 2010; Dennis et al., 2011); and brain activation during sentence, artificial grammar, and syntax violation processing (Folia, Forkstam, Ingvar, Hagoort, & Petersson, 2011; Kos et al., 2012; Whalley et al., 2011). Why or how these common variants could be influencing brain structure or function is unknown, and further neuromolecular investigations are needed to understand these links.

Taken together these data support multiple roles for *CNTNAP2* in migration, neuronal connectivity, and synapse activity that may underlie the phenotypic differences observed in individuals carrying rare or common genetic variants in this gene.

5. Function of Language-Related Genes: ARHGEF39

Common variation in *ARHGEF39* was only recently associated with endophenotypes of language impairment (Devanna et al., 2018) and thus relatively little is known about the function of this gene. However, it warrants further discussion here as it represents an unusual example of a noncoding variant with clear functional consequences that may be relevant for language disorder.

As we have seen in sections 3 and 4, rare coding variants that disrupt protein sequence can have severe consequences and thus lead to disorder. However, noncoding variation may also contribute to disorder if it is located in a region of the noncoding genome that is responsible for regulating gene expression. Such noncoding regulatory variants are particularly good candidates for complex language disorders because these disorders are likely to be caused by the additive effects of subtle changes to multiple genes, rather than disruption of a single coding gene.

A recent study aimed to discover such noncoding regulatory variants in children with language impairment, focusing specifically on one type of regulatory region, known as a 3′-untranslated region (or *3′UTR*) of genes (Devanna et al., 2018). 3′UTRs control how much

protein is expressed from a gene via multiple mechanisms including by interacting with regulatory molecules known as *microRNAs* (Grimson et al., 2007). From 43 affected children, a variant was identified in the 3′UTR of the *ARHGEF39* gene, which was predicted to disrupt this regulatory process. This variant was the alternative allele of a common SNP (rs72727021), which made it possible to determine whether it was associated with quantitative measures of language impairment in a larger cohort of 983 individuals from 285 families affected by language impairment (Devanna et al., 2018). The alternative allele of the rs72727021 SNP had a frequency of 12.3% in the language-impaired children in this cohort, as compared to a frequency of around 10.8% in unselected European populations and was significantly associated with reduced performance on tests of nonword repetition[6] in the language-impaired children (Devanna et al., 2018).

5.1. What Does *ARHGEF39* Encode? *ARHGEF39* is a member of the *ARHGEF* family of Rho guanine nucleotide exchange factors—enzymes that catalyze activation of guanosine triphosphatases and act as adapter proteins in a number of cellular reactions. Little is known regarding the specific function of ARHGEF39, however other members of this family of proteins regulate a range of processes such as gene expression, cell migration, cell growth and dendritic outgrowth (Goicoechea, Awadia, & Garcia-Mata, 2014; Newey, Velamoor, Govek, & van Aelst, 2005; van Aelst & Cline, 2004; van Aelst & D'Souza-Schorey, 1997).

5.2. Why Does Variation in *ARHGEF39* Associate with Disorder? The SLI-associated rs72727021 variant is located in the 3′UTR of the *ARHGEF39* gene and was predicted to disrupt the interaction between 3′UTR and a microRNA molecule (Devanna et al., 2018). MicroRNAs bind to 3′UTR regions of messenger RNAs, resulting in assembly of a protein complex that causes reduced translation of the messenger RNA and, as a consequence, lower protein levels (Chen & Rajewsky, 2007). Because this interaction is mediated via complementary base pairing, the sequences bound by microRNAs have been well defined. Thus, clear predictions of binding sites and effects of variants can be made *in silico* (Lewis, Burge, & Bartel, 2005). The rs72727021 SNP was located in a binding site for microRNA-215, and its presence was predicted to alter microRNA binding and protein expression (Devanna et al., 2018), however direct functional tests in cell lines were needed to confirm these effects.

By introducing the 3′UTR of *ARHGEF39* together with microRNA-215 into cell lines, it was possible to see that the reference sequence (the A allele that is common in the general population) was downregulated by microRNA-215. By contrast, the alternative 3′UTR sequence (carrying the SLI-associated alternative C allele) was not downregulated (see figure 41.3A) (Devanna et al., 2018). Comparable disruption of gene regulation and expression were also observed in the human brain (see figure 41.3B). In postmortem human tissue samples, individuals with the A allele had lower expression of *ARHGEF39* than individuals with the C allele (in heterozygous or homozygous state) across multiple regions of the brain, including the cortex (Devanna et al., 2018). Taken together, these data suggest that the presence of this single change in a noncoding DNA region disrupts regulation of *ARHGEF39* expression. Why elevated *ARHGEF39* expression may be related to language impairment is currently unknown, but functional studies into this gene in the future may shed light on this putative new language-related gene.

Interestingly, *ARHGEF39* is not the only member of the *ARHGEF* gene family that has been implicated in language impairment or neurodevelopmental phenotypes. A common variant thought to affect *ARHGEF19* expression is associated with language impairment (Nudel et al., 2014), *ARHGEF6* has been linked to intellectual disability (Kutsche et al., 2000), and *ARHGEF9* mutations have been found in individuals with developmental delay, intellectual disability, and seizures (de Ligt et al., 2012; Harvey et al., 2004; Kalscheuer et al., 2009; Lemke et al., 2012; Lesca et al., 2011; Marco et al., 2008; Shimojima et al., 2011), as well as ASD and speech delays (Bhat et al., 2016). These data may point to a larger role for the *ARHGEF* family in neurodevelopment and language-related circuitry and supports the call for greater studies to be performed on the neuromolecular functions of these genes.

6. Finding New Language-Related Genes via Neuromolecular Approaches

Although there are infrequent examples where mutations in a single gene like *FOXP2* can cause language impairments, it is clear that there is no single "gene for language," rather there are interconnected networks of genes that contribute to complex phenotypes underlying the human capacity for language. It is therefore essential that, rather than only considering individual genes, gene networks be considered as a whole to obtain a complete understanding of the molecular-genetic underpinnings of language. There are many ways to identify gene networks related to language. One way is to use known language-related genes, such as *FOXP2*,

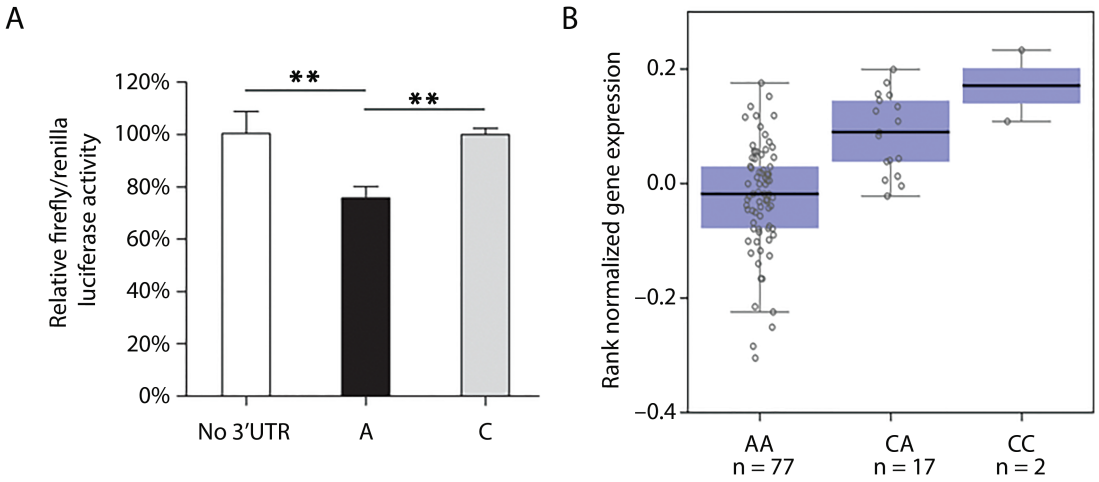

FIGURE 41.3 The presence of the rs72727021 SNP in the 3′UTR of the *ARHGEF39* gene results in altered regulation in human cell lines (** = $p<0.01$) (A) and postmortem human cortex tissue (B). The alternative allele was significantly associated with nonword repetition in language-impaired children and led to increased expression in both model systems. Figures adapted from Devanna et al. (2018).

as an entry point to identify the wider molecular networks within which they are embedded. Another way is to perform hypothesis-free discovery of networks by exploring the molecular properties of relevant circuits, tissues, and/or behaviors.

6.1. MOLECULAR WINDOWS INTO LANGUAGE Language-related genes can be used as a molecular window into language pathways by understanding their wider molecular networks. These networks include transcription factors that regulate expression, interacting proteins, and downstream molecular cascades (e.g., resulting from enzymatic reactions, signaling cascades, or gene regulatory mechanisms).

Exploration of the molecular networks related to *FOXP2* has shown the power of this approach (Fisher & Scharff, 2009). Proteins that interact with FOXP2 have proven strong candidates for language-related disorders. In performing its function as a transcription factor, FOXP2 interacts with other regulatory factors in order to bind DNA and regulate gene expression (Li, Weidenfeld, & Morrisey, 2004; Wang, Lin, Li, & Tucker, 2003). The closely related protein, FOXP1 (also a FOX transcription factor), is expressed in overlapping brain regions with FOXP2, including parts of the cortex (motor cortex layer IV) and striatum (caudate nucleus and putamen) where these proteins are able to dimerize to regulate gene expression (Ferland et al., 2003; Hisaoka, Nakamura, Senba, & Morikawa, 2010; Li et al., 2004). Mutations in *FOXP1* have been found in individuals displaying motor impairments, intellectual disability, autism, and speech/language impairments (Bacon & Rappold, 2012; Bacon et al., 2015; Carr et al., 2010; Chien et al., 2013; Hamdan et al., 2010; Horn, 2011; O'Roak et al., 2011; Sollis et al., 2016; Vernes, MacDermot, Monaco, & Fisher, 2009).

Identifying the downstream targets of FOXP2 has facilitated the identification of new candidate genes and identified molecular links with existing candidate genes for language-related disorders. *CNTNAP2* was the first example of a FOXP2 target gene used as a candidate for language disorder (Vernes et al., 2008). *CNTNAP2*'s status as a FOXP2 target led to its association with SLI and represented the first molecular link between language-related disorders (see section 4).

DISC1 was a well-studied candidate gene for schizophrenia when it was identified as a direct target of FOXP2 (Spiteri et al., 2007), suggesting a possible molecular link between speech/language disorder and schizophrenia (Farrell et al., 2015). Schizophrenia is a neuropsychiatric disorder with a range of clinical symptoms including auditory hallucinations, delusions, and social and communicative impairments (Kuperberg, 2010). Impaired verbal communication is a key feature of schizophrenia and may manifest in many ways, such as alogia (poverty of speech), disorganized speech pattern, misassociation of words and phrases, use of neologisms or nonwords, and production of meaningless sentences (Kuperberg, 2010). *DISC1* disruptions have also been linked to ASDs (Crepel et al., 2010; Kilpinen et al., 2008; Williams et al., 2009; Zheng et al., 2011). FOXP2 was found to bind the promoter of the *DISC1* gene in embryonic human basal ganglia tissue (Spiteri et al., 2007). In functional cell experiments, FOXP2 was shown to bind to the promoter to downregulate the

expression of *DISC1*, and this repression was disrupted when patient mutations of *FOXP2* were introduced (Walker et al., 2012). Using induced pluripotent stem cell models, loss of *DISC1* expression was shown to affect synapse formation and synaptic activity and have knock-on consequences for large networks of neuronal genes (Wen et al., 2014).

Many more studies are needed to clarify how *FOXP2* genetic networks relate to language disorders and to elucidate the molecular links between diverse disorders involving language phenotypes, but these examples give good reasons to expect this approach to yield valuable insights in the coming years.

6.2. Hypothesis-Free Network Identification It is possible to identify language-related molecular networks without prior hypotheses about the genes involved. One way to do this is to survey the genes expressed in specific brain regions to understand the molecular pathways acting during development or function of these regions. This can be done by surveying known language-related regions of postmortem human brain (see parts V and VI of this volume), or circuitry in animal brains underlying language-relevant behaviors (see part VIII of this volume). Molecular networks can be surveyed by transcriptome analysis, that is, RNA-Sequencing, which will identify every gene being expressed in the tissue at a given time point, together with the levels of expression for each gene (from very high to very low). By surveying multiple individuals/animals and different time points or behaviors, relationships between genes can be extracted from these data following the principal that genes that are tightly coupled in their expression patterns across samples will be likely to act together (Langfelder & Horvath, 2008). Linking this to the known functions of these genes facilitates the building of large-scale, molecular networks of functional relevance to the tissue/behavior under study.

This approach has been used in vocal learning songbirds to identify gene networks regulated during singing (Hilliard, Miller, Fraley, Horvath, & White, 2012; Hilliard, Miller, Horvath, & White, 2012). By surveying key regions of the song circuitry in singing and silent brains using transcriptomics and *in silico* network building methods, large-scale molecular networks were built that were driven by, and involved in, singing behavior. This showed that specific parts of the song circuitry displayed distinct molecular profiles and identified new genes that may be involved in such behavior across species (Hilliard, Miller, Fraley, et al., 2012; Hilliard, Miller, Horvath, & White, 2012). More recently, bats have emerged as a promising mammalian model to study vocal learning. A comparable network building approach was used to identify molecular networks in vocal-related regions of the brain (see figure 41.4) (Rodenas-Cuadrado, Chen, Wiegrebe, Firzlaff, & Vernes, 2015). This hypothesis-free approach highlighted a molecular pathway underlying glutamatergic synaptic function in the periaqueductal gray of the bat brain—a region that, across mammals, is crucial for the control of vocalizations. Activation or blockade of this glutamatergic synaptic pathway in mammals can induce or prevent vocalizations in mammals, respectively (Rodenas-Cuadrado et al., 2015). As such, this unbiased network building approach made it possible to identify a gene network that may be mechanistically important for vocal-motor control in mammals and demonstrated the potential of such approaches to identify gene networks involved in communicative behavior.

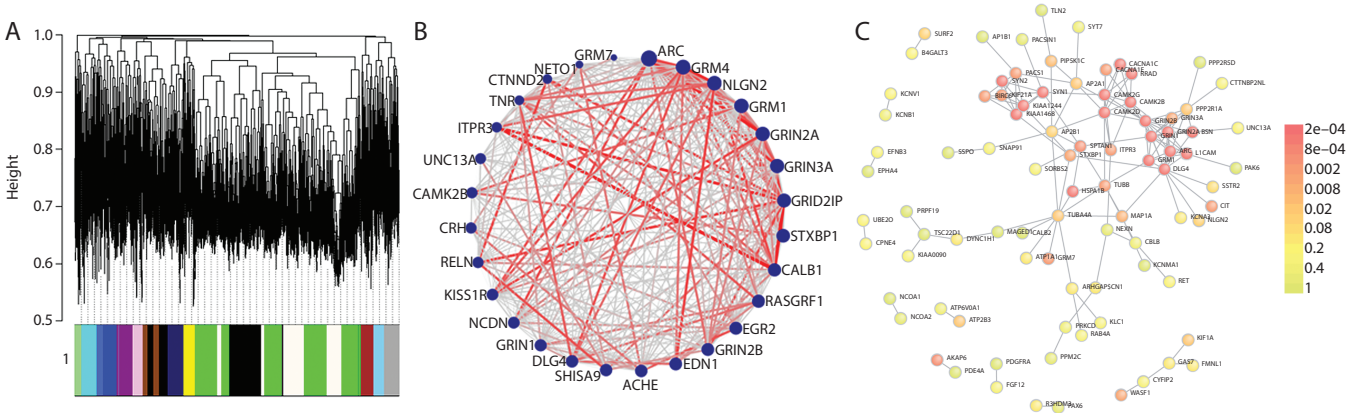

Figure 41.4 Molecular networks can be identified by surveying gene expression in specific brain regions and using network clustering methods (A) to identify highly interconnected gene networks (B) with known functional relevance (C). Example figures adapted from Rodenas-Cuadrado et al. (2015).

Further such studies in animal models linking gene networks to speech/language relevant behaviors or from postmortem human tissue (e.g., Konopka et al., 2012) are likely to reveal a genome level view of molecular networks and identify new candidate genes underlying speech, language, and related disorders (see also Johnson & White, chapter 8 of this volume).

7. Conclusion

The use of neuromolecular approaches as outlined in this chapter can help to elucidate how genes contribute to human speech and language and language-related disorders. By coupling such approaches with crucial research to determine brain networks involved in language, clinical studies of disorders, and animal behavior studies it will be possible to form an integrated view from gene to brain and behavior of the underlying biology of human language.

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NOTES

1. Although it should be noted that over time and after many divisions, the properties of the cells change and often look less like their source tissue if kept dividing for too long (e.g., years)
2. One micron is equivalent to one millionth of a meter. For perspective, the width of human hair is estimated to be between 20 and 200 μm.
3. Following standard nomenclature, genes are denoted in italics, proteins in regular font. Uppercase letters denote the human version of the gene (i.e., *FOXP2*), lowercase the mouse version of the gene (i.e., *Foxp2*).
4. *FOXP2* is also expressed in non-neuronal tissue such as the lungs and heart, see (Schroeder & Myers, 2008).
5. It is from its membership in the FOX family that *FOXP2* gets its name.
6. Children with language impairment commonly perform poorly on the nonword repetition task, making it a common metric in cohorts such as this.

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