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The "New Math" of Neuroscience: Genetic Tools for Accessing and Electively Manipulating Neurons

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> *When an individual develops from an egg, the one dimensional information contained in the linear sequence of genes on the chromosomes is somehow translated into a two-dimensional blastula, which later folds to produce a precise three-dimensional array of sense organs, central nervous system, and muscles. Finally, the ensemble interacts to produce behavior, a phenomenon that requires four dimensions, at the least, to describe. The genes contain the information for the circuit diagram, but little is known about the relationship between this primary information and the end result. How the tags of specificity are parceled out among the neurons so that they form the proper network, or even what kinds of molecules carry the specificity are, at present, complete mysteries. The problem of tracing the emergence of multidimensional behavior from the genes is a challenge that may not become obsolete so soon.*
>
> (Benzer 1971)

> *Three main approaches are needed to unscramble a complicated system. One can take it apart and characterize all the isolated bits…then one can find exactly where each part is located in the system in relation to other parts. Finally, one must also study the behavior of the system and its components while interfering very delicately with its various parts to see what effect such alterations have on behavior at all levels. If we could do this all with our brains, we could find out how they work in no time at all.*
>
> (Crick 1988)

4.1 Introduction

New genetic approaches, exemplified by current work on motor control in the mouse and fruit fly, *Drosophila melanogaster*, are rewriting the playbook for analyzing the structure and function of neural circuits in both invertebrates and vertebrates. A key factor has been advances in our understanding of the developmental mechanisms that pattern the nervous system, which has been facilitated by methodological advances on two fronts. The first is the ability to manipulate and monitor the activity of neurons

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using genetic tools. The second is the developing capacity to undertake these manipulations in highly restricted sets of neurons in the nervous system. We outline here how genetic strategies are being deployed in flies and mouse, focusing on their application to motor control. Similar efforts are underway in other genetic model systems such as the nematode *Caenorhabditis elegans* and zebrafish.

From Behavioral Mutants to Genes Much of the impetus for studying the interaction between genes and behavior came from the work of Seymour Benzer in the 1970s. His lab catalyzed two key developments: (1) the use of chemical mutagenesis to identify neurological mutants in *Drosophila* using simple behavioral assays and (2) the establishment of a working group of scientists to map the gene responsible for Huntington's disease, a severe degenerative motor disorder in humans. The insights from this research gave birth to the idea that one can analyze neuron function and dysfunction at the level of a gene or neuron, thereby essentially fulfilling the first requirement Crick prescribed for "unscrambling a complicated system" by allowing access to the individual parts of the nervous system, namely neurons and the genetic code that controls their identity and function.

The initial approach that Benzer and his colleagues used to probe behavior in *Drosophila* combined mutagenesis with elegant behavioral screens. In screening mutagenized flies based on their geotactic and phototactic response, Benzer and colleagues identified a number of genes including the *period* and *sevenless* genes (Benzer 1967; Hafen et al. 1994). While the primary goal for these early genetic screens was to find where the gene was expressed and thereby identify the cell types responsible for generating the observed behavior or neural deficit, it soon became evident that such hope was premature. In many cases, although the mutants displayed a specific behavioral defect, the corresponding gene was expressed rather broadly. For example, the *period* gene is broadly expressed in the central nervous system (CNS) and in non-neuronal tissues. Because of this, identifying the neurons responsible for the behavioral changes that occur in mutant animals remains a major challenge in the field.

From Genes to Defined Neurons The step from gene expression in the nervous system to identifying which neurons contribute to a particular behavior remains a key issue for mapping neuronal circuits in invertebrates and vertebrates alike. What was previously lacking was the ability to manipulate and monitor cellular activity in clearly identifiable neurons of the brain. It is recent progress on this front that is having a huge impact. This issue can be divided into two tasks. One is to identify and characterize genes that are expressed in subsets of neurons, which can then be used to alter the properties of the neurons, e.g., increase or decrease neuron activity. The other is to find ways to express these genes in specific neurons in the brain, with the ultimate goal being able to target every neuron singly.

4.2 Restricting Gene Expression to Specific Neurons

Two general approaches, driven by the genetic approaches available in flies and mice, have been used to identify genes that are expressed in subsets of neurons and thus describe neural networks on the level of neuron identity and synaptic connectivity.

Table 4.1 Summary of methods for monitoring and altering neuron activity.

Reporters	Permanent effectors	Conditional effectors
Fluorescent proteins GFP and GFP color variants, Kaede-GFP tdTomato, SypHTomato *Genetically encoded calcium indicators* GCaMP variants, TN-XXL	*Activity/Transmission blockers* Tetanus toxin light chain (TeNT) Inwardly rectifying K^+ channel (Kir2.1) *Activator* Voltage gated Na^+ channel (NaChBac) *Cell ablation* Diphtheria toxin (DTX) Diphtheria toxin receptor (DTR, mouse) Reaper (Rpr, fly) Head involution defective (Hid, fly)	*Optogenetic* Channelrhodopsin and variants (ChR) Halorhodopsin (HR) *Chemogenetic* Allatostatin, hM4D, hM3D (mouse) PX2-purinoreceptor (PXP2, fly) Histamine gated Cl^- channel (Ort, fly) *Thermogenetic* Temperature sensitive Shibire (Shi, fly) Transient receptor Potential A1 channel (TrpA1, fly)

The first of these is to use molecular genetic techniques to hijack the cis-regulatory elements of genes that are expressed in restricted or specific populations of neurons and use these elements to direct the expression of anatomical tracers or reporter proteins in the neuron subset (e.g., GFP, tdTomato, tau-lacZ, Brainbow, sypHTomato, GRASP (Table 4.1)). This approach has been used extensively to visualize the morphology and connectivity of particular neuronal populations. Another approach is to use heterologous genetic "driver" proteins (Gal4, Cre, Flp, TetR) in binary genetic approaches to drive the expression of various effector or reporter proteins (Table 4.1, Figs. 4.1–4.3). These effector/reporter proteins come in multiple types: (1) proteins that regulate neuronal activity and synaptic transmission, e.g., tetanus toxin light chain subunit (TeNT), the potassium channels Kir2.1 and Shibire; (2) optogenetic proteins, e.g., channelrhodopsin and its variants, halorhodopsin, Arch, and Jaws; (3) chemogenetic proteins, e.g., P2X2, Ort, the allatostatin receptor (AlstR), Designer Receptor Exclusively Activated by Designer Drugs (DREAADs) (hM4D, hM3D), and genetically-encoded activity reporters such as GCaMP and TN-XXL (Table 4.1).

4.2.1 Promoter Bashing, Enhancer Trapping: Binary Systems for Targeted Gene Expression

Two approaches have been used to create genetic drivers by capturing cis-regulatory elements. The first is identifying the regulatory elements that control the expression of a particular gene and using these elements to generate transgenic "driver" animals. This has been especially useful in *Drosophila*. Although this approach is useful in some

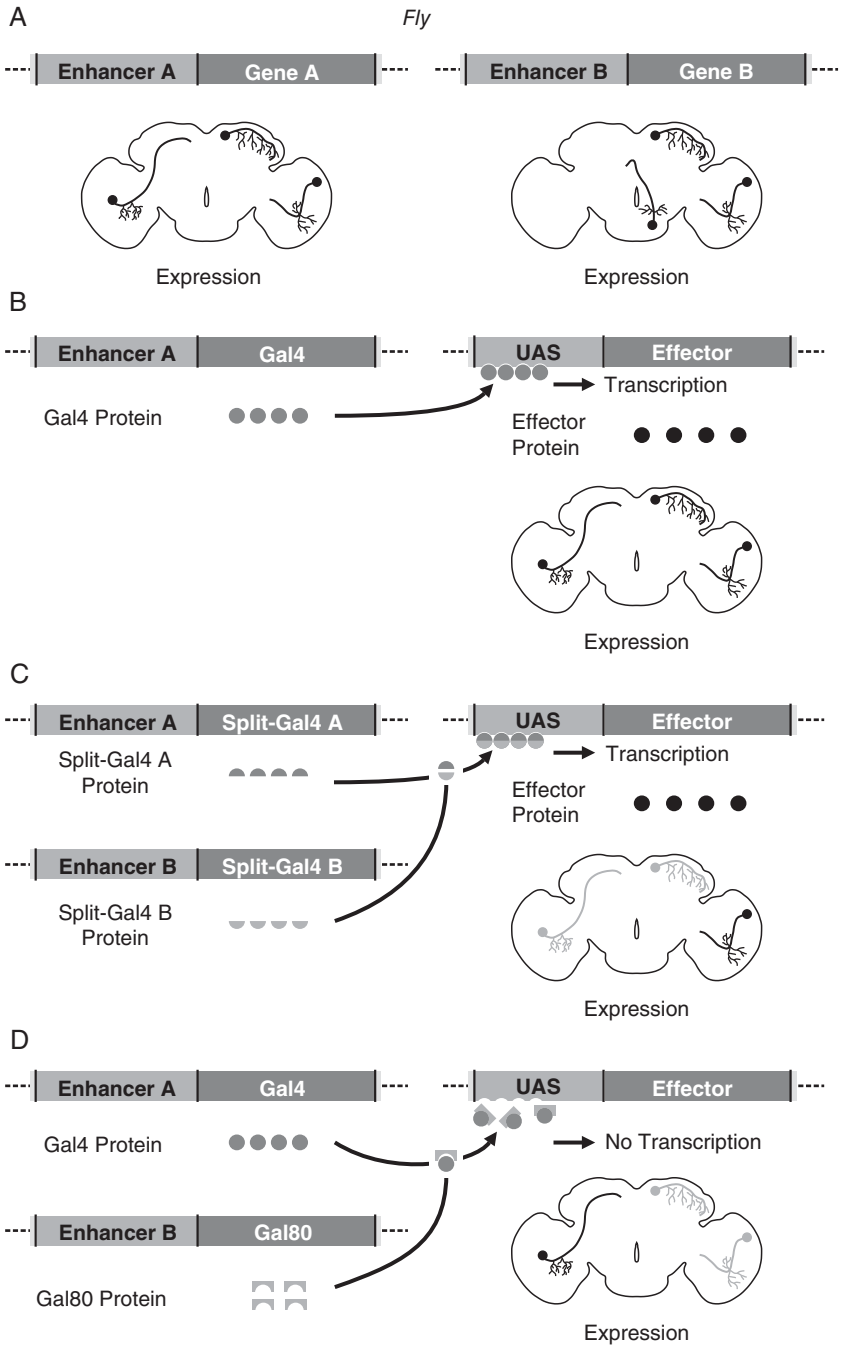

Figure 4.1 Intersectional approaches targeting specific neuronal cell types in *Drosophila*. (A) Schematic drawing of two protein encoding genes, each comprising two functional regions: a cis-regulatory region (Enhancer) and a protein coding region (Gene). Each gene has a distinct protein expression pattern in the adult *Drosophila* brain. (B) The Gal4-UAS system. Enhancer A regulates the expression of the transcriptional activator Gal4 that is able to bind to the UAS (Upstream Activation Sequence) to induce the expression of the target gene (Effector). The Gal4-UAS system is commonly used to induce expression of target genes to specific cells. (C) The Split-Gal4 system. Two Gal4 subunits are needed to induce transcription of the target gene (Effector). The target gene is expressed only in cells in which an overlapping expression pattern of both cis-regulatory regions (Enhancer A/B) occurs. (D) The Gal80 system. The cis-regulatory region of Enhancer A drives the expression of the transcriptional activator Gal4 that initiates the transcription of the target effector gene. Expression of the effector gene is restricted to cells that express Gal4 but not Gal80, which inhibits Gal4. The Split-Gal4 and Gal80 approaches are intersectional strategies used to restrict transcription of the target gene to a certain subset of a known expression domain. The anatomical drawings in B-D show the expression pattern of the target gene (Effector) based on the expression domains of Enhancer A/B (A).

situations in mouse, expression is often highly variable and may not fully recapitulate the endogenous expression pattern. The second approach, which uses gene trapping or insertion by homologous recombination to hijack a gene's regulatory sequences, has proved more productive and reproducible.

In *Drosophila*, the most widely used binary system is the Gal4-UAS system, established by Brand and Perrimon (1993) (Fig. 4.1). This approach is based on two transgenic lines of flies, one (the "driver") in which the yeast transcriptional activator Gal4 is expressed in a select group of cells, and another (the "effector/reporter") that bears a tandem array of UAS (upstream activation sequence) Gal4 binding sites fused to the effector gene of choice. When the lines carrying each allele are crossed, the target gene is expressed in progeny carrying both alleles in a cell type specific manner that is determined by the cis-regulatory sequences controlling the Gal4 transcriptional activator. Additional binary systems have been subsequently developed, including the LexA/LexAop system (Lai and Lee 2006) and the QF/QUAS system (Potter et al. 2010).

In mice the most common approach for expressing effector/reporter proteins in neurons has been to use the binary Cre/LoxP recombination system to recombine out a transcriptional stop sequence surrounded by two loxP sites (LoxP-stop-LoxP (LSL); called a "floxed" stop sequence because it is "flanking/flanked by LoxP"). The enzyme Cre recombinase recognizes and cuts the DNA at the two LoxP sites, after which DNA ligase rejoins the two strands, hence excising the intervening stop sequence. The floxed stop sequence is located upstream of the coding region of the reporter/effector protein of choice (Fig. 4.2). Excision of the stop sequence leads to expression of the reporter in cells expressing Cre. The Cre driver is typically expressed in a cell or tissue specific manner, with the effector/reporter being expressed either ubiquitously or in only a small group of cells, including specific populations of neurons or neuronal subtypes. The yeast derived recombinase Flippase (Flp) and its Flippase Recognition Target site (FRT) (Dymecki and Kim 2007; O'Gorman et al. 1991) has also been employed for this purpose. In *Drosophila*, Flp is often used to generate genetic mosaics in which a small number of cells express GFP, allowing visualization of small number of neurons in isolation. A third recombination system, the Dre recombinase (Dre)/Rox recognition site system is now beginning to be used in mice (Anastassiadis et al. 2009, 2010). These recombination systems can be used, either individually or in combination with

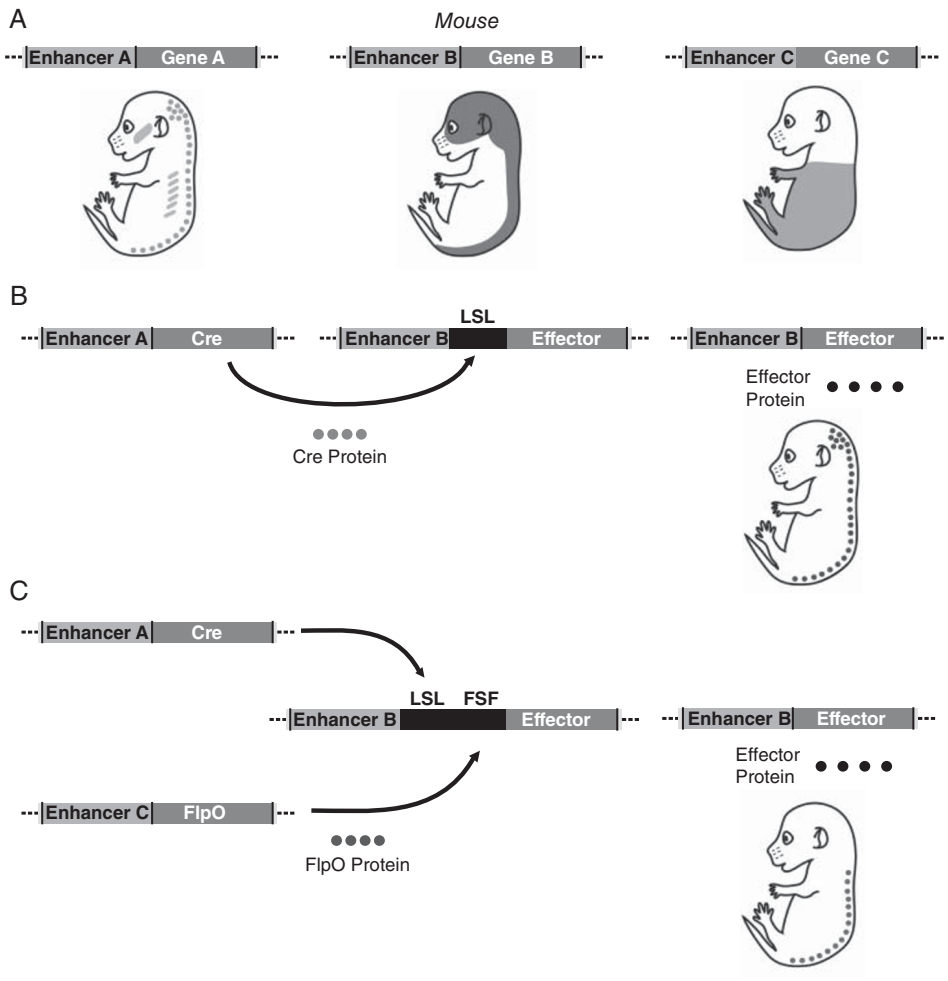

Figure 4.2 Intersectional approaches to target specific neuronal populations in mouse. (A) Schematic drawing of three genes with their enhancer regulatory regions that show different expression patterns in the embryonic or adult mouse, compare to Fig. 4.1. (B) Expression of Cre under the control of the gene A enhancer sequence enables recombination of the Lox-stop-Lox sequence resulting in expression of the effector protein in cells displaying co-expression of genes A and B. (C) Intersection of Cre and Flp recombinases that recombine tandem stop sequences flanked by loxP (LSL) and FRT (FSF) sites allow further spatial control of effector protein expression (see Britz et al. 2015; Dymecki and Kim 2007).

each other, to target multiple populations of neurons simultaneously. Although there are currently very few drivers and reporters that use Dre/Rox recombination, this is changing rapidly.

Generation of Driver Lines In *Drosophila*, gene trapping using P-element mediated gene insertion has been used extensively to generate cell type specific drivers, although in many instances these drivers capture large and diverse cohorts of neurons. Numerous consortiums have developed libraries of transgenic lines in an effort to cover all the

neurons in *Drosophila*, the most comprehensive being the one from HHMI Janelia (Pfeiffer et al. 2008). In this approach, 925 genes of *Drosophila* were selected based on their putative function and expression in the CNS. From each of these genes, overlapping fragments 3 kb upstream and downstream of the coding regions were used to generate a collection of 7000 transgenic Gal4 lines (Jenett et al. 2012). An anatomical data base of the collection in which each Gal4 line was crossed to an effector line carrying UAS-GFP and stained for GFP expression in the CNS is publically available. A similar approach was used by other consortiums to generate different sets of Gal4 lines (Vienna *Drosophila* Resource Center (VDRC), Kyoto *Drosophila* Genetic Resource Center (DGRC)).

An alternative strategy, based enhancer trapping, is exemplified by the InSITE (Integrase Swappable In vivo Targeting Element) fly collection. In this strategy, enhancer trap lines were generated in which a modularly designed transgene system was integrated randomly into the genome of *Drosophila*. The InSITE-system uses three different site-specific recombination systems that enable the user to convert a Gal4 enhancer trap line into several different transcriptional activated enhancer trap lines (LexA, QF, or split-Gal4) simply by crossing fly lines (Gohl et al. 2011). This genetic platform facilitates the interchange between different binary systems.

In mouse, most cell specific drivers generated to date use Cre. These Cre lines are either traditional or bacterial artificial chromosome (BAC) transgenics (Gong et al. 2003, 2007; Gordon and Ruddle 1983), or knockins (insertions of genetic sequences at specific genetic loci) generated by homologous recombination (Cappechi 1989). Mouse lines for two other recombinase systems, Flp/FRT and Dre/Rox, are beginning to come on line (Anastassiadis et al. 2010; Dymecki and Kim 2007). However, efforts to develop the Gal4 system in mouse have proved problematic, in large part due to unstable expression of the UAS recognition sites used to drive effector gene expression. The Gal4-UAS binary system is beginning to be used in zebrafish to map neural circuits (Scott 2009; Scott et al. 2007), including those in the spinal cord controlling locomotion.

4.2.2 Intersectional Strategies

The binary approaches outlined above often show limited selectivity with regards to the targeting of specific cell types in the nervous system. To overcome this, a number of intersectional approaches have been devised that further restrict effector protein expression, both spatially and temporally. In *Drosophila*, two modifications to Gal4-UAS mediated transcriptional regulation are used to increase the specificity of this binary system. The first of these, the split-Gal4 approach, divides the Gal4 transcriptional activator into two subunits that can re-associate when expressed in the same cell (Fig. 4.1C). Individually, the Gal4 subunits cannot activate expression of a UAS containing target gene, but do so if both subunits are co-expressed in the same cell (Luan et al. 2006). The second approach uses Gal80, a repressor protein that inhibits Gal4's ability to activate the effector gene. In this approach, if line 1 expresses the transcriptional activator Gal4 and line 2 expresses the Gal4 inhibitor Gal80, expression of the effector gene is restricted to the portion of the line 1 expression domain not part of the expression domain of line 2 (Fig. 4.1D) (Suster et al. 2004).

Flp/FRT Intersectional Systems Flp-mediated FRT recombination, when used in combination with the Gal4-UAS binary system, represents an alternative binary system in which

recombination instead of transcriptional regulation is used to control gene expression (compare to Cre/loxP system described above). To further extend the use of Flp/FRT recombination, libraries of transgenic enhancer trap Flp recombinase fly lines have been generated that reproducibly target specific sets of cells. In some instances, Flp expression can be neuron cell type specific, as exemplified by an enhancer trap Flp line whose expression is restricted to 1–2 bilateral serotonergic neurons in the brain (Alekseyenko et al. 2014). This line was used to show that these neurons are involved in aggression in adult male flies. The Flp/FRT recombination system is also being employed in mice to gain access to more defined populations of neurons. Examples of this are the use of tissue or cell specific Flp drivers in combination with Cre to ablate specific populations of neurons in the medulla and spinal cord (see Section 4.4). The Flp/FRT system can also be used in mice where the overlapping expression patterns of two or more genes enables one to uniquely target a small cohort of neurons or a particular neuronal cell type (Fig. 4.2C) (Bourane et al. 2015; Britz et al. 2015; Dymecki and Kim 2007; Ray et al. 2011).

4.2.3 Temporally Inducible Systems

In many instances temporal control is required in addition to spatial regulation. Temporal variants of the Gal4/UAS-systems are the TARGET and Gene Switch system. These allow Gal4 expression to be controlled by temperature or application of RU-486 (Mifeprestone), respectively. The TARGET system uses a temperature-sensitive allele of Gal80 under the control of the ubiquitously expressed tubulin promotor. At room temperature, Gal80 inhibits Gal4 and the effector gene is not expressed. At 30°C, Gal80 is inactivated, and Gal4 can now activate the effector gene (McGuire et al. 2003). The Gene Switch system uses a modified version of Gal4 that includes the ligand-binding domain for the human progesterone receptor, which is activated by RU-486. This modified Gal4 binds to the UAS sequence and activates transcription only upon application of ligand, RU-486 (Osterwalder et al. 2001). A further variation of the Gene Switch system is the Gal4-ER system, in which Gal4 is induced by the application of estradiol (Han et al. 2000). Both inducible Gal4/UAS systems are used to limit the expression of a target gene to the experimental time window so as to exclude potential interfering effects of the gene being expressed at earlier points in development (McGuire et al. 2004).

In mice, the estrogen receptor binding domain (ERT2) fused to Cre provides a degree of temporal control over Cre activity and Cre-dependent expression (Leone et al. 2003; Metzger and Chambon 2001). With the Cre-ERT2 allele, Cre recombinase is only active when animals are exposed to the estrogen agonist tamoxifen. This results in the recombining out of the Cre specific LSL or FLEX (a more advanced technique that allows turning on one gene while turning another off, Schnütgen et al. 2003) sequences that are blocking transcription, thereby turning on expression of the downstream effector/reporter protein. A drawback with this approach is that the effector/reporter gene, once activated, cannot be switched off. Using this driver system with effector/reporter systems that utilize DREADDs or optogenetic proteins, which have their own built in system for temporal control, is therefore particularly helpful when analyzing neuronal function.

The Tetracycline (Tet) and Hybrid Tet-Cre Systems The advantage of tetracycline (Tet) inducible systems is increased temporal control over gene expression. This has obvious

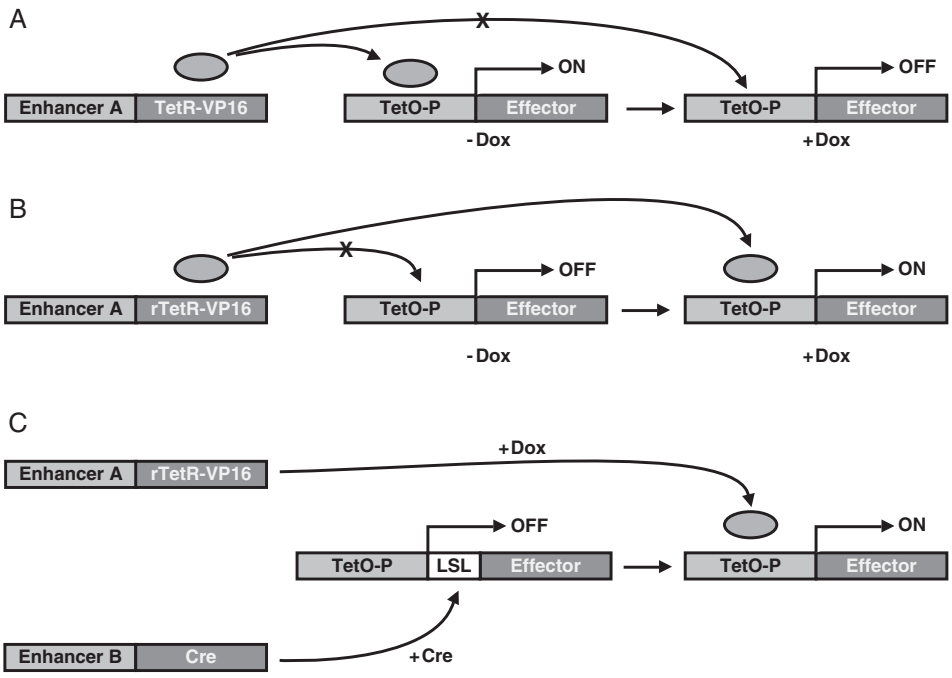

Figure 4.3 Regulation of gene expression in mice using the tetracycline antibiotic repressor-operator system. (A) Simple binary Tet-Off activation system. The Tet-repressor domain fused to the VP16 transactivation domain binds to the TetO enhancer upstream of the effector protein coding region. Upon binding TetR-VP16, the gene encoding the effector protein is turned on. Expression can be controlled by application/injection of doxycycline (Dox), which prevents binding of the TetR-VP16 protein to the TetO enhancer. (B) Alternative binary TetR-On system. In this instance the reverse TetR is fused to a VP16 transactivation domain. In the presence of doxycycline, the rTet-VP16 protein binds to the TetO-P enhancer-promoter to activate effector gene expression. (C) Binary Cre Tet-On system. Intersectional expression of Cre and rTetR-VP16 enables spatially and temporally restricted expression of the effector protein.

advantages when one wants to acutely manipulate neuronal activity by transiently expressing Tetanus toxin or Kir2.1 in neurons, as opposed to constitutively expressing them with site-specific recombinase systems. The Tet inducible system is used in two primary configurations, Tet-On (rtTA) or Tet-Off (tTA) (Fig. 4.3A and B). In both configurations, effector/reporter gene expression is controlled by upstream TetO regulatory sequences that bind the Tet-activator (TA) protein (Corbel and Rossi 2002; Gossen and Bujard 2002). More recently, a combined Tet-off/Tet-On system has been developed that allows for more stringent control of gene expression. The major advantage of the combined Tet system is that it largely overcomes the leakiness encountered with the simpler Tet-On system. The hybrid TetR-Cre system provides a further layer of control over gene expression in which Cre recombination can be used to restrict expression of either the TA protein or the downstream TetO-regulated effector/reporter (Fig. 4.3C). This is important given the leaky expression transgenic mice often display and the observation that a single gene rarely imparts cell type specificity. While this approach has thus far received limited use, it has a number of

advantages with regard to temporal control and levels of gene expression that can be obtained, approaching in some instances the levels seen with viral vector approaches (Madisen et al. 2015).

4.3 Tracing, Manipulating, and Monitoring Neurons

The ability to drive the expression of different reporter/effector proteins (Table 4.1) in specific populations of neurons is now facilitating a range of analyses in neurons from morphological and neuroanatomical tracing to manipulating and monitoring neuronal activity. These techniques can be used in conjunction with conventional electrophysiological recordings and behavioral analyses.

4.3.1 Tracing Neuronal Projections and Connections with Fluorescent Reporters

Green fluorescent protein was the first genetically encoded reporter to be used as a molecular genetic tool in neurobiology by Martin Chalfie, who expressed it in *Caenorhabditis elegans* to investigate sensory neuron morphology (Chalfie et al. 1994). The brightness and photostability of GFP has since improved enormously, and new variants developed with yellow (YFP), blue (BFP), and red (RFP) emission spectra (Zhang et al. 2002). The versatility of these fluorescent proteins has been further enhanced by fusing them to peptide sequences that target them to specific subcellular compartments, for example nls::GFP (nuclear), mCD8::GFP (membrane), Syb::GFP and pHlourin (presynaptic sites), and Dscam17.1::GFP (post synaptic sites). Additional photoactivatable versions of GFP include Kaede, which is converted by UV light exposure to a red fluorophore. While photoconversion of Kaede has been used to trace individual neurons in *Drosophila* and zebrafish larvae (Chen et al. 2012; Hatta et al. 2006; McLean and Fetcho 2009; Mizuno et al. 2003), its utility in organisms where the CNS is not transparent is limited.

A number of red-shifted fluorescent reporters have been developed that provide better optical access in *in vivo* and intact neural preparations due to the reduced diffraction and scattering of longer wavelength light. These offer a number of advantages for visualizing neurons in the mouse CNS, particular those more than 50–100 μm below the surface of the tissue. tdTomato, a modified version of DsRed, is now widely used in mice (Shaner et al. 2004). tdTomato has an emission intensity similar to GFP and mCherry. More importantly, it localizes to neuron soma and processes, allowing better morphological characterization and tracing of connections. A number of mouse strains harboring Cre-dependent fluorescent reporters such as GFP, tdTomato, and Brainbow are being used to analyze neuron morphology, axonal projections, and connectivity (Buffelli et al. 2003; Livet et al. 2007; Madisen et al. 2010). However, visualizing individual neurons with these reporters remains problematic due to the large number of neurons typically marked when Cre recombination alone is used. This has been addressed to some extent by developing intersectional reporters that are Cre- and Flp-dependent or Cre- and Dre-dependent (Madisen et al. 2015).

More recently, a set of fluorescent reporter proteins have been developed that allow visualizing synapses and synaptic activity. These fusion proteins typically contain a domain from a SNARE protein or protein that is enriched at synapses fused to GFP or

tdTomato. Examples include pHlourin (Miesenböck et al. 1998) and sypH-Tomato (Li and Tsien 2012). They provide an anatomical readout of putative synaptic contacts, and are a proxy for active synapses. In invertebrates, "GFP reconstitution across synaptic partners" (GRASP) is used to identify putatively synaptically connected neurons (Feinberg et al. 2008; Gordon and Scott 2009), where it provides a stringent indication of functional connectivity. The availability of viral tracing technologies for mapping synaptic connectivity in mice has resulted in GRASP failing to take hold in this species.

4.3.2 Viral Tracers for Mapping Neural Connections

A variety of neurotrophic viral tracers have been developed to facilitate mapping synaptic connections. These tracers are most used in mammals because of the lack of neurotrophic viruses in *Drosophila*. Synaptic connectivity maps in *Drosophila* and *C. elegans* are instead based on anatomical mapping and reconstruction studies, approaches that are cumbersome in rodents and primates. Three viral approaches are currently used in mice to map neural network synaptic connections: (1) adeno-associated virus (AAV) carrying various reporter cargos such as GFP and its variants (Wouterlood et al. 2014); (2) alpha herpes viruses (HSV-1 and PRV; Card and Enquist 2014) and (3) rabies viruses (Callaway 2008; Ugolini 2010), the last of which includes variants that only cross a single synapse (Callaway and Luo 2015). PRV and rabies, which are transported in a retrograde direction, have been used extensively in mice to trace interneuronal inputs to somatic motor neurons (Coulon et al. 2011; Jovanovic et al. 2010; Lanuza et al. 2004; Ugolini 2010) and other CNS neurons (Campbell and Herbison 2007; Card and Enquist 2014; DeFalco et al. 2001).

No reliable anterograde viral tracers exist that can be used for mapping circuits in mice, although attempts to generate such reagents are underway in several labs. The selective expression wheat germ agglutinin (WGA) or WGA-Cre remains the best approach for transsynaptic anterograde tracing of neuronal connections (Braz et al. 2002; Yoshihara et al. 1999). However, the low levels of WGA expression that can be obtained in transgenic reporter mice and the lack of specificity with regard to Cre drivers have impeded widespread use of WGA as a genetically-encoded anterograde tracer. Utilizing Cre-dependent expression of WGA in AAV viruses to increase expression levels may be a viable solution to both issues.

Pseudorabies virus (PRV) is a double stranded virus that has been engineered to express Cre, GFP, RFP, and β-galactosidase (Card and Enquist 2014). A number of Cre-dependent PRV viruses have been generated that allow specific populations of neurons to be targeted and inputs to these neurons mapped (Card et al. 2011; DeFalco et al. 2001). The use of the Brainbow reporter addresses in part a major drawback of using PRV to trace neuronal connections, the inability to restrict transport to a single synapse. The Brainbow-expressing strain of PRV (PRV263), after undergoing Cre-mediated recombination, expresses either YFP (yellow) or mCerulean (blue) instead of tdTomato (red), allowing one to distinguish between neurons that are presynaptic to Cre-expressing neurons and those presynaptic to neurons that do not express Cre (Card et al. 2011).

Rabies viruses have been used to trace motor networks in multiple species including non-human primates, guinea pigs, rats, and mice. A complication with non-pseudotyped "wildtype" rabies virus is the difficulty in identifying the order of

connectivity, which is determined largely by the kinetics of viral infection (Ugolini 2010). A powerful advance for mapping neuronal circuits in rodents was the development of pseudotyped rabies viruses that allow single synapse tracing (Callaway 2008; Wickersham et al. 2007a,b). The first step in achieving this advance was deleting the gene encoding the rabies glycoprotein, RG, from the viral genome. Although these viruses can reproduce in cells in which they are present, they cannot normally (see next) infect other rodent cells because their viral surfaces lack RG, which is required for the virus to bind to rodent neurons. They are introduced into specific neurons, and made able to infect only neurons immediately pre-synaptic to those neurons, in a three-step process.

First, the gene for the envelope protein (EnvA) for the avian leukosis virus (ALV) is introduced into the viral genome, thus producing viruses with the EnvA protein embedded in the viral membrane (EnvA pseudotyped virions). EnvA shows very high specificity for its receptor, TVA, which is not expressed in mammals. Thus this step alone does not allow the modified virus to infect rodent cells. The second step is to introduce the TVA gene into a specific population of rodent neurons, which allows the pseudotyped virus to infect these neurons, and only these neurons. At this stage one can visualize the infected neurons, but not their presynaptic partners. The third step is to introduce the RG gene into the cells also expressing the TVA gene. The RG protein will thus be present in the cytoplasm of these cells and the pseudovirus can therefore produce virions containing the RG protein. These virions can infect neurons making synapses onto the TVA and RG expressing neurons, but cannot infect any further neurons because the presynaptic neurons do not express RG.

A number of genetic strains of mice have been developed in which RG and TVA expression can be restricted to specific neuron populations. These include the *R26*CAG$^{\text{LSL-RG-TVA}}$ (Takatoh et al. 2013) and *R26*$^{\text{ds-HTB}}$ strains of mice (Bourane et al. 2015; Stam et al. 2012), in which TVA and RG expression depends on either Cre alone (*R26*CAG$^{\text{LSL-RG-TVA}}$) or Cre and Flp (*R26*$^{\text{ds-HTB}}$). Recent studies have begun to use this approach to trace connections from genetically defined neurons in the medulla and hindbrain that are involved in motor control (Bourane et al. 2015; Stanek et al. 2014).

Rabies viruses lacking RG can also be used to trace premotor interneuron connections to specific motor neuron populations by co-injecting RG-minus pseudorabies virus virions that nonetheless have an RG-plus membrane in combination with an AAV virus that expresses the RG protein (Stepien et al. 2010). This technique depends on the fact that the AAV virus has two life cycle pathways: in the presence of a helper virus such as adeno or herpes virus AAV enters a lytic pathway in which it produces virions whereas without a helper virus it enters a lysogenic pathway in which it inserts into the host DNA and does not produce virions. The Stepien et al. procedure works as follows: RG-minus rabies pseudovirus is grown in a host containing the RG gene, and thus pseudo-rabies virions whose membrane contain RG are produced. These are injected into the muscle along with AAV viruses containing the RG gene. Both are taken up by the motor neurons innervating the muscle (the pseudo rabies viruses at the neuromuscular junction synapses because their coats contain RG provided by the host, and the AAV viruses because they are normally infective of mammalian cells). There is no helper virus in the motor neurons and the AAV viruses therefore insert into the motor neuron genome and produce RG in the motor neuron cytoplasm. This allows RG-containing rabies pseudovirus virions to be released and taken up by the motor neurons' presynaptic partners.

These, however, cannot further transmit the pseudovirus because the AAV virus is not producing virions, and hence the presynaptic neurons do not become infected with it.

A potential difficulty with this approach is that it can lead to infection of sensory neurons and anterograde transynaptic labeling in the spinal cord of neurons post-synaptic to the sensory neurons (Zampieri et al. 2014). This anterograde movement of the pseudovirus appears to occur in sensory neurons due to differences in axonal transport in sensory and motor neurons, and the trans-synaptic labeling appears to occur at axo-axonic synapses of sensory afferents onto their post-synaptic partners, which limits the number of post-synaptic neurons likely labeled. This observation does, nonetheless, raise a concern in interpreting pseudorabies labeling work.

4.3.3 Manipulating Neuronal Function

A powerful and rapid development in recent years has been the ability to activate or silence neuronal activity with genetically-encoded proteins that alter neuronal excitability or synaptic transmission (Table 4.1). This was initially achieved using genes that constitutively modify neuronal activity. Many early studies utilized tetanus toxin light chain (TeNT), which cleaves the synaptic vesicle protein synaptobrevin and inhibits transmitter release at chemical synapses (Sweeney et al. 1995). Another approach has been to use the inward rectifying potassium channel (Kir2.1), which hyperpolarizes targeted neurons by increasing K^+ conductance and thus reduces action potential firing (Nitabach et al. 2002). Both systems have been effectively exploited in invertebrate and mammalian systems. By contrast, studies that employ excitatory channels such as the low-threshold, slowly inactivating voltage-gated sodium channel (NaChBac) have been undertaken only in flies. NaChBac increases the effect of excitatory synaptic inputs, resulting in hyperexcitation of NaChBac-expressing neurons (Nitabach et al. 2006). More recently a range of optogenetic and chemogenetic reagents have been added to the arsenal of genetically-encoded effectors. These reagents have a significant advantage over TeNT, Kir2.1, and NaChBac in that the timing of activation and inactivation can be controlled, in the case of optogenetic effectors very precisely.

Optogenetic Methods Channelrhodopsin (ChR) and its variants are now regularly used to activate neurons (Luo et al. 2008; Zhang et al. 2007). These proteins are nonselective cation channels that depolarize neurons and were first discovered in *Chlamydomonas reinhardtii*, where they function as phototaxis photoreceptors (Nagel et al. 2003, 2005). Absorption of 470 nm light by ChR induces a photocycle with several kinetic intermediates, some of which form a non-selective cation channel with a reversal potential near 0 mV. ChR has been incorporated into *Drosophila* using the Gal4-UAS system. However, in order to function, ChR requires all-*trans*-retinal, which is not synthesized by *Drosophila* and must be supplied orally. Mice synthesize all-*trans*-retinal and supplementation is not necessary. ChR has fast opening and closing kinetics that provide a high degree of temporal resolution for photostimulation. The low conductance of ChR was improved in ChR2 (Pulver et al. 2009). Recently developed versions include ones with improved photoactivation kinetics (Chronos), a red-shifted excitation spectrum (Chrimson and ReaChR), and ChR 2-XXL, which functions without retinal supplementation (Dawydow et al. 2014; Klapoetke et al. 2014; Lin et al. 2013).

Halorhodopsin (HR) is a light-gated ion pump from *Halobacteria* that transports chloride ions across the cell membrane independent of the electrochemical gradient. Like ChR, it has seven transmembrane domains and requires all-*trans*-retinal. Illumination with light at a wavelength of 570 nm isomerizes the chromophore, inducing a photocycle that pumps one chloride ion into the cell. Experiments in cultured hippocampal neurons show that HR activation hyperpolarizes the cell, leading to the inhibition of neuronal activity or elimination of single action potentials during rhythmic neuronal activity (Han and Boyden 2007; Zhang et al. 2007). Like ChR, HR has rapid kinetics, low probability of interfering with endogenous proteins, and almost no leak conductance.

Because it is a pump, early versions of HR induced only small hyperpolarizations that made utilization *in vivo* difficult. In particular, since typical CNS neurons receive a high number of synaptic inputs, HR's hyperpolarizing current can be overridden by basal synaptic input, resulting in HR's activation only reducing, not stopping, neuron firing. In addition, ectopic expression of HR leads to the protein accumulating in the endoplasmic reticulum rather than the cell membrane, reducing HR's hyperpolarizing effect. Later versions of HR, enhanced *Natronomonas pharaonis* Halorhodopsin (eNpHR) (Gradinaru et al. 2008; Zhao et al. 2008), overcame this problem by adding N-terminal signal peptide and C-terminal endoplasmic reticulum export sequences that increase shuttling of HR to the cell membrane, thereby improving the inducible hyperpolarizing current of HR. Further modification of eNpHR by adding the trafficking signal of the potassium ion channel Kir2.1 increased the HR photocurrent 20-fold (Gradinaru et al. 2010) and shifted the absorbance spectrum to 620–680 nm. Future approaches could combine HR and ChR expression in one neuronal circuit to remotely control the activity of these neurons.

Chemogenetic Methods Chemogenetic approaches are now being used extensively in flies and mice to manipulate neuronal activity. The two primary systems used in *Drosophila* are the P2X purinoceptor 2 ($P2X_2$) ATP-gated non-selective cation channel for excitation, and the histamine-gated chloride channel Ort for inhibition. DREADDs based on the human muscarinic receptors hM3 and hM4 are increasingly being used for chemogenetic excitation and inhibition of neurons in mice (Ambruster et al. 2007; Urban and Roth 2015). The insect allatostatin peptide receptor AlstR has also proved to be an effective way to silence neurons (Gosgnach et al. 2006; Tan et al. 2006) However, bioavailability of the peptide ligand for AlstR, particularly *in vivo*, can be problematic. The muscarinic DREAADs therefore remain the preferred approach for manipulating the activity of genetically defined neurons in mice *in vivo*.

$P2X_2$ can be activated by local application of ATP. $P2X_2$ activation opens a large (30 pS) conductance that lasts for over 100 ms (Ding and Sachs 1999) and induces membrane depolarization and action potential firing in hippocampal neurons (Zemelman et al. 2003). Temporal resolution of $P2X_2$ stimulation can be improved by using photoactivatable caged ATP in which ATP is linked to a photolabile blocking group. Illumination uncages the ATP from the group and activates $P2X_2$ in $P2X_2$ expressing neurons. Although $P2X_2$ has a low probability of channel opening in the absence of ATP, $P2X_2$ leak currents in the absence of ATP nonetheless reduce the lifespan and affect the behavior of flies expressing high $P2X_2$ levels, despite *Drosophila* having no endogenous ATP receptor (Lima and Miesenböck 2005). In mammals ATP can interfere with endogenous purine receptors.

Ort is a large conductance, slowly desensitizing, homomeric channel and a reliable method to chemogenetically silence neurons. *In vivo* whole-cell recordings in *Drosophila* show that Ort activation reversibly reduces input resistance, lowers membrane potential below spike threshold, and nearly eliminates spontaneous and evoked neuronal activity in antennal lobe projection and local neurons (Liu and Wilson 2013). Ort was more effective in silencing neuronal activity in the olfactory system of *Drosophila* than *shibire^{ts}* (*shi^{ts}*) and halorhodopsin, both of which have been previously shown to be reliable genetic tools for inactivating neuronal populations in *Drosophila* (Inada et al. 2011; Okusawa et al. 2014). Ort use is limited to CNS regions lacking an endogenous histamine receptor.

The muscarinic DREADDs are modified G protein coupled receptors (GPCRs) that come in two basic forms, the Gi coupled hM4D receptor (inhibitory) and the Gq coupled hM3D receptor (excitatory). Both receptors are activated by clopazine-N-oxide (CNO), which is inert and does not activate any known mammalian muscarinic receptor. hM3D and hM4D were both generated by modifying the 3rd and 5th transmembrane domains of the hM3 and hM4 receptors. This renders hM3D and hM4D insensitive to acetylcholine and highly sensitive to CNO. hM3D, when activated by CNO, mimics the activity of the native muscarinic receptors and increases neuronal excitability by cell depolarization, often resulting in increased burst firing. hM4D functions by activating G protein-coupled inwardly rectifying potassium channels, which hyperpolarize the neuron and reduce firing in response to ligand mediated activation. While both DREADDS are highly effective in manipulating neuronal activity in mice *in vivo*, their activation lacks the temporal precision that can be obtained by optogenetic methods. As with all GPCRs, they also desensitize in prolonged presence of ligand, which may be limiting in studies requiring prolonged (>1–2 hours) silencing or activation.

Two approaches have been used to target DREADD expression to specific neurons. The first uses FLEX-AAV viruses to target expression, while the second employs intersectional knockin transgenic mice that express hM4D following Cre- and Flp-mediated recombination (Bourane et al. 2015; Ray et al. 2011). Each approach has its own advantage, namely high expression with AAV and high reproducibility with the transgene. Importantly, the phenotypes observed following CNO-hM4D silencing closely match those seen with other manipulations, such as neuronal ablation (Bourane et al. 2015) and silencing with TeNT, indicating that the DREADDS provide an excellent method for selectively manipulating neuronal activity.

Thermogenetic Methods The transient receptor channel A (TrpA) belongs to the Trp superfamily of ion channels, a superfamily highly conserved throughout the animal kingdom from worms to flies to mammals (Ramsey et al. 2006; Venkatachalam and Montell 2007). The TrpA subfamily comprises three channels: TrpA1, painless, and pyrexia, all directly activated by temperature. TrpA1 is activated by temperature above 27°C whereas painless and pyrexia respond only to noxious temperature stimuli above 39°C. The *TrpA1* gene encodes a temperature- and voltage-gated non-selective cation channel. The TrpA1 protein consists of six transmembrane domains with a pore building loop between the 5th and 6th transmembrane domains. The N-terminus is thought to be the heat-responsive element (Zhong et al. 2012).

The *Drosophila* TrpA1 channel (dTrpA1) is responsible for warm temperature sensation and regulates thermotactic behavior in *Drosophila* (Hamada et al. 2008). dTrpA1

is a reliable, versatile molecular genetic tool for inducibly manipulating neuronal activity. Moreover, dTrpA1 expression can be directed to specific neuron types using the binary Gal4-UAS system. dTrpA1 can be activated using a Peltier-driven heating device (Schoofs et al. 2014), a heatable perfusion system, or infrared laser (Marella et al. 2012) and in behavioral experiments with a heating chamber or fly arena with a temperature control.

The heterologous TrpM8 channel is a potential genetic tool to overcome issues associated with endogenous expression of dTrpA1 in *Drosophila*. TrpM8 is the mammalian cold- and ligand-gated nonselective cation channel mice use for cold sensation (McKemy et al. 2002). TrpM8 is activated at temperatures below 18°C or by the application of menthol. In flies, the heterologous TrpM8 channel has been successfully used to investigate the neuronal network for wing expansion (Peabody et al. 2009) and memory formation (Krashes et al. 2009).

A particularly reliable method to silence neuron activity is temperature-sensitive *shibire* (shi^{ts}). The *Drosophila shibire* gene encodes for dynamin, which participates in endocytosis and synaptic vesicle recycling. In neurons expressing shi^{ts} dynamin is inactive at restrictive temperatures above 29°C, reversibly inhibiting synaptic transmission (Kitamoto 2001). Post-synaptic targets of shi^{ts} expressing neurons therefore receive no synaptic input from these neurons at the non-permissive temperature. A disadvantage of this method is that shi^{ts} expression increases sensitivity to high temperatures which could result in developmental defects. Effective use of shi^{ts} also requires high expression levels. Blocking synaptic transmission with neurons having substantial stores of synaptic vesicles requires increasing temperature and/or incubation time.

Neuronal ablation provides a complementary approach for assessing the contribution that molecularly- or genetically-defined populations of neurons make to behavior and circuit function. In *Drosophila*, the two commonly used systems utilize the apoptosis inducing genes *reaper* (*rpr*) and *head involution defective* (*hid*) (Busto et al. 1999; McNabb et al. 1997) to ablate neurons. In mice, the most common approach is to use diphtheria toxin (DTX) to kill neurons. This can be done in one of two ways: (1) expression of the diphtheria toxin A subunit (DTA) directly in the cells of interest (Gosgnach et al. 2006) or (2) selective expression of the diphtheria toxin receptor (DTR) in target neurons followed by administration of the toxin (Britz et al. 2015; Buch et al. 2005). Cell types defined by expression of a cell surface receptor with a known ligand, e.g., the NKR1 receptor that binds substance P or the gastrin releasing peptide receptor that binds bombesin/GRP, can be selectively ablated using ligands conjugated to saporin (Bourane et al. 2015; Gray et al. 2001). The advantage of receptor-mediated killing via DTR or saporin is that the experimenter has control over the timing of neuronal ablation. As with the silencing methods described above, targeting the "killer" protein to specific populations of neurons can be achieved using the intersectional genetic approaches described in Section 4.2.2.

4.3.4 Monitoring Neuronal Activity

Electrophysiology has been the most widely used method for monitoring neuronal activity in many model organisms including mice. In *Drosophila*, recording from brain neurons is demanding because of the small size of the brain and neurons (Olsen and Wilson

2008). With the advent of labeling cells with GFP more labs are beginning to undertake whole cell recordings in flies. Recording from identified neurons in the mouse *in vivo* is also problematic, necessitating the use of *ex vivo* slice or *en bloc* preparations. With the exception of the PreBötzinger complex, which can be preserved and studied in a slice, these recordings have not yielded much information about the organization and activity of motor circuitry in mice, aside from determining the cellular properties and conductances of neurons.

A complementary approach is to use calcium indicators to monitor the changes in cytoplasmic [Ca^{2+}] that occur during neuron activity (Grienberger and Konnerth 2012). These indicators can detect activity, spiking events, and even post-synaptic potentials. The first generation of calcium indicators were synthetic indicators, such as Oregon Green Bapta-1 (OGB1) and Fura-2, which need to be loaded into cells. These chemical indicators have been largely superseded by genetically encoded calcium indicators (GECIs) that can be easily and noninvasively targeted to specific neuronal cell types using the genetic expression systems described above.

GECIs comprise a calcium binding domain fused to one or two fluorescent proteins. In single fluorescent protein GECIs, the fluorescence emission is due to reversible conversion of the fluorescent protein as a function of Ca^{2+} binding. In two fluorescent protein GECIs, calcium binding induces fluorescence resonance energy transfer between the two fluorescent proteins, and the ratio of the two emissions is measured. The most commonly used GECIs are the single fluorescent protein calcium indicators, GCaMP and its variants, of which the latest, GCaMP6, is capable of single spike resolution and high Ca^{2+} signals (Chen et al. 2013). GCaMP comprises a circular enhanced GFP core flanked on one side by the calmodulin M13 peptide and on the other by calmodulin. Ca^{2+} binding leads to a Ca^{2+}-dependent increase in fluorescence that can be monitored by a photomultiplier tube coupled to a confocal microscope. The temporal resolution of calcium imaging cannot compete with intracellular recordings, although they are fast approaching millisecond resolution. Nonetheless, GECIs are powerful tools for measuring the activity of single neurons or neural ensembles in behaving animals or neurons difficult to access by electrophysiological methods.

In *Drosophila*, GCaMP-based monitoring of gustatory neurons has shown that specific gustatory receptor neurons selectively respond to either sweet or bitter compounds and mediate acceptance or avoidance behavior (Gordon and Scott 2009; Marella et al. 2006). In mice, 2-photon imaging of GECI signals has been used to monitor neural activity in the hippocampus and cortex, both in slice preparations and *in vivo* (Dombeck et al. 2010), and Komiyama et al. (2010) have recorded calcium signals in the motor cortex of mice performing an olfactory discrimination task.

In vivo imaging of calcium activity is now possible in the spinal cord following bulk labeling of dorsal neurons with OGB1. To date, this imaging is restricted to cells located close to the dorsal aspect of the spinal cord (Farrar et al. 2012; Johannssen and Helmchen 2010). The development of Gradient Index Rod (GRIN) microendoscopes may enable imaging neurons located more ventrally in the spinal cord and medulla where the motor networks for walking, breathing, and chewing are located. Imaging motor network activity in spinal cord and hindbrain is currently limited to *in vitro* tissue preparations where rhythmic motor activity can be induced by drugs, K^+, or electrical or light stimulation

(Hinckley and Pfaff 2013; Kam et al. 2013; Koshiya and Smith 1999; O'Donovan et al. 2005; Thoby-Brisson et al. 2005).

4.4 Case Studies

Flies and mice are fast becoming models for investigating the neural networks that underlie sensorimotor behaviors in invertebrates and vertebrates. We outline below several studies relevant for motor control, with an emphasis on basic strategies and the tools used.

Escape Behavior in Flies: Remote Control of Behavior through Genetically Targeted Photostimulation of Neurons Lima and Miesenböck (2005) investigated the hierarchical organization of the motor circuit underlying the escape behavior in flies using two promotor-fusion lines to drive $P2X_2$ expression in subsets of neurons that make up the giant fiber-escape circuit (Fig. 4.4). Two highly restricted Gal4 lines were used. The first, ShakB-Gal4, was expressed in 11 pairs of neurons in the thoracic ganglion, seven of which are direct or indirect targets of the giant fiber (GF) neurons that mediate the stereotyped escape behavior, namely the tergotrochanteral motor neurons (TTMns) that innervate the jump muscles and peripherally synapsing interneurons (PSIs) that innervate the dorsal longitudinal flight muscle motor neurons (DLMns) (Fig. 4.4B). ShakB-Gal4 was not expressed in the GF neurons. The second, an enhancer trap line Gal4-c17, drove $P2X_2$ expression in the GF neurons (Fig. 4.4C) and in eight peripheral sensory neurons. Because the caged ATP was directly microinjected into the CNS, the eight peripheral neurons were not activated by photostimulation. Whereas photostimulation of control flies lacking $P2X_2$ expression showed no escape response (Fig. 4.4A), uncaging ATP in both Gal4 lines expressing $P2X_2$ elicited the typical escape behavior (Fig. 4.4B, C). Most importantly, photoactivation of the seven paired thoracic neurons triggered the escape behavior in decapitated flies (Fig. 4.4D), demonstrating these neurons are in and of themselves capable alone of executing the motor program.

Feeding Behavior in Flies: Selection of Motor Programs for Suppressing Food Intake and Inducing Locomotion in the Drosophila Brain The *hugin* gene encoding the neuropeptide hugin (Fig. 4.5A) is expressed in 20 neurons in the subesophageal ganglion of *Drosophila* larva (Fig. 4.5B). When a 1.5 kB fragment from the *hugin* promotor driving Gal4 expression in all 20 *hugin* neurons was used to activate dTrpA1, the motor program for food intake ceased and the motor program for crawling was induced (compare Fig. 4.5C and D). By further subdividing the promotor, Schoofs et al. (2014) generated two different promotor fragment Gal4 fusion constructs that targeted either the 16 *hugin* neurons that project to the ring gland, higher brain centers, and pharynx (Fig. 4.5E), or the four *hugin* neurons that project to the ventral nerve cord (VNC) (Fig. 4.5F). Activation of the 16 *hugin* cells selectively reduced the cibarial dilator muscle (CDM) motor pattern, which is involved in food intake (Fig. 4.5E). By contrast, activating the four *hugin* VNC cells induced locomotion with little effect on the CDM motor pattern (Fig. 4.5F), showing that the two motor programs can be decoupled (Fig. 4.5G), thus providing insight into the neuronal circuits underlying the action selection of different motor programs.

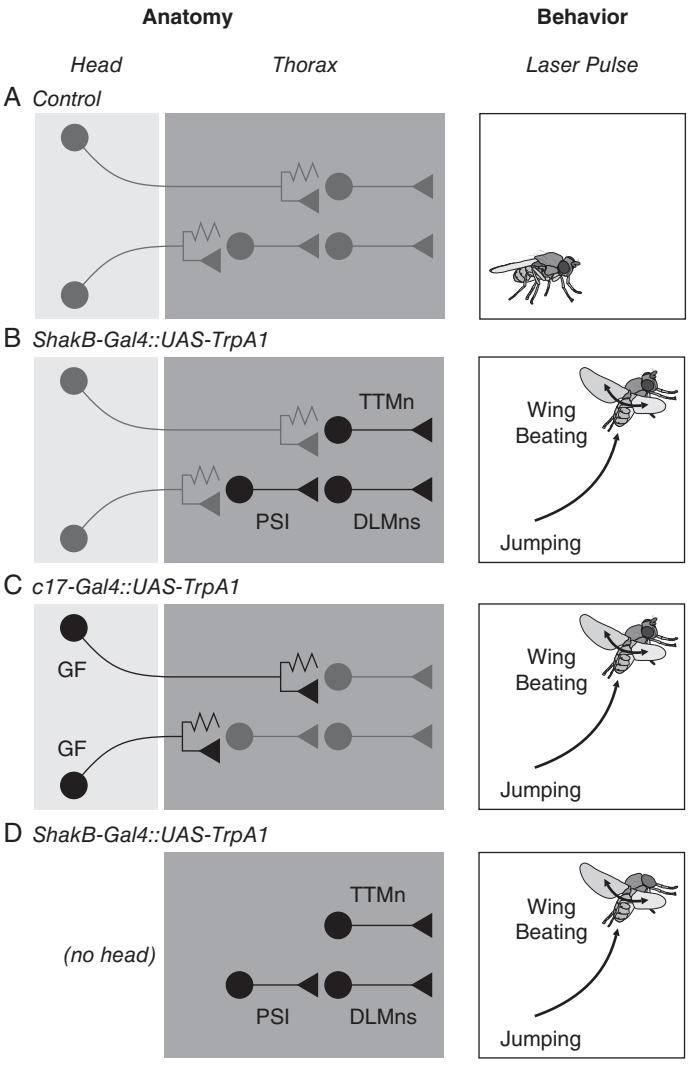

Figure 4.4 Activation of escape behavior in *Drosophila* by genetically-targeted photostimulation. Left column: Simplified diagrams of the neural network underlying the escape behavior indicating which neurons expressed $P2X_2$ under the genetic control of Gal4 (black). The neural network consists of paired giant fibers in the brain (GF) whose axons project to the thoracic ganglion. The GF neurons are connected to the tergotrochanteral muscle motor neurons (TTMns; responsible for jumping) and to peripherally synapsing interneuron (PSI). The PSI directly drive the dorsal longitudinal muscle motor neurons (DLMns) responsible for flight. A 150 ms laser pulse was used to uncage microinjected ATP and activate the $P2X_2$ channel (excitatory). (A) A laser pulse had no effect on control flies not expressing $P2X_2$. (B) A laser pulse triggered escape behavior in a fly expressing $P2X_2$ in the PSI-TTMn-DLMn group (*shakB-Gal4*). (C) A laser pulse triggered escape behavior in flies expressing $P2X_2$ in the GF neurons (*Gal4-c17*). (D) A laser pulse triggered escape behavior in decapitated flies expressing $P2X_2$ in the PSI-TTMn-DLMn group (*shakB-Gal4*). Panels in left column from Lima and Miesenböck (2005) with permission.

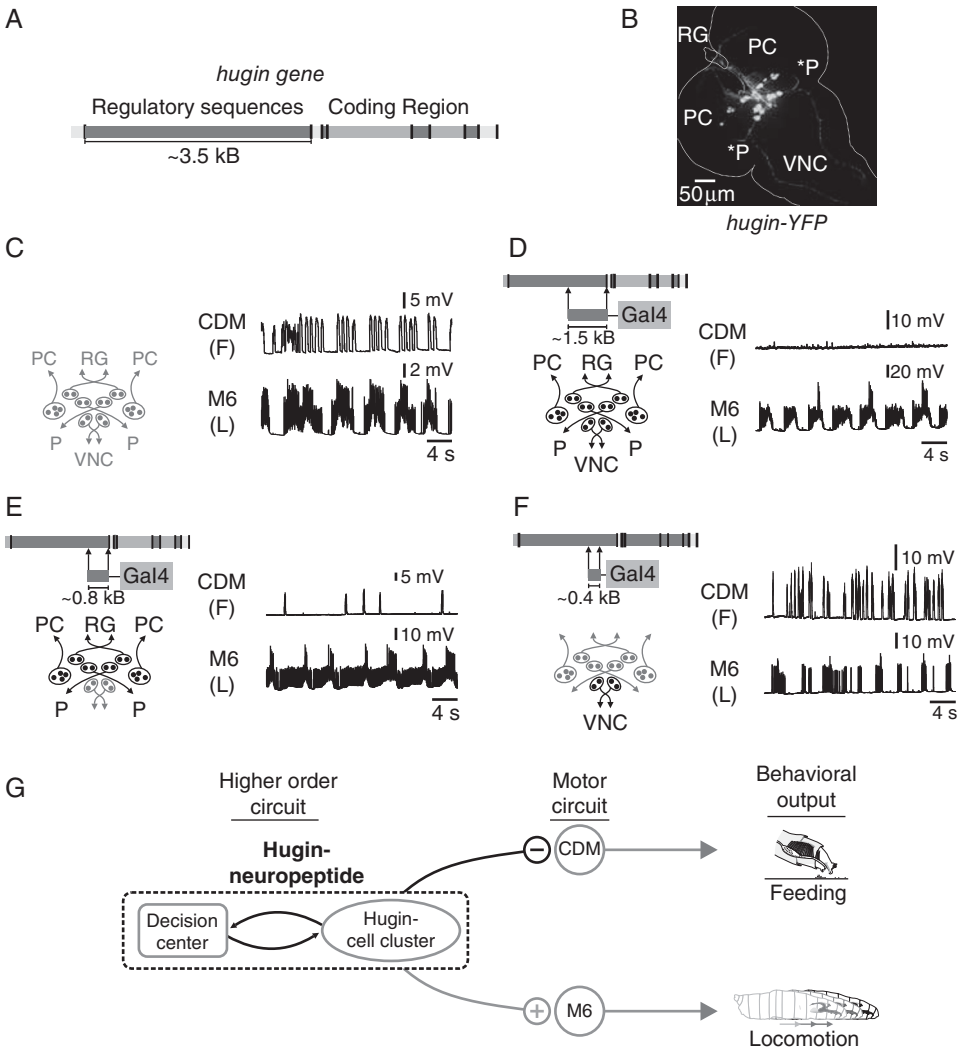

Figure 4.5 Contribution of *hugin*-expressing neurons to motor program selection. (A) Schematic of the *hugin* gene with its 3.5 kB cis-regulatory region. (B) eYFP expression driven by the *hugin*-promotor in larval central nervous system of *Drosophila*. The *hugin* cluster comprises 20 neurons in the larval subesophageal ganglion that can be divided into four subclusters according to their projection targets: ring gland (RG, 2 cells per hemisphere), protocerebrum (PC, 4 cells per hemisphere), ventral nerve cord (VNC, 2 cells per hemisphere) and pharynx (P, 2 cells per hemisphere). (C) Intracellular muscle recording of a cibarial dilator muscle (CDM) and longitudinal body wall muscle M6 in a wild type larva; CDM activity reflects food intake (F) whereas M6 activity reflects locomotion (L). (D) Left: driving the expression of temperature-sensitive cation channel dTrpA1 in all 20 hugin neurons by HugS3-Gal4 line (1.5 kb fragment of the cis-regulatory region). Right: excitation of the 20 hugin neurons by dTrpA1 stops CDM activity and increases M6 activity. (E) Left: driving the expression of temperature-sensitive cation channel dTrpA1 in only the 16 *hugin* neurons projecting to the ring gland, protocerebrum, and pharynx by the Hug0.8-Gal4 line (0.8 kb fragment of the cis-regulatory region). Right: excitation of the 16 hugin neurons by dTrpA1 reduces only CDM activity. (F) Left: driving the expression of temperature-sensitive cation channel dTrpA1 in only the 4 hugin neurons projecting to ventral nerve cord by the HugVNC-Gal4 line (0.4 kb fragment of the cis-regulatory region). Right: excitation of the 4 hugin neurons by dTrpA1 has no effect on CDM activity but slightly increases M6 activity. (G) Model for motor program selection by the hugin cell cluster. C-F adapted with permission from Schoofs et al. (2014).

Flexion–Extension Motor Behaviors in Mice: Intersectional Ablation of Inhibitory Neurons in the Spinal Cord Until recently, isolating small populations of neurons in mice to examine their role in motor control has proved difficult. In a recent study, Britz et al. (2015) used an intersectional approach to assess the contribution of two classes of inhibitory neurons to flexor-extensor locomotor behaviors. The V1 and V2b IN classes had previously been shown to be essential for flexor-extensor control (Zhang et al. 2014). However, because En1 (V1 INs) and Gata3 (V2b INs) are expressed outside of the spinal cord, their contribution and role in adult animals could not be assessed. To overcome this an intersectional approach was employed in which Flp recombinase was localized to the caudal spinal cord thus allowing expression of DTR via the overlapping expression of Cre and Flp (Fig. 4.6A–C). Moreover, by using the neuronal *Tau* promotor to target DTR expression to neurons, cell killing could be restricted to the V1 and V2b INs in the lower cervical, thoracic, and lumbar levels of the cord.

Juvenile mice displayed marked changes in hindlimb movement (Fig. 4.6D), with mice lacking V1 INs showing hyperflexion of the hindlimb and ankle joint and V2b IN ablated mice displaying a hyperextension phenotype. This phenotype was also seen when ablation was performed in adult mice, with V1 IN-ablated mice showing prolonged flexor (TA) EMG activity during walking and V2b IN-ablated mice showing ectopic extensor (GS) EMG activity during swing (Fig. 4.6E, F). The studies thus revealed distinct and specific role for both classes of neurons during locomotion.

Medullary V2aINs and the Executive Control of Movement in Mice: Descending V2a Neurons that Regulate Locomotor Activity (Bouvier et al. 2015) and V2a Propriospinal Neurons that Provide an Efferent Copy of Motor Activity (Azim et al. 2014) Two studies have used conditional genetic approaches to investigate the role of discrete populations of V2a-derived medullary INs in regulating movement. In both studies, spatial control of V2a neuronal activity was attained by stereotactic injection of FLEXed AAV viruses that contained ChR2, TeNT, and DTR. The V2a neurons were accessed via their selective expression of Chx10 (Vsx2). By targeting V2a reticular neurons in the rostral medulla, Bouvier et al. (2015) showed that these neurons, or a subpopulation of them, function as command neurons to halt ongoing locomotion. *Chx10*^{Cre}-dependent ChR2 expression allied with targeted illumination was used to activate these neurons, upon which locomotion stopped (Fig. 4.7A, B). Moreover, activation of these neurons is not only sufficient but necessary, as selective inactivation of V2a "stop" neurons with TeNT reduced arrest during various locomotor tasks (Fig. 4.7C).

Azim et al. (2014) used a similar strategy to investigate the role of V2a propriospinal neurons (PNs) that innervate the lateral reticular nucleus (LRN), a precerebellar structure that provides an efferent copy of motor actions to the cerebellum. Selective ablation of these neurons degraded forelimb reaching movements (Fig. 4.7D), consistent with previous studies in cats and primates. By activating ChR2 expression in the V2a propriospinal axonal processes that project to the LRN, Azim et al. (2014) showed that the efferent copy of the reaching action the LRN relays to the cerebellum ensures smooth goal-directed movement of the forelimb (Fig. 4.7E). These two studies, together with the work of Britz et al. (2015), illustrate how cutting edge genetic approaches are currently being employed to interrogate the motor system at a cellular level.

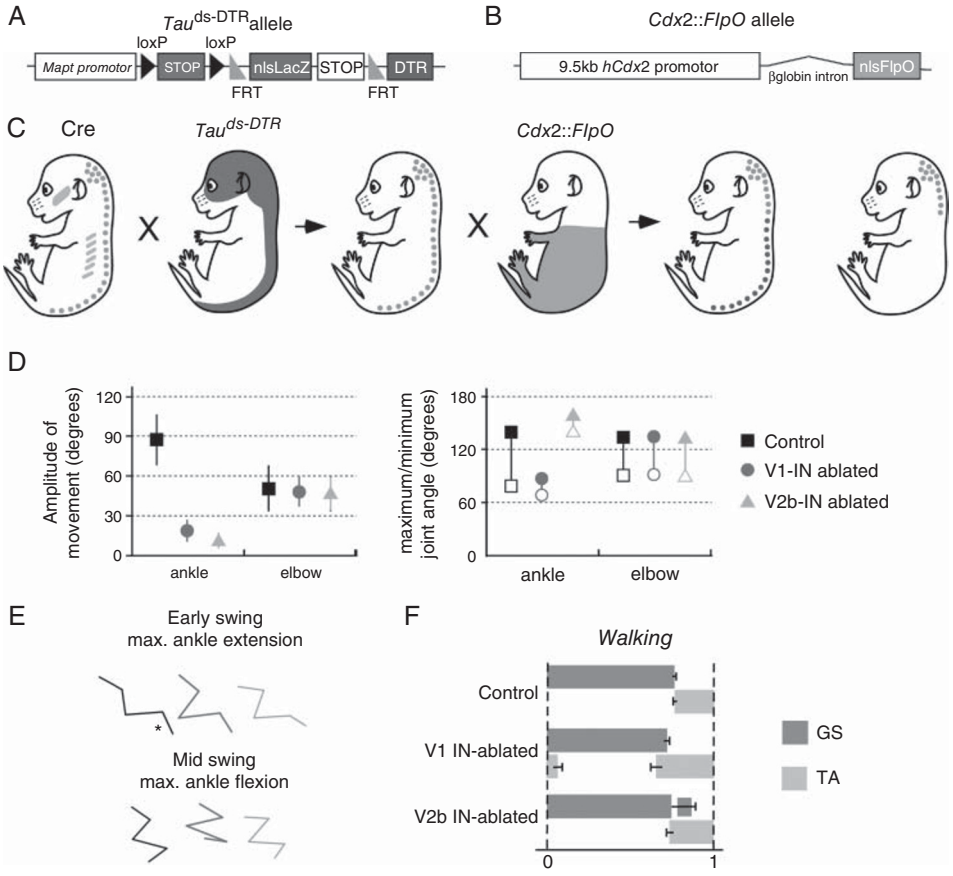

Figure 4.6 Role of V1 and V2 interneurons in controlling hindlimb flexor-extensor movements. (A) The targeting vector for Cre and Flp dependent expression of the diphtheria toxin receptor (DTR). DTR expression was controlled by regulatory sequences from the *Tau* (*Mapt*) gene. Tau expression is largely restricted to neurons. (B) The transgene for the *Cdx2::FlpO* allele used to restrict Flp expression to the caudal torso, including spinal cord. (C) Schematic showing the combinatorial approach used to restrict DTR expression to either V1 ($En1^{Cre}$) or V2b ($Gata3^{Cre}$) INs in the caudal spinal cord. Upon intrathecal injection of DTX, V1 or V2b INs expressing DTR are killed. (D) Summary of kinematic analysis from juvenile P7 air stepping mice showing changes in movement amplitude and angle of the ankle (experimental) and elbow (control) joints. (E) Stick diagram showing average joint angles for the hindlimbs of adult mice during walking at early swing/late stance and mid swing. The ankle joint is indicated with an asterisk. Gray scale identification same as in D. Mice lacking V2b INs show an increased ankle joint angle at the late stance/early swing transition, which is associated with a delay in the transition from stance to swing. At mid swing the ankle joint in mice lacking V1 INs is more acute than in the control. (F) EMG activity in ankle flexor (TA) and extensor (GS) muscles is altered, indicating V1 INs are required to suppress flexor motor activity at the beginning and end of stance (extension), while V2b INs are required to suppress extensor motor neuron activity during swing (flexion). Figure adapted, with permission, from Britz et al. (2015).

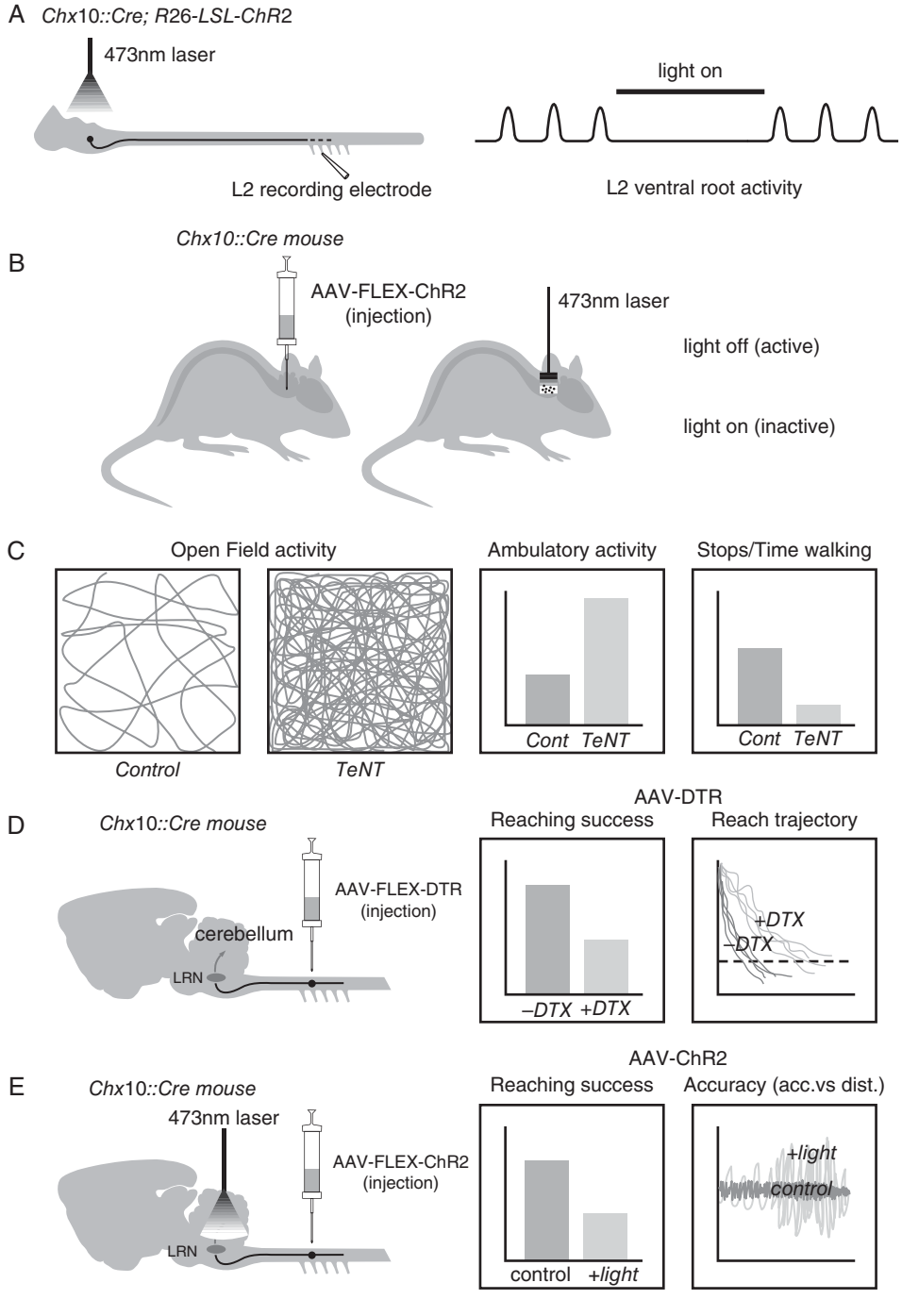

Figure 4.7 Control of movement by hindbrain and cervical V2a neurons. (A) Schematic showing that activation of Chx10-derived V2a "stop" neurons in the medulla regulate locomotor CPG activity. *In vitro* optogenetic activation of medullary V2a neurons in *Chx10::Cre; R26-LSL-ChR2* mice leads to a cessation of rhythmic locomotor activity in the isolated spinal cord preparation. The trace on the right, a rectified electroneurogram of the L2 ventral root, shows rhythmic locomotor activity. (B) *In vivo* optogenetic activation of V2a stop neurons produces locomotor arrest in mice when ChR2 is selectively expressed and activated in medullary V2a neurons. A Cre dependent AAV virus expressing ChR2 (AAV-FLEX-ChR2) was injected stereotactically into different motor regions of the medulla. Cells expressing Cre under the control of Chx10 regulatory sequences (*Chx10::Cre*) undergo recombination, resulting in the expression of ChR2. ChR2 is activated by a 473 nm laser light source. (C) Expression of tetanus toxin light chain (TeNT) in medullary V2a neurons increases locomotor activity as assessed by the open field test. Graphs show changes in ambulatory activity and the number of stop events during locomotion following inactivation of medullary V2a neurons with TeNT. (D) V2a propriospinal neurons in the cervical spinal cord project to the lateral reticular nucleus (LRN), which in turns projects to the cerebellum. Ablation of LRN-projecting V2a propriospinal neurons was accomplished by injecting a FLEXed-conditional DTR into the cervical cord of *Chx10::Cre* mice to selectively target V2a neurons. Treatment with diphtheria toxin (DTX) kills V2a propriospinal neurons, leading to a deficit in reaching (middle panel) that is accompanied by increased numbers of misses of the target zone (area under the dashed line in right panel). (E) Following injection of AAV-FLEX-ChR2 into the cervical spinal cord, ChR2-induced activation of the ascending branch of V2a propriosinal projection (+light) decreases reaching success (middle panel) and forepaw movement accuracy (right panel), indicating that an accurate LRN-mediated efference copy of V2a neuron activity is required for skilled reaching movements. All panels original, but based on data in Bouvier et al. (2015) and Azim et al. (2014).

4.5 Future Perspective

In recent years one method has rapidly become a powerful tool in biological research, named "Targeted genome editing or genetic engineering" (Sander and Joung 2014). Core elements of this technology are the bacterial immune mechanisms against invading viral DNA, the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas-systems. Basically, the system generates a RNA-sequence that binds to the invading DNA and directs a nuclease to cleave it. Charpentier and Doudna adapted this bacterial mechanism to perform site-specific DNA cleavage (Jinek et al. 2012). Today, the CRISPR/Cas-system is being used for site-specific DNA modification, e.g., insertion, deletion, and sequence changes, not only in classical genetic organisms like mice, zebrafish, nematodes, and fly, but also in organisms with limited genetic accessibility. This new genetic technology promises to impact the field of neurosciences in a fundamental way.

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