Introduction

What Do We Mean When We Say “Neural Development”?

Development unfolds smoothly over time but can be divided for experimental analysis into successive stages, each with its own defining events. Some of these events have clear beginnings and endings, although others may be protracted, sometimes unexpectedly so. For example, myelination of axons in the human brain, a key event that supports behavioral development by increasing the rate of action potential transmission, begins approximately 24 weeks after conception and then continues for decades. In general, the earliest events are most easily categorized as discrete stages shared by almost all members of a species, whereas later events are best described as ongoing processes, the exact details of which are unique to each brain. This is particularly true in long-lived species such as humans, but the inherent ability of nervous systems to refine neural circuitry across the life span is evident even in short-lived invertebrates. The neuroscientist Martin Heisenberg and colleagues were reflecting on data obtained from neuroanatomical studies of fruit fly brains, not human brains, when they were inspired to write, “An individual’s life experience can... be encoded in the volume of selected neuropil regions.”

What Is in This Book and How to Use It

After a brief presentation of methods (this chapter), an overview of human development (Chapter 2), and an introduction to animal models (Chapter 3), the subsequent chapters consider the molecular mechanisms of selected earlier and later events (Chapters 4 and 6), neurogenesis (Chapter 5), and formation of synapses (Chapter 7). Glial cells are the focus of Chapter 8. Chapter 9 describes the postembryonic maturation of the nervous system via metamorphosis in some species and adolescence in others. In Chapter 10 the focus shifts to human intellectual disabilities. This chapter attempts to build a case that at least some forms of human intellectual disability reflect reversible differences in developmental processes rather than permanent...
deficits. This chapter was inspired by my personal connections with two outstanding neuroscientists—William T. Greenough at the University of Illinois at Urbana-Champaign and Linda L. Restifo of the University of Arizona, a researcher who is also a physician. Many other outstanding investigators work in this field, but it was Greenough’s studies of the fragile X protein in the context of his life’s work on experience-driven brain plasticity and Restifo’s studies of mental retardation genes in Drosophila that forced me to rethink my views on human intellectual disability.

Each chapter has notes. Some provide additional background information on the topic being discussed. This information may be useful and/or interesting, but it has been placed in the notes because I believe it is not essential for an understanding of neural development. The notes are probably most helpful if they are consulted the first time you read the chapter. Some notes link specific results recounted in the text to specific references. The full references can be found in the chapter-by-chapter reference lists that appear at the end of the book. These reference lists also include pertinent reviews and commentaries that provide additional context if you are interested in the history of developmental neuroscience. Short chapter-by-chapter lists of trustworthy online resources can also be found at the end of the book. These are intended to provide additional graphic material and technical details as well as links to selected patient information web sites. This material is also nonessential. It is included to allow you to follow up a specific interest, either as you read or in the future.

Students who want to go further will find that there are numerous points of entry into the research literature. The student can begin with the end-of-chapter suggestions for Investigative Reading. Each of these readings is introduced by a short question based on the chapter. The answer to the question (one answer; the student may well come up with a superior alternative) is contained in the recommended reading. Students are encouraged to try to answer the questions on their own before going online to retrieve the article. Note that only partial citations are provided at the end of each chapter. This is because the titles of the articles often give the answers away! The journal articles listed in the Investigative Reading sections are freely accessible online, and full citations are provided at the end of the book.

This text is designed to provide a concise introduction to nervous system development. This goal will be achieved, in part, by a nearly exclusive focus on the central nervous system (the brain and spinal cord in vertebrates, the brain and nerve cord in invertebrates). We’ll venture into the peripheral nervous system primarily in Chapter 7, where I use the neuromuscular junction to describe how synapses form. Topics intentionally shortchanged for the sake of brevity include the history of embryology, the neural crest, development of vertebrate sense organs, and the emerging story of microRNAs (miRNAs) as posttranscriptional regulators of development. In addition,
methods for studying development of the nervous system

The modern neuroscientist's tool kit is stocked with powerful tools for studying the structure and function of the nervous system. While the tools of the electrophysiologist (intra- and extracellular recordings of neuronal electrical activity) and the neuroanatomist (many variants of microscopy) are still in heavy use, many developmental neuroscientists routinely incorporate measures of gene expression and functional brain imaging into their studies. Others just as routinely use genetically engineered (transgenic) animals.

The following sections introduce key techniques used to study nervous system development. Some researchers specialize in a particular technique, but many investigators work in teams and combine multiple approaches to answer research questions. Students who delve into the primary research literature are often amazed at the number of techniques required to generate the data contained in a single paper. This is one of the reasons that many modern research papers feature lengthy author lists.

Birthdating

All cells, including neurons, are produced by division of other cells. The time at which the cell division occurs that produces a particular neuron is referred to as that neuron's birthdate. Knowing neuronal birthdates is important for understanding the sequence of events that builds a neural circuit or a brain. Birthdating is also important for exploring the capacity of mature brains to add new neurons. Neurons themselves do not divide—part of becoming a neuron involves saying farewell to the cell cycle—so the challenge to the developmental neuroscientist wishing to determine a birthdate is to catch the neuron in the act of being produced by a progenitor cell that by definition is not itself a neuron.

If an animal is small and transparent, the process of cell division can be observed directly using a microscope. Otherwise, developing tissues may be fixed (preserved by chemical treatment), sectioned into thin slices (section thickness is typically measured in micrometers, μm), and attached to glass slides for viewing with a microscope. Stains may be applied to the sections to enhance detection of dividing cells. A combination of hematoxylin and eosin reveals key features of many tissues, including nervous tissue, because hematoxylin stains nuclei blue and eosin stains most other structures red or pink. DNA stains aid the identification of mitotic profiles by making condensed metaphase chromosomes readily visible. The Feulgen stain is tradi-
tionally used to mark DNA for viewing with a standard bright-field microscope. Modern biologists with access to a fluorescence microscope can choose from an array of colorful dyes that bind to DNA.

A drawback to searching for mitotic profiles in tissue is that the window for detecting these profiles is often so brief that the likelihood of catching a neuron in the act of being born is small. An alternative approach also relies on detection of DNA, but instead of staining all of the nuclear DNA present in a tissue, the investigator labels only new DNA. This is accomplished by providing special DNA precursors to cells as they copy their nuclear DNA prior to cell division. These precursors do not occur naturally in cells. Because the precursor provided is incorporated into new DNA, any neurons born during the time the precursor was present contain labeled DNA and can therefore be distinguished from cells born when the precursor was not present.

In classic studies, living animals were injected with the nucleoside thymidine linked to a radioactive atom (a nucleoside is a purine or pyrimidine base attached to a ribose sugar molecule; a radioisotope commonly used to label nucleosides is tritium, a radioactive isotope of hydrogen). The distribution of radioactivity in a tissue section prepared from the treated animal was subsequently detected by applying the section to a photographic emulsion. The radioactive decay particles emitted from the radioisotope exposed the film. At the end of an exposure period typically measured in months, the location of nuclei with radiolabeled DNA was revealed by developing the emulsion using darkroom chemicals. This method of detecting the distribution of a radioisotope in tissue is known as autoradiography.

Tritiated thymidine (3H-thymidine) was used in neuronal birthdating studies through the 1970s. Its use has been superseded by a method based on detection of bromodeoxyuridine, a synthetic nucleoside that is an analog of thymidine. Antibodies can be purchased that bind specifically to bromodeoxyuridine. Labels attached to these antibodies make the position of bromodeoxyuridine within a tissue section readily evident using standard techniques of light microscopy (fig. 1.1). Bromodeoxyuridine is commonly referred to by its nickname, BrdU, pronounced bee-are-dee-you. Oval spots representing BrdU-labeled nuclei flash before the mind’s eye of a neuroscientist who hears the term neuronal birthdating.

Birthdating methods that rely on incorporated nucleosides work only when the investigator can introduce the marker at the appropriate stage without perturbing normal development. Depending on the species, this may be accomplished by injecting or feeding or by immersing the entire animal in a solution containing BrdU. An alternative approach relies on immunodetection of endogenous molecules expressed by dividing cells. This circumvents the need to introduce a marker. Antibodies are available that recognize proteins expressed during the cell cycle. These include antibodies that bind to proliferating cell nuclear antigen (PCNA) and a nuclear protein...
called Ki-67. These proteins are not expressed by mature, postmitotic neurons, but they are good markers for progenitor cells and for newborn neurons, as they persist for several hours after mitosis before being metabolized.

One disadvantage of relying on the immunolabeling of endogenous proteins as markers for mitosis is that the antibodies used in these studies may not recognize proteins from a broad range of species. For example, antibod-
ies raised against a fragment of human Ki-67 nuclear protein will likely cross-react with similar proteins expressed during the cell cycle in other mammals but are unlikely to bind even to related proteins in fish, birds, or insects. The challenges imposed by the need to introduce BrdU into developing tissues are often outweighed by the fact that this marker can be used to birthdate neurons (and other cells) in absolutely all animals, from hydoras to humans.

Tissues exposed to BrdU can be chemically fixed for analysis shortly after introduction of the marker. This provides a snapshot of the cell divisions occurring at a specific point in development. Another approach is to allow the BrdU-treated animal to survive for some length of time after the marker is introduced. Because neurons do not divide, incorporated BrdU will persist in the nuclear DNA. The incorporated BrdU can be detected as long as the animal (or the neuron) lives.

**Lineage Analysis**

Lineage analysis refers to tracing the origin of a particular cell or cell type back through a series of successive cell divisions. This method differs from birthdating techniques in that the result is a family tree rather than a birthdate. Of course, the lineage of every cell in the body can be traced back to the fertilized egg (zygote), so it is not necessary to do experiments to prove this. It is the later portions of the lineage that are interesting to developmental neuroscientists, because these represent points at which developmental mechanisms act to limit a cell’s fate.

The optical microscopes of the nineteenth century permitted biologists to observe living tissues directly to determine cell lineage. Direct observation remains a powerful tool for lineage analysis in small transparent or translucent embryos. In the 1970s and 1980s, direct observation of cell divisions was used to determine the lineage of every cell in *Caenorhabditis elegans*, a nematode worm. Researchers used the technique of differential interference contrast (DIC) microscopy to enhance the contrast of the unstained living embryos and larvae they examined. But determination of cell lineage by direct observation is impossible in many animals, either because the embryo is not transparent or because development occurs inside an egg with an opaque shell or inside the mother’s body. As a consequence, cell lineage determinations are often based on introduction of a marker into suspected progenitor cells. For example, a small amount of dye can be injected into a cell. If that cell subsequently divides, the resulting daughter cells will each contain some of the dye. It can be inferred that the injected cell was the parent of the pair of dyed cells. If one or both of the dyed daughter cells divides, the dye will also be found in the granddaughters of the injected cell.

A disadvantage of the direct injection method is dilution of the marker. Whatever dye was injected will be partitioned between pairs of progeny and
hence diluted upon each successive division. Eventually the lineage marker will be so diluted that it will become undetectable. As a consequence, the simplest versions of this method cannot be used for analysis of long lineages.

But what if we could introduce a marker that replenished itself in the daughter cells after each division? Avoiding dilution would make it possible to trace long cell lineages. This approach is exploited by two powerful methods for determining cell lineage. The first is based on retroviruses that carry marker genes. The second produces marked cells by a process of gene recombination.

The genes of retroviruses are encoded in RNA instead of DNA. When a retrovirus infects a cell, the RNA-based genome of the virus is reverse transcribed and then integrated into the DNA of the infected cell. If the infected cell is a progenitor cell, its daughters inherit the viral genes along with the genes of the progenitor cell. Progenitor cells are typically infected by injecting the retrovirus into the extracellular fluid near the target cell or cells.

Natural retroviruses are altered for use in cell lineage tracing in the following ways. First, they are modified so that they cannot replicate. This modification prevents the virus from infecting neighboring cells, which may also be progenitors. Without this modification, it would be difficult to be certain that an inferred cell lineage was correct. Second, the retrovirus is modified so that it carries a reporter gene in addition to its own viral genes. A reporter gene produces a product that is easy to measure or see under a microscope. Two commonly used reporter genes code for the enzymes horseradish peroxidase (not surprisingly, a peroxidase enzyme produced by horseradish plants) and β-galactosidase (an enzyme encoded by the lacZ gene of E. coli). The presence of the retrovirus in a particular cell is revealed by supplying the tissue with the appropriate enzyme substrate; for example, the organic compound X-gal forms a blue product in the presence of β-galactosidase.

A second method for inserting a permanent marker into specific cell lineages is based on introduction of genes encoding site-specific DNA recombinases and their target sites into the genome of the species being studied. Because this method relies on transgenic animals with deliberately modified genomes, it is often used to study development of the nervous systems of genetic model organisms such as mice or fruit flies.

Site-specific DNA recombinases catalyze cleavage and rejoining of DNA strands at specific target sites. The recombinases used for experimental analysis of cell lineage have such large targets that they are unlikely to occur at random in an animal genome. This is the case for the Cre-LoxP system from the bacteriophage P1 (a bacteriophage is a virus that infects bacteria) and the FLP-FRT system from yeast. Both the Cre-LoxP and the FLP-FRT recombinase systems have been used to mark the progeny of specific neural progenitor cells (fig. 1.2). The introduced marker is permanent, so lineages marked in the embryo can be analyzed in the adult, even if the progeny have changed their morphology or migrated away from their place of birth. Because the
logic of Cre-LoxP and FLP-FRT studies is similar, only the Cre-LoxP system is described here.

Cre, so named because it causes recombination, binds to a 34-base-pair target sequence called the locus of crossing over in bacteriophage P1, or simply Lox. If two directly repeated Lox targets are present in DNA, the Cre recombinase excises the intervening DNA, which is sometimes said to be floxed (flanked by LoxP). If the excised DNA coded for stop of transcription, Cre action permanently turns on transcription of a previously silenced downstream gene. And if the previously silenced but now constitutively (continuously) expressed downstream gene encodes a marker such as β-galactosidase, that marker becomes a lineage tracer.

In practice, use of DNA recombinase systems for lineage tracing requires extensive prior knowledge of the genes expressed in the progenitor cell populations being studied. This is because the expression of Cre recombinase must be limited to a particular progenitor cell or progenitor cell population if the results are to be interpretable. This is accomplished by placing Cre expression under control of promoter and enhancer elements from a gene with expression known from other studies to be restricted to the progenitor cells of interest.

![Diagram of Cre-LoxP system](image)

Figure 1.2. Use of the Cre-LoxP system for cell lineage tracing. One of the parent mice shown in (a) expresses the Cre recombinase enzyme under the control of a cell-type-specific promoter; the other parent is a reporter mouse in which a marker gene (lacZ in this example) is separated from a constitutively active promoter by a transcriptional stop sequence. The stop sequence is flanked by LoxP sites. Because LoxP is the target recognized by Cre recombinase, any cells in the offspring (b) that express Cre undergo removal of the stop sequences, which results in transcription of the reporter gene. The cells in which LoxP recombination occurs and any progeny they produce will be permanently marked. GFP is another popular Cre-dependent reporter gene. If the Cre recombinase gene is fused to a domain that binds a specific ligand (for example, a hormone), the Cre recombinase enzyme will be expressed only when the ligand is present. Arrows indicate the direction of transcription; triangles indicate the position of the LoxP sites.
You may have noted that use of the *Cre-LoxP* system for lineage analysis requires not one but two transgenes—in our example, the first transgene is the *Lox*-stop of transcription-*Lox-lacZ*, and the second is Cre recombinase driven in tandem with a developmental gene. If, for example, such a study is performed in mice, this means that two different lines of transgenic mice must be developed and then mated to produce double transgenic offspring. One quarter of the resulting offspring will have *lacZ* expression permanently turned on by Cre-mediated recombination. In practice, many different types of Cre transgenic mice, reflecting known patterns of developmental gene expression, can be interbred with a single strain of *Lox* mice.3

**Microscopy**

Microscopes are essential tools for developmental neuroscientists. Stereomicroscopes are commonly used in many laboratories for viewing whole animals and dissecting tissues of interest for subsequent analysis. Compound bright-field microscopes, which magnify light passed through sections of specimens using an objective and eyepieces, permit individual cells to be viewed directly. Eukaryotic cells in general and neurons in particular are often so large (relatively speaking) that it is easy to view not only the cells themselves but also large organelles through a compound microscope. The colored reaction products obtained when chromogenic (color-generating) substrates are supplied to cells labeled with enzyme markers are also easily seen with compound microscopes. A major limitation of bright-field microscopy, however, is the requirement that the sections be relatively thin—most studies are performed on sections thinner than 40 μm, sometimes much thinner. Tissues prepared for bright-field microscopy are therefore rather two-dimensional and, almost always, long dead. Development, by contrast, is three-dimensional and dynamic. Although generations of hardworking neuroscientists skillfully reconstructed key events in nervous system development by analyzing static, two-dimensional images, spectacular advances in microscopy and computer-based image acquisition and analysis now make it possible to visualize living cells as they make critical developmental decisions.

The development of confocal laser scanning microscopy (often referred to simply as confocal microscopy) for fluorescent markers is particularly noteworthy. Conventional fluorescence microscopes illuminate a tissue section with light of one wavelength that induces fluorescence in the sample. The resulting emitted light, which is of a longer wavelength than the illuminating light, is detected by the researcher through the microscope objective and eyepieces. Conventional fluorescence microscopy is valued for its sensitivity (a high signal-to-noise ratio) but shares with bright field microscopy the requirement for thin sections: unless the sections are thin, conventional fluorescent images are bright but blurry. Confocal microscopy also relies on
fluorescent markers but uses point-by-point illumination with a focused laser beam paired with a moving (scanning) pinhole to eliminate out-of-focus emitted light. The scanning is controlled by a computer, and images can be recorded in the z plane (depth of the tissue) as well as the x and y planes. The images acquired by laser scanning can be digitally stacked to produce a three-dimensional, high-resolution view of the specimen. Confocal images of cells and tissues are astonishingly crisp. Many well-characterized fluorescent molecules (fluorophores) are commercially available, permitting investigators to design sophisticated experiments using multiple markers.

Near or at the top of many biologists’ lists of favorite fluorophores is green fluorescent protein (GFP). GFP occurs naturally in the *Aequorea victoria* jellyfish as a component of this species’ bioluminescent light organs. Note that many fluorophores are small molecules rather than proteins. This means that they are not encoded directly in the genome and therefore cannot be used to make fluorescent transgenes. But, as its name indicates, GFP is a protein. The cloning of the gene for GFP in 1992 allowed biologists to add the gene to other organisms in the form of transgenes coding for fusion proteins. A fusion protein is created by joining two genes that originally coded for separate proteins. If one of the fused genes is the gene for GFP, fluorescence can be detected whenever the cell produces the fusion protein. The GFP gene can also be attached to the regulatory region that drives expression of another gene so that a cell fluoresces whenever the other gene is being expressed. Today many variants of GFP (many of which are not green) are available as reporter molecules. For example, the gene encoding GFP can substitute for *lacZ* as the reporter gene in the Cre-Lox cell lineage analysis system described in the preceding section. The developers of GFP as a tool for studies of gene expression were Osamu Shimomura, Martin Chalfie, and Roger Tsien. They were recognized with the Nobel Prize in Chemistry in 2008.

**Gene Expression**

The two most complex cell populations in your body are those of your nervous system and your immune system. It is true that many lymphocytes are genetically unique, but from the neuroscientist’s perspective this diversity is accomplished by a sly bit of trickery involving DNA recombination during lymphocyte maturation. The nervous system achieves its diverse populations of neurons and glial cells not because these cells contain recombined genes but because at any given time they express different combinations of the genes encoded in the genome. This means that understanding nervous system development (and development in general) requires a clear accounting of transcriptional regulation in specific cell populations. At one time this task would have been conceptualized primarily in terms of identification of transcription factors, the protein signals that interact directly with nuclear
DNA to turn specific genes on and off. Transcription factors have not diminished in importance and are in fact a major focus of this book, but other factors such as noncoding RNAs (ncRNAs) and epigenetic modifications of chromatin are now recognized as essential to a complete understanding of cell differentiation.

Neuroscientists have just begun the hard work of listing the genes expressed at different stages of development of the nervous system. The challenge is great. First, multiple time points must be studied to capture all phases of development of a neuron's mature phenotype: neurogenesis, migration, growth of dendrites and axons, synaptogenesis, and establishment of neurotransmitter signaling systems. Second, the intrinsic plasticity of nervous systems means that even neurons that have differentiated a mature phenotype vary their gene expression profiles over multiple time scales. Third, cell populations within the nervous system are typically intermingled, so neurons from different lineages with different phenotypes can be neighbors. This mosaic quality of the brain means that tissue extract–based methods for assessing gene expression, such as DNA microarrays and whole-transcriptome shotgun sequencing (RNA-seq), tell only part of the story. Information flow in neural circuits depends on polarized synaptic connections rather than proximity. Studies of gene expression in the brain therefore require a merger of anatomical and molecular approaches.

A direct way of asking if a particular protein is expressed by a particular nerve cell or population of nerve cells at a particular point in time is to make the protein visible by tagging it with an antibody molecule. Different antibody molecules bind with specificity to different epitopes—typically fragments of proteins—and can themselves be tagged with fluorophores or enzyme markers. Identification of proteins in tissue samples or cell extracts using labeled antibodies is referred to as immunolabeling. Most techniques are based on chemically fixed tissues, because such fixation preserves the spatial distribution of proteins in the living cells. These techniques are referred to as immunohistochemistry or sometimes simply IHC (fig. 1.3). Both polyclonal antibodies (found in the serum of an animal exposed to the target protein) and monoclonal antibodies (found in the medium of hybridoma cells formed by fusing B lymphocytes from an animal exposed to the target protein with immortal tumor cells) are widely used in neuroscience research.

Antibodies can also be used to identify proteins present in extracts or homogenates of nervous tissue. A mixture of proteins can be separated by molecular mass using gel electrophoresis (passing an electrical current through a gel onto which the protein has been placed, or loaded). The separated proteins are in turn transferred to a membrane that is incubated in a solution containing the antibody; the antibody binds to any protein on the membrane that contains its epitope, and such binding (revealed by visualization of a marker) is evidence that the protein of interest was present in the tissue
Use of immunolabeling to study neuronal gene expression. Immunolabeling provides a method for identifying cells that contain specific protein epitopes. The major steps are shown in sequence; actual experiments typically take days to complete, and each step in the procedure must be optimized to achieve meaningful results. The primary antibody, which may be obtained from any antibody-producing species other than the one being studied, binds to an epitope on the target protein; the secondary antibody binds to the primary antibody. The secondary antibody is modified by attachment of an enzyme or a fluorophore so that the presence of the target protein can be detected in the tissue by microscopy. For example, if the primary antibody was made in a mouse, the secondary antibody must be an anti-mouse antibody made in another species, such as a goat or a donkey. Note that a primary antibody made in a mouse cannot be confidently used to localize proteins in mouse tissue, because the anti-mouse secondary antibody would bind both to the primary antibody being used in the study and to endogenous mouse immunoglobulin molecules. Important control procedures include processing tissue with the primary antibody omitted and incubating the primary antibody with an excess of the target protein before applying it to tissue. This latter control is referred to as preabsorption. A preabsorbed antibody should not produce signal in tissue; if it does, the resulting immunolabeling will be difficult or impossible to interpret. The term wholemount refers to the processing of an entire embryo or, in some cases, the entire central nervous system, without sectioning.
from which the extract was prepared. This procedure is referred to as Western blotting or immunoblotting (the two terms mean the same thing).

Given that DNA makes RNA makes protein and proteins get the job done, students are sometimes surprised to learn that many studies of gene expression do not involve identification of proteins. Instead, the presence of messenger RNA (mRNA) in an extract prepared from a tissue sample is commonly taken as evidence that a particular gene is being expressed at that time in that cell population. This approach is popular in part because sequencing of nucleic acids is more easily and cheaply accomplished than sequencing of proteins. Probes for detection of specific mRNA sequences are easily synthesized, and the tiny amounts of mRNA present in individual cells or small pieces of tissue can be amplified (copied) for study by reverse transcribing the mRNA to its complementary DNA (cDNA). By contrast, proteins are more difficult (and costly) to sequence, are typically detected using antibodies produced by immunizing animals with proteins (which take weeks to months to produce), and cannot be readily copied for study. The number of predicted protein sequences available as a result of completed genome sequencing projects vastly outstrips the availability of antibodies targeted to specific proteins.

A standard method for detection of mRNA is Northern blotting. This procedure begins with isolation of total mRNA from a biological sample. Many kits are available that make the processes of tissue disruption and RNA stabilization efficient and reproducible, even for small samples. The extracted mRNA is loaded onto a gel and separated into fragments of different sizes by electrophoresis. The separated mRNAs are then transferred (blotted) to a filter. Specific mRNAs are identified by exposing the filter to a solution containing a radiolabeled single-stranded RNA or DNA probe complementary to the mRNA from the gene of interest. This radioactive probe can be used to expose X-ray film. Because only the target mRNA is visible on the film, the visible bands reveal how much of the target RNA was present in the sample. Standards can be electrophoresed and blotted side by side with the tissue-derived RNA so that the size of the visible band can be estimated.

Northern blotting is a tried and true method based on procedures many students will have an opportunity to perform in a laboratory class, but most researchers analyzing gene expression today prefer a fluorescence-based method called real-time quantitative reverse-transcription polymerase chain reaction, or real-time qRT-PCR (fig. 1.4). As its name indicates, real-time qRT-PCR is based on monitoring the progress of a PCR reaction in real time. Fluorescent reporter molecules fluoresce with increased intensity as the PCR product accumulates, allowing quantitation of the number of starting copies of mRNA. (The original mRNA, however, is no longer present because it was reverse transcribed into cDNA after being extracted from the sample to take advantage of the superior stability of DNA.) A single instrument runs the PCR reactions and monitors the fluorescent signal in multiwell plates. Many instruments run plates that contain 384 or even more wells, which means
Select target gene and housekeeping gene

Design primers and probe (software package)

Extract RNA from tissue

Quantify extracted RNA (spectrophotometer)

Assess quality of RNA (agarose gel)

cDNA reaction (reverse transcription)

PCR reaction
1. Polymerization
2. Probe displacement
3. Probe cleavage

Inspect raw data

Final data analysis (calculations)

**Figure 1.4.** Use of real-time qRT-PCR to study neuronal gene expression. Several methods are widely used. The method depicted uses specific DNA probes that have a reporter fluorophore (R) attached at one end and a quencher fluorophore (Q) attached at the other end. The reporter fluorophore emits a detectable fluorescent signal only when it is cleaved from the probe. This occurs in the presence of an appropriate primer because DNA polymerase extends the forming DNA strand during the PCR reaction. The reaction takes place and the fluorescence is detected in an instrument typically referred to as a real-time PCR machine. The number of PCR reaction cycles required to produce detectable reporter fluorescence is related to the number of transcripts for that gene initially present in the tissue sample (more transcripts = fewer PCR cycles required to reach threshold). Gene expression is quantified by comparing the number of cycles required to reach the detection threshold for the gene of interest to the number of cycles required for a stably expressed housekeeping gene to be detected. Commonly used housekeeping genes are 18s rRNA, β-actin mRNA, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Choice of an appropriate housekeeping gene is particularly important for developmental studies, because a hallmark of development is changing gene expression as cells grow and differentiate.
that gene expression in hundreds of samples can be analyzed by a single researcher in a single afternoon.

The wonderful efficiency of real-time qRT-PCR may not compensate for the loss of spatial resolution inherent in methods based on analysis of isolated RNA, especially for researchers investigating the development of complex tissues such as those of the nervous system. Another tool for analysis of gene expression is in situ hybridization (fig. 1.5). In this method, target mRNA sequences are localized to specific cells in tissue sections, tissue pieces, or even whole embryos. This is accomplished by exposing the target sequence (in the tissue) to a complementary nucleotide probe (either cRNA or cDNA). Under the appropriate conditions, hybridization of the probe with the target occurs (in the context of molecular biology, the term hybridization means that a double-stranded nucleic acid forms as a result of complementary base pairing). The probe is tagged so that it can be recognized after hybridization. The most typical tags are radiolabeled nucleotides or digoxigenin, a steroid molecule found exclusively in parts of plants in the genus Digitalis (foxgloves). Because digoxigenin does not naturally occur in animal tissues and because it is highly immunogenic (which means that antibodies that bind strongly and selectively to digoxigenin are readily available), it provides an excellent nonradioactive label widely used by neuroscientists.

Tissue-based methods of studying gene expression leverage the information produced by more than a century of careful anatomical studies of development. It is therefore difficult to overstate the contribution of in situ hybridization to our knowledge of development. Although the tissue samples used for in situ hybridization are all fixed specimens (i.e., they are dead), analysis of carefully staged sequences of samples reveals the dynamic changes in gene expression that are the essence of development. This method has been partly superseded by the use of transgenic markers for gene expression in models such as the fruit fly and the mouse, but in situ hybridization remains in wide use by researchers working with other species.

In summary, developmental neuroscientists often study mRNA location and abundance because they can do so efficiently and quantitatively. The starting point for an RNA-based study of gene expression may be no more than a nucleotide sequence from a database. By contrast, detection of proteins typically requires production of specific antibodies. Making an antibody is not a particularly expensive endeavor (and it is typically done by contract with an academic or commercial facility), but it is time-consuming and unpredictable, two features guaranteed to make it unpopular with busy scientists.

**RNA Interference**

Once an investigator has a spatial and temporal map of a gene expressed in the nervous system during development, the next goal is often to test the
**Figure 1.5.** Use of in situ hybridization to study neuronal gene expression. In situ hybridization is a method for identifying cells that contain specific mRNA sequences. This method exploits the single-stranded state of mRNA within living cells. Probes must be antisense to the target mRNA that the investigator wishes to localize. Both radiolabeled oligonucleotide DNA probes and riboprobes (cRNA, complementary to the sequence of the target mRNA) are used for in situ hybridization studies. For riboprobes, RT-PCR is used to produce a DNA template that can be used to generate the riboprobe by in vitro transcription. A labeled ribonucleotide triphosphate (for example, digoxigenin-11-UTP or biotin-16-UTP) is added to the transcription reaction so that the synthesized RNA probe can be detected after it has been hybridized to the target mRNA. Different tissues require different protocols and, as in the case of immunolabeling, each step in the procedure must be optimized. An important control is to perform the procedure with the sense probe, which, in contrast to the antisense probe, should not yield specific signal.
tissue destruction. In genetic model organisms (several of which are described in Chapter 3), a normal gene can be replaced with an engineered mutant gene that does not express a functional version of the protein being studied. The ability to achieve targeted gene knockdowns accounts for much of the popularity of genetic model organisms. But targeted gene knockdowns can also be achieved in other species using the technique of RNA interference, commonly referred to as RNAi. RNAi methods are based on the surprising fact that double-stranded RNA (dsRNA) complementary to a target gene inhibits the activity of the target gene. The selective knockdown of gene expression initiated by introduction of artificial dsRNA into a cell or tissue is the result of the activation of a ribonuclease enzyme called Dicer, which cleaves the dsRNA into short fragments that in turn bind to the target mRNA and cause its cleavage so that it cannot be used for transcription. The gene-silencing action of endogenous dsRNA was first observed in plants and is now recognized as a regulatory mechanism shared by all eukaryotic cells. Gene knockdowns have been achieved in many different species of experimental animals using dsRNA-mediated interference. The discoverers of the mechanisms of RNAi, Andrew Fire and Craig Mello, were recognized with the Nobel Prize in Physiology or Medicine in 2006.7

Morpholino antisense oligonucleotides can also be used to reduce the expression of specific genes. Morpholinos are short, single-stranded oligonucleotide chains designed to be complementary to a target mRNA. Similar to DNA and RNA, they contain nitrogenous bases. But the bases in morpholinos are attached to a morpholine ring instead of a deoxyribose or ribose. The linkage between adjacent subunits in a morpholino is also different than in endogenous nucleic acids.

Morpholinos reduce gene expression by a different mechanism than dsRNAs. Instead of activating the Dicer ribonuclease, morpholinos bind to their target mRNAs in the cytoplasm and block translation. Morpholinos offer the advantages of being specific, relatively nontoxic, and extremely stable in living cells and tissues. For reasons that are not fully understood, morpholinos are most effective in frogs and zebrafish, and they have been used primarily in these animals. Many developmental neuroscientists now incorporate either RNAi- or morpholino-based gene knockdowns into their research. Examples will be found in many of the following chapters.

Human Brain Imaging
And now for something completely different.8 The study of development at the cellular and molecular levels of analysis ultimately relies for meaning on our knowledge of neural circuit structure. The remarkable progress of recent decades in our understanding molecular and cellular mechanisms of development should not obscure the gaps in our knowledge of how the human brain develops, particularly during childhood and adolescence. The devel-
opment of noninvasive pediatric (and even prenatal) neuroimaging is bridging these gaps and raising new questions.

Computer-assisted techniques that can be used to image the living human brain for the purpose of medical diagnosis can also be used to study brain development. The technique of computerized axial tomography uses multiple X-rays to map tissue density to produce maps of the brain referred to as CAT or CT scans. This technique is useful for detecting certain forms of brain damage or developmental abnormalities, but the resulting images are not detailed enough to track subtle longitudinal changes. Magnetic resonance imaging (MRI) techniques use powerful magnets to map brain density. Because MRI images are higher in resolution (the distinguishability of two adjacent points without blurring) than CAT scans, they reveal more meaningful neuroanatomical detail. MRI studies have revealed previously hidden dynamics of brain development, thereby generating new questions for cellular and molecular biologists to answer. For example, MRI studies of the cortices of a sample of more than 300 children and adolescents provocatively revealed that significant differences in rate of change of cortical thickness were correlated with performance on standardized intelligence quotient (IQ) tests. The factors regulating these changes remain to be determined.

Several methods go beyond the descriptive neuroanatomical maps produced by MRI to provide glimpses of the brain at work. Positron emission tomography (PET) detects the distribution of injected radioactive tracers within the living brain, allowing neuroscientists to infer which brain regions have the highest levels of metabolic activity under particular test conditions. PET scans have provided new information about the brain, but the use of PET in children has been limited because of the requirement for intravenous injection of the radioactive tracer. Health concerns also limit the use of X-ray-based techniques in humans in general and children in particular, because exposure to X-rays is linked to death of cells undergoing mitosis at the time of exposure and the later development of cancers in exposed tissues. The development of functional MRI (fMRI) scanning in the 1990s solved this problem by using oscillating magnetic field gradients to monitor changes in the ratio of oxygenated hemoglobin to deoxyhemoglobin for the purpose of detecting localized changes in brain oxygen use. This type of fMRI is referred to as blood oxygenation level-dependent fMRI, or BOLD. The BOLD fMRI response is informative because increases in blood flow (hemodynamic response) and oxygen consumption are correlated with increased intensity of synaptic signaling. One of the first BOLD fMRI studies of children was published in 1995. Three boys and three girls between the ages of 9 and 11 years had their brains imaged using BOLD fMRI while they performed a simple memory task requiring them to press a button whenever a particular sequence of letters appeared on a screen. The researchers specifically sought to understand the normal development of prefrontal circuitry. Other fMRI techniques continue to be developed, but BOLD fMRI is the
leading technique currently used to study development of the human nervous system. BOLD fMRI offers the opportunity to determine the brain basis of age-related changes in behavior in both typically and atypically developing individuals. Note that studies of different age groups naturally present different challenges. Artifacts caused by head movements are most common in fMRI studies of preadolescents, but sleepiness sometimes interferes with the collection of reliable fMRI data from teenagers!

Longitudinal studies of human cortical development are described in Chapter 9. Note that noninvasive brain imaging is a rare example of a research tool that is more effectively applied to large brains (such as human brains) than to the smaller brains of popular animal models for the study of development. Only recently have advances in fMRI made it possible to study the brains of smaller species. For example, BOLD fMRI studies of zebra finches listening to recordings of their own song (the birds were anesthetized during the experiment) revealed a previously unknown right hemisphere lateralization of responses to a bird’s own song. These pioneering studies were performed using adult birds, but they raise the possibility that the brain events responsible for the development of singing, a behavior essential for avian social communication and reproduction, will one day be studied by making repeated BOLD fMRI measurements on individual birds as they learn to sing.

Another variant of MRI useful for developmental neuroscience is diffusion tensor imaging (DTI). This method exploits the tendency of water molecules to diffuse along nerve fiber tracts rather than crossing them and is therefore useful for visualizing the development of white matter (tracts of myelinated nerve fibers) in the brain. For example, DTI has been used to monitor the postnatal development of the corpus callosum, the large bundle of nerve fibers that connects the right and left hemispheres of the human brain.

The Future

New methods continue to be developed to monitor and manipulate the function of neural circuits in the living brain. These novel approaches to classic questions, such as the changes that occur in synaptic structure as a result of experience (learning) can also be applied to problems in nervous system development. A particularly exciting new method introduces light-sensitive cation channels into neurons. This type of channel—an example is channelrhodopsin-2, a light-sensitive plasma membrane protein native to a species of single-celled algae—opens in response to blue light, with the result that any illuminated neuron containing the channel rapidly depolarizes. The ability to stimulate neurons without the need to penetrate them with traditional microelectrodes offers opportunities for minimally invasive manipulation of signaling in small embryos poorly suited for traditional electrophysiology. For example, rhythmic waves of electrical activity can be
recorded from the spinal cords of chick and mouse embryos. Decreasing this
spontaneous electrical activity by application of drugs that block the GABA\textsubscript{A} category of neurotransmitter receptors leads to disturbances in the position of motoneuron axons as they grow toward their muscle targets. Researchers asked if the axon pathfinding errors resulted specifically from the change in the pattern of electrical activity by observing axons growing in chicken embryos treated to express the channelrhodopsin-2 protein in the developing spinal cord.\textsuperscript{13} Flashes of light were able to drive neural activity in the embryos even when the GABA\textsubscript{A} blocker picrotoxin was present. The result supported the view that normal patterns of spontaneous neural activity are required to guide axons to their embryonic muscle targets.

Notes

3. A list of mouse strains expressing Cre plus accessible explanations of Cre-recombinase systems is available on the web site of the Jackson Laboratory, an independent, nonprofit organization that has been supplying mice for research since 1933.
4. Free online tutorials and webinars in microscopy are offered by many microscope manufacturers.
5. The Molecular Probes Handbook, available via the Invitrogen web site, provides a comprehensive and frequently updated review of fluorophores developed for research in the life sciences.
8. This useful phrase is borrowed from the title of a 1971 feature film released by Monty Python, a legendary British comedy group.
11. See Poirier et al. (2009).

Investigative Reading

1. To study neurogenesis in the adult brain, you inject a group of mice with the thymidine analog, BrdU. You then prepare slides of brain sections and perform immunofluorescent colabeling with an antibody to BrdU and another to the cell cycle marker Ki-67. What percentage of your BrdU-labeled cells do you expect to coexpress the Ki-67 marker? Conversely, what percentage of Ki-67-labeled cells do you expect to contain incorporated
BrdU? What is the evidence that the newborn cells that you identify using antibodies to BrdU and Ki-67 are neurons?


2. Huntington’s disease is an incurable inherited disease of the nervous system. The disease typically appears between the ages of 30 and 50; it is progressive, meaning that the symptoms worsen with time. Patients suffer from uncontrollable movements, cognitive decline, and eventually dementia. At autopsy, brains from Huntington’s patients have neurodegeneration in several regions. The most severe damage occurs in a part of the brain called the striatum, which controls behavior and coordinates movement. The mutation that causes Huntington’s disease has been identified. The affected gene in humans, named the Huntingtin (*HTT*) gene, codes for an abnormally long version of the Huntingtin protein (HTT). It is not yet known how this mutant protein kills neurons. It is also not known why some areas of the brain are more vulnerable than others. One way to understand the mechanisms by which a mutant protein damages cells is to study the function of the normal protein in animal models. The genome of the fruit fly *Drosophila melanogaster* contains a gene that encodes a homologous protein called *d htt*. Researchers used reverse transcription–PCR to show that the *d htt* gene is expressed in adult fruit flies, but these studies were performed on RNA extracts of whole flies. You want to know if the *d htt* gene is expressed in the fly nervous system; you also want to know if the protein is expressed in the cytoplasm or the nucleus. What methods do you use?


3. **Challenge Question:** You are using a mouse model to study a human disease called spinal muscular atrophy (SMA), an incurable inherited disease characterized by progressive muscle weakness caused by the deaths of spinal motoneurons. You find that motoneurons from your mutant mouse have reduced expression of β-actin, a cytoskeletal protein. β-actin is enriched in growth cones, the tips of growing axons. Your goal is to test the hypothesis that β-actin is required for the formation of the neuromuscular junction that normally occurs when growth cones reach their muscle targets. What technique do you use to delete β-actin from mouse motoneurons without affecting any other cell population?