Topic Introduction

Experimental Methods for Examining Synaptic Plasticity in *Drosophila*

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The *Drosophila* neuromuscular junction (NMJ) ranks as one of the preeminent model systems for studying synaptic development, function, and plasticity. In this article, we review the experimental genetic methods that include the use of mutated or reengineered ion channels to manipulate the synaptic connections made by motor neurons onto larval body-wall muscles. We also provide a consideration of environmental and rearing conditions that phenocopy some of the genetic manipulations.

BACKGROUND

The *Drosophila* larval NMJ has been extensively studied as a model system for examining all aspects of synaptic development, function, and plasticity. Several excellent and up-to-date reviews of this system have been published over the last few years (Budnik and Ruiz-Canada 2006; Collins and Diantonio 2007). In this article, we provide an overview of the genetic methods most often used for manipulating the NMJ for the purpose of studying synaptic development and plasticity. There is a multitude of mutations that yield NMJ phenotypes. Here we focus on the better understood genetic approaches: those that directly affect neurophysiological functions to alter levels of membrane excitability; constructs that alter either presynaptic transmitter release or postsynaptic receptor function; and manipulations of second messengers and signal transduction cascades, cell adhesion molecules (CAMs), retrograde growth factors, and transcription factors.

In addition, we consider environmental and rearing conditions that phenocopy the genetic approaches that affect synaptic growth and function. The article is organized around seven tables that summarize the major experimental tools, the genes that are involved, comments about the method, source material, and relevant references.

METHODS FOR MANIPULATING SYNAPTIC CONNECTIONS

Membrane Excitability

We begin with a review of the genetic tools that are commonly used to manipulate membrane excitability, summarized in Table 1. Two approaches have been adopted. The earliest methods made use of mutations that affect either the expression or function of ion channels. The more recent second approach is to use the directed expression of modified ion channels in neurons or muscles. These channels have often been reengineered to introduce useful properties, such as altered activation voltages or green fluorescent protein (GFP) tagging.

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TABLE 1. Methods to alter membrane excitability

Effect	Tool (gene symbol)	Comment	Commonly used alleles (allele type: stock #) ^a	Reference(s) ^b
Increased excitability	<i>Shaker (Sh)</i>	KV1.2 K ⁺ channel	<i>Sh</i> ¹⁴ (null: 3563); <i>Sh</i> ⁷ (anti); <i>Sh</i> ²¹ (hypo); <i>Sh</i> ¹⁶ ; <i>Sh</i> ⁵ (111)	Budnik et al. (1990)
	<i>ether-a-gogo (eag)</i>	K ⁺ channel subunit	<i>eag</i> ^{sc29} (null: 1442); <i>eag</i> ¹ (hypo: 3561); <i>eag</i> ^{4PM}	Budnik et al. (1990)
	<i>Hyperkinetic (Hk)</i>	Channel subunit; oxidoreductase	<i>Hk</i> ¹ (3562); <i>Hk</i> ² (55)	Budnik et al. (1990)
	<i>comatose; Ca-P60A (comt; Ca-P60A)</i>	Temperature-dependent seizure	<i>Comt</i> ⁶ ; <i>CaP60A</i> ^{Kum170}	Hoeffer et al. (2003)
	Dp <i>para</i> +	Duplication of chromosomal region containing para locus	Dp(1;4)r+1 (5273)	Budnik et al. (1990)
	UAS-SDN	Shaker dominant negative		Mosca et al. (2005)
	UAS-eag-DN	eag dominant negative	(8178)	Broughton et al. (2004)
	UAS-TrpA1	Warm-activated trp channel	(26263, 26264)	Hamada et al. (2008)
	UAS-TRPM8	Cold-activated trp channel		Peabody et al. (2009)
	UAS-NaChBac	Bacterial Na channel		Nitabach et al. (2006)
	UAS-P2X ₂	Light activation of channel agonist		Lima and Miesenbock (2005)
	UAS-Channel-rhodopsin-2 (UAS-ChR2)	Light-gated cation channel		Schroll et al. (2006), Ataman et al. (2008)
Decreased excitability	<i>maleless (mle)</i>	Regulates Na ⁺ channel expression	<i>mle</i> ^{nap-ts1} (GOF)	Budnik et al. (1990), Jarecki and Keshishian (1995)
	<i>paralytic (para)</i>	Na ⁺ channel	<i>para</i> ^{lk2} (null); <i>para</i> ^{ts1} (hypo: 1572)	Budnik et al. (1990), Jarecki and Keshishian (1995)
	<i>Temperature-induced paralytic E (tipE)</i>	Channel subunit	<i>tip</i> ^{E1}	Jarecki and Keshishian (1995)
	UAS-Kir2.1	Inward rectifier K ⁺ channel		Baines et al. (2001), Paradis et al. (2001)
	UAS-EKO	Voltage-gated reengineered Sh channel		White et al. (2001)
	UAS-dORK	Constitutively open K ⁺ channel		Nitabach et al. (2002)

^aThe allele nomenclature and annotation of allele type listed in this and subsequent tables is derived from FlyBase. Please refer to FlyBase (<http://flybase.org/>) for synonymous alleles. The stock number listed is from the Bloomington *Drosophila* Stock Center (<http://flystocks.bio.indiana.edu/>). Abbreviations of allele type: null, amorph; hypo, hypomorph; anti, antimorph; GOF, gain of function.

^bIn general, the references cited in this and subsequent tables refer to examples of the use of the genetic tools at the neuromuscular junction (NMJ). In cases in which a tool has not been used at the NMJ, the reference cited refers to the original characterization/development of the genetic tool.

Ion Channel Mutations

In general, these approaches involve mutations that have one of three primary effects: They alter the main conductance subunit of the channel itself, such as *paralytic (para)* or *Shaker (Sh)*; they affect other genes required for channel function, such as *temperature-induced paralytic E (tipE)*; or they affect the expression of the ion channel subunits, such as *maleless (mle^{nap-ts1})*. An important property of many of the classical ion channel mutations is the availability of temperature-sensitive (ts) conditional alleles. In most cases, the ts mutations have a modest phenotype at the permissive temperature; however, at the restrictive temperature a full-fledged phenotype is obtained. The conditional quality of the ts mutations is of value for studies of neural plasticity and function. For example, paralytic hypoactivity mutations that would otherwise be lethal at the embryonic stage are used to acutely suppress activity at later times of development for phenocritical analyses.

Elevated membrane excitability is obtained using loss of function of ion channels that carry outward currents that repolarize the membrane voltage, such as the K⁺ channel mutations *Sh* and *ether-a-gogo (eag)*, which are often used in combination (*eag Sh*). Hyperexcitability can also be obtained by increasing the dosage of Na⁺ channels, for example, by a duplication of the *para* gene. The most powerful bouts of acute hyperexcitability involve mutant combinations of ts “seizure” mutations, such as the *comatose Kum* double mutations *com^{ts}; CaP60A^{kumts}*. At the restrictive temperature these animals experience brief bouts of very high-frequency action potential firing, followed by paralysis (Hoeffer et al. 2003). In general, presynaptic membrane hyperexcitability leads to the

expansion of the synapse, including an increase in the number of synaptic boutons and nerve terminal branches, as well as increased neurotransmitter release (Budnik et al. 1990; Schuster et al. 1996b).

Reduced membrane excitability is often achieved by loss of function of channels carrying inward currents, most commonly the Na^+ channel *para*, or through loss of function of genes required for Na^+ channel function or expression, such as *tipE* or *mle^{nap-ts1}*. In contrast to the growth effects observed with hyperactivity, reduced neural activity impairs the proper refinement of synaptic connections at the NMJ (Jarecki and Keshishian 1995; White et al. 2001). Although hypoactive mutants have relatively normal NMJ growth, their individual synaptic boutons are significantly smaller (Lnenicka et al. 2003).

Expression of Ion Channels

The directed expression of modified ion channels to manipulate neural excitability represents the most promising direction for controlling neural activity, in both a constitutive and a conditional manner. The tools are expressed as upstream activating sequence (UAS) effectors, controlled by either conventional Gal4 drivers or by inducible methods, such as the steroid-activated GeneSwitch Gal4 drivers (Osterwalder et al. 2001; Roman et al. 2001). Three channels have been widely used to suppress membrane excitability: UAS-EKO, UAS-Kir2.1, and UAS-dORK. Each is a K^+ channel that shunts the membrane voltage toward the equilibrium potential of potassium (E_K), a voltage that is usually more negative than the resting voltage. Whereas the dORK channel introduces a constitutively open K^+ shunt, both the Kir2.1 and EKO channels have voltage dependence (Baines et al. 2001; Paradis et al. 2001; White et al. 2001; Nitabach et al. 2002). Kir2.1 is a modified GFP-tagged inward rectifier channel, and it is most effective near the resting membrane voltage (Baines et al. 2001; Paradis et al. 2001). In contrast, EKO is an extensively modified variant of the fast activating Shaker K^+ channel, in which the fast inactivation function has been disabled. Although EKO has a constitutive K^+ conductance at the resting voltage, it rapidly activates with depolarization, intercepting the membrane voltage to drive it back toward E_K when the cell initiates an action potential. The construct also has a GFP tag, to help monitor its localization. As EKO is based on a native K^+ channel (Shaker), it also localizes to membrane sites in which the Dlg adapter protein is found, such as the subsynaptic reticulum of the NMJ (White et al. 2001).

Several effective tools have also been introduced for acutely elevating membrane excitability. For example, both the *eag* and *Sh* proteins can be suppressed by dominant negative constructs (Broughton et al. 2004; Mosca et al. 2005). SDN (Shaker Dominant Negative) is a truncated Shaker subunit that abolishes *Sh* function when expressed in neurons or muscles. It functions as a dominant negative construct, presumably by disrupting the proper assembly of the *Sh* channel during its biogenesis (Mosca et al. 2005). However, SDN will have an effect only in cells that express the Shaker channel. Moreover, the degree of increased excitation is limited to that achieved by a *Sh* mutant. As an alternative, a more general tool for elevating membrane excitability is NaChBac, a bacterially derived Na^+ channel that depolarizes any cell in which it is expressed (Nitabach et al. 2006).

Several recently introduced approaches allow the investigator to control neural excitability in a conditional fashion using either light or temperature. These involve the expression of either the light-activated protein Channelrhodopsin-2 (ChR2) or the temperature-activated channels TrpA1 and TRPM8 (Schroll et al. 2006; Ataman et al. 2008; Hamada et al. 2008; Peabody et al. 2009). These tools allow the investigator to elevate membrane excitability transiently in targeted neurons or in muscles, on illumination with the appropriate wavelength or by a temperature shift. A caveat of ChR2 is that *Drosophila* requires that retinal be provided to assemble a functional light-gated channel (Schroll et al. 2006; Ataman et al. 2008). An alternative and effective method for light activation involves P2X₂, a purine receptor that can be activated by using photolytically cleaved ligands. However, this approach requires that the caged ligand be provided to the animal before light activation (Lima and Miesenböck 2005).

Finally, the researcher has the option of using the directed expression of double-stranded UAS RNA interference (RNAi) constructs to knock down specific ion channels (Dietzl et al. 2007).

When combined with inducible Gal4 expression systems, this knockdown can be performed in a conditional manner in the cells of interest, bypassing early lethality that might result from constitutive channel knockdown. The increasing availability of these lines for a wide range of *Drosophila* ion channels provides a powerful alternative to the several methods described above. The use of transgenic RNAi lines is also a powerful tool for loss of function studies for any of the signaling cascades described later in the article.

Overall, a wide range of options is available to the researcher interested in manipulating neural excitability, but care must be exercised in selecting the appropriate tool for the task at hand. If the goal is to examine the effects of long-term changes in membrane excitability, any of the classic ion channel mutations are a good choice. However, these will usually affect neurons and/or muscles throughout the organism, complicating cell-specific analyses. Also, to have a direct effect, the gene being mutated must be expressed in the neuron or muscle of interest. For example, the para Na⁺ channel is not expressed in muscles, whereas the Shaker K⁺ channel is not expressed in all neurons nor at all developmental stages. A second consideration is to control for genetic background effects, because many synaptic phenotypes have limited penetrance or result in modest changes in NMJ size or function. One solution to this problem is to use genetic suppression as a control. Thus the triple mutant *eag Sh mle^{nap-ts1}* line is often used as a control for *eag Sh*, in which the reduced Na⁺ channel expression due to *mle^{nap-ts1}* suppresses the membrane hyperexcitability phenotype caused by *eag Sh* (Budnik et al. 1990).

The conditionally activated channels such as UAS-ChR2, UAS-TrpA1, or UAS-TRPM8 have tremendous power, and they are expected to become the methods of choice when rapid and acute excitation of specific neurons is desired. It is likely that corresponding methods will be introduced for acute suppression of activity, also based on optical methods, such as those that involve the use of halorhodopsins. An exciting prospect for the investigator will be animals in which distinct wavelengths of light could be used to acutely elevate and suppress membrane excitability in specific neurons.

A protocol is available for **Monitoring Membrane Excitability in *Drosophila* Expressing Modified Shaker Constructs** (Olsen and Keshishian 2012).

Synaptic Transmission

The next class of genetic tools target chemical synapses, with the goal of either elevating or decreasing synaptic transmission (Table 2). As we saw with methods used to manipulate membrane excitability (Table 1), these tools make use of both classic mutations as well as a variety of transgenic constructs.

Functional plasticity at the NMJ typically involves changes in either quantal content (the amount of neurotransmitter quanta released per action potential) or quantal size (the postsynaptic response due to the release of a single transmitter quantum). There are numerous manipulations that affect

TABLE 2. Methods to alter synaptic transmission

Effect	Tool (gene symbol)	Comment	Commonly used alleles (allele type: stock #)	Reference
Decreased presynaptic release	UAS-shi ^{ts1}	Temperature-sensitive Dynamin mutation		Kitamoto (2001)
Decreased postsynaptic sensitivity	UAS-TNT	Tetanus toxin light chain		Sweeney et al. (1995)
	<i>DGluRIIA</i>	Mutations in postsynaptic glutamate receptor subunit	<i>DGluRIIA^{AD9}</i> ; <i>DGluRIIA^{SP16}</i>	Petersen et al. (1997)
	UAS-DGluRIIA M614R	Channel dead glutamate receptor subunit		Diantonio et al. (1999)
	UAS-DGluRIIB	Wild-type glutamate receptor subunit		Diantonio et al. (1999)
Increased postsynaptic sensitivity	UAS-DGluRIIA subunit	Wild-type glutamate receptor		Petersen et al. (1997)

either parameter, as will be discussed in later sections. However, here we focus on genetic tools that affect either the exocytosis/endocytosis machinery of the synaptic terminal or neurotransmitter receptors in muscle in a direct, specific, and well-controlled fashion.

Tools Targeting Presynaptic Function

The first widely used genetic tool for controlling synaptic transmission was the *ts* allele of the dynamin gene *Shibire* (*Shi^{ts}*). At the restrictive temperature, *Shi^{ts}* animals rapidly collapse and remain paralyzed until cooled to the permissive temperature (Grigliatti et al. 1973). For acute blockade of chemical synaptic transmission throughout the nervous system, this is a powerful tool, but it has been supplanted by the introduction of a UAS *Shi^{ts}* effector, permitting dominant, cell-specific, and conditional silencing of synaptic release on shifting to the restrictive temperature (Waddell et al. 2000; Kitamoto 2001). For example, the rapid activation and conditional nature of the construct has allowed researchers to examine with impressive detail when cell-specific synaptic activity is required during the establishment of long-term memories (Waddell et al. 2000). However, because dynamin function is required for membrane turnover and neuronal growth, suppressing function for prolonged periods during development is problematic, especially when the structural plasticity of synapses is being studied.

A direct approach for silencing synaptic transmission is to disrupt the SNARE (soluble NSF attachment protein receptor) complex involved in vesicle exocytosis. The method of choice uses tetanus toxin light chain (TetTxLC), expressed in a cell-specific manner under the control of the UAS-TNT effector. TetTxLC is a protease that degrades the vSNARE synaptobrevin, resulting in a complete blockade of evoked synaptic transmission (Sweeney et al. 1995). The silencing is essentially irreversible; therefore, to restrict expression to specific postembryonic stages of development requires the use of inducible Gal4 expression systems, such as GeneSwitch or the Gal80-based TARGET system. Even so, the researcher should be aware that all existing inducible systems have a low level of leakiness. Given the very potent nature of TetTxLC, there may be an effect on evoked transmission even in the absence of induction. Expression of inactive forms of the tetanus toxin light chain can be used as a control.

Tools Targeting Postsynaptic Function

The suppression and elevation of the postsynaptic response at the NMJ is most directly achieved by altering glutamate receptor function. *Drosophila* body-wall muscles express two tetrameric DGluRII receptor complexes, which differ by whether they include a DGluRIIA or DGluRIIB subunit. Quantal size increases in proportion to the ratio of DGluRIIA to DGluRIIB. Thus, an effective method to suppress synaptic drive at the NMJ is to use either the *DGluRIIA* mutation or alternatively to drive expression of the dominant negative transgene UAS *DGluRIIA*-DN. Quantal size can also be decreased by driving expression of DGluRIIB, once again reducing the ratio of DGluRIIA/DGluRIIB. It was through the use of these lines that the first evidence for a retrograde homeostatic regulation of presynaptic neurotransmitter release at the NMJ was discovered (Petersen et al. 1997; Diantonio et al. 1999). Quantal size is effectively increased by driving expression of DGluRIIA.

Signal Transduction and Second Messengers

Cyclic AMP

The cyclic AMP (cAMP) cascade is a potent regulator of both functional and structural synaptic plasticity at the NMJ. The genetic tools most widely used to manipulate the levels of intracellular cAMP at the synapse are the adenylyl cyclase *rutabaga* (*rut*) and the cAMP phosphodiesterase *dunce* (*dnc*). Levels of cAMP can be increased by using *dnc* mutations or expression of UAS-*rut*, whereas low levels of cAMP can be achieved by using *rut* mutations or UAS-*dnc* (Table 3). Loss-of-function mutations of either *rut* or *dnc* disrupt short-term plasticity, as measured by paired pulse facilitation (PPF) or posttetanic potentiation (PTP) (Zhong and Wu 1991). However, the increased levels of

TABLE 3. Methods to manipulate signal transduction and second messenger systems

Effect	Tool (gene symbol)	Comment	Commonly used alleles (allele type: stock #)	Reference(s)
Increased cAMP	<i>dunce (dnc)</i>	Phosphodiesterase mutation	<i>dnc^{M14}</i> (null: 4714); <i>dnc^{M11}</i> (null); <i>dnc¹</i> (hypo: 6020) (9405; 9406)	Zhong and Wu (1991), Zhong et al. (1992) Zhong and Wu (2004)
	UAS-rut	Used for overexpression of wild-type adenylyl cyclase		
Decreased cAMP	<i>rutabaga (rut)</i>	Adenylyl cyclase mutation	<i>rut¹</i> (9404)	Zhong and Wu (1991), Zhong et al. (1992)
	UAS-dnc	Used for overexpression of wild-type phosphodiesterase		Cheung et al. (1999), Sanyal et al. (2002)
Decreased Ca ²⁺	<i>cacophony (cac)</i>	Presynaptic CaV2.1 Ca ²⁺ channel	<i>cac^{L13}</i> ; <i>cac^{HC129}</i> ; <i>cac^S</i> ; <i>cac^{NT27}</i>	Kawasaki et al. (2000), Rieckhof et al. (2003), Frank et al. (2006)
	UAS-PV (parvalbumin)	Intracellular Ca ²⁺ buffer		Harrisingh et al. (2007)
Increased CaMKII activity	UAS-CaMKII ^{T287D}	Constitutively active CaMKII		Jin et al. (1998), Koh et al. (1999)
Decreased CaMKII activity	UAS-Ala UAS-CaMKIINtide	CaMKII inhibitory peptide CaMKII inhibitory peptide		Jin et al. (1998), Koh et al. (1999), Haghighi et al. (2003)
Increased PKA activity	UAS-PKAact	Constitutively active PKA catalytic subunit		Davis et al. (1998)
Decreased PKA activity	UAS-PKAinh	PKA regulatory subunit with mutation in cAMP binding site		Davis et al. (1998)
Increased PAR protein activity	UAS-PKM UAS-par-1	Persistently active form of aPKC		Ruiz-Canada et al. (2004), Zhang et al. (2007)
Decreased PAR protein activity	<i>aPKC</i>	Atypical protein kinase C	<i>aPKC^{k06403}</i> (10622); <i>aPKC^{Exc55}</i> ; <i>aPKC^{EX48}</i>	Ruiz-Canada et al. (2004)
	UAS-DN-PKM	Dominant negative kinase dead aPKC		Ruiz-Canada et al. (2004)
	<i>par-6</i>	Component of PAR-aPKC complex	<i>par-6^{D226}</i>	Ruiz-Canada et al. (2004)
	<i>bazooka (baz, par-3)</i>	Component of PAR-aPKC complex	<i>baz⁴</i> (3295)	Ruiz-Canada et al. (2004)
	<i>par-1</i>	Serine threonine kinase	<i>par-1^{k06323}</i> (10615); <i>par-1^{W3}</i> ; <i>par-1^{D-16}</i>	Zhang et al. (2007)

presynaptic cAMP in *dnc* mutant- or UAS-rut-expressing animals enhance evoked transmitter release and lead to an expansion of the synaptic arbor (Zhong and Wu 1991, 2004; Zhong et al. 1992). Furthermore, the activity- and environment-dependent expansion of the synaptic arbor is tightly regulated by presynaptic cAMP levels (Zhong and Wu 2004).

Ca²⁺

Calcium signaling is required for the proper function of evoked transmitter release and is an important player in different forms of synaptic plasticity. A variety of alleles of the presynaptic Ca_v2.1 Ca²⁺ channel *cacophony (cac)* have been used to impair calcium influx at the NMJ (Table 3). Null mutations of *cac* are lethal but heteroallelic combinations and a variety of hypomorphic alleles can circumvent this problem. Reduced *cac* function causes a decrease in the size of the synaptic arbor. This effect is likely not a direct result of reduced synaptic transmission, because other mutants that inhibit transmitter release do not have synaptic growth phenotypes (Rieckhof et al. 2003). Normal *cac* function is also required for the homeostatic up-regulation of transmitter release that is induced when postsynaptic GluR function is impaired (Frank et al. 2006). Another tool that could be used to reduce the levels of intracellular Ca²⁺ at the synapse is the transgenic expression of the intracellular Ca²⁺ buffer protein parvalbumin (UAS-PV) (Harrisingh et al. 2007) (Table 3). This tool, to our knowledge, has not yet been used at the NMJ.

CaMKII

The role of CaMKII at the NMJ has been investigated using transgenic constructs that inhibit its activity (UAS-Ala and UAS-CaMKIINtide) or produce constitutive activation of the enzyme

(UAS-CaMKII^{T287D}) (Table 3). A role for CaMKII in regulating the synaptic localization of the PDZ protein Discs large (Dlg) and bouton morphology was shown by altering CaMKII activity both pre- and postsynaptically (Koh et al. 1999). Postsynaptic CaMKII has been shown to negatively regulate the retrograde signaling that controls the homeostasis of transmitter release (Haghighi et al. 2003).

Protein Kinase A

The role of protein kinase A (PKA) in postsynaptic muscles has been investigated using transgenic tools that increase (UAS-PKAact) or inhibit (UAS-PKAinh) PKA activity (Table 3). These studies have shown that postsynaptic PKA regulates quantal size in a GluRIIA-dependent fashion. Increased PKA activity decreases quantal size, whereas inhibition of PKA leads to an increase in quantal size. These effects are eliminated in the absence of the GluRIIA subunit (Davis et al. 1998).

Proline- and Acidic Amino Acid-Rich Proteins

Experiments have shown roles for the baz/par-6/aPKC complex as well as the par-1 serine-threonine kinase at the NMJ. The tools available to study the proline- and acidic amino acid-rich (PAR) proteins include loss of function mutations (*par-6*, *baz*, *aPKC*, *par-1*) and transgenic lines that express dominant negative (UAS-DN-PKM), persistently active (UAS-PKM), or wild-type forms of these proteins (UAS-*par-1*) (Table 3). Because null mutations in the baz/par-6/aPKC complex are embryonic lethal, hypomorphic alleles of *aPKC* and heterozygous mutation of *baz* and *par-6* have been used (Ruiz-Canada et al. 2004). Both loss- and gain-of-function manipulations of the baz/par-6/aPKC complex impair synaptic growth and disrupt postsynaptic GluR distribution (Ruiz-Canada et al. 2004). The regulation of the cytoskeleton by the baz/par-6/aPKC complex is important for the normal growth of the synapse but the specific role of baz/par-6/aPKC during activity-dependent plasticity is not well understood. Loss of function and overexpression studies have shown that *par-1* negatively regulates synapse formation and synaptic strength via its ability to phosphorylate Dlg (Zhang et al. 2007).

CAMs and Growth Factors

CAMs

The growth and plasticity of the larval NMJ is directly influenced by both CAMs expressed at the synaptic contacts and by at least two transsynaptic growth factor signaling systems.

An important insight into the regulation of NMJ growth emerged from the discovery that the immunoglobulin cell adhesion molecule Fasciclin 2 (Fas2) is abundant at the NMJ, and that its expression scales inversely with membrane excitability. Thus, in hyperactive mutant backgrounds, in which the size of the NMJ increases substantially, the level of synaptic Fas2 decreases significantly (Schuster et al. 1996b). Alleles of Fas2 that reduce expression to the levels found in hyperactive mutants also have significantly expanded NMJs (Schuster et al. 1996b). This observation shows that the reduction of synaptic Fas2 observed with elevated neuromuscular activity is sufficient to account for most of the resulting NMJ overgrowth. A well-characterized allelic series of Fas2 mutants is available, with phenotypes ranging from mild hypomorphs to complete nulls (Table 4) (Grenningloh et al. 1991).

TABLE 4. Methods to alter cell adhesion

Effect	Tool (gene symbol)	Comment	Commonly used alleles (allele type: stock #)	References
Increased Fas2 levels	UAS-Fas2			Schuster et al. 1996(a,b), Davis et al. (1997)
Decreased Fas2 levels	<i>Fasciclin 2</i> (<i>Fas2</i>)	Series of mutant Fas2 alleles that express different levels of Fas2	<i>Fas2</i> ^{EB112} (null) > <i>Fas2</i> ^{e76} (hypo) > <i>Fas2</i> ^{e86} (hypo) > <i>Fas2</i> ^{e93} (precise P-element excision)	Grenningloh et al. (1991), Schuster et al. (1996a,b), Davis et al. (1997)

Fas2 levels can also be elevated by expression of the UAS-Fas2 effector in either motor neurons or muscles. Although overexpression of Fas2 in muscle generally suppresses NMJ growth, it should be noted that a balanced overexpression of Fas2 on both sides of the synapse results, surprisingly, in the expansion of the NMJ (Ashley et al. 2005).

Growth Factors

The transsynaptic signaling systems that affect the size of the *Drosophila* NMJ include the TGF- β growth factor Glass bottom boat (Gbb), a member of the BMP family, and the Wnt growth factor Wingless (Wg). Both growth factors have been shown to be essential for normal NMJ development (Aberle et al. 2002; Marques et al. 2002; Packard et al. 2002; McCabe et al. 2003). In addition to mutant alleles of the Gbb ligand and its type II receptor Wishful Thinking (Wit), the TGF- β signaling cascade can be manipulated using a variety of transgenic lines (Table 5). It should be noted that *wit* and *gbb* mutations are generally not homozygous, but are used in heteroallelic combinations, such as *gbb*¹/*gbb*² and *wit*^{A12}/*wit*^{B11}.

For gain of function using directed cell-specific expression, the most common approach has been to drive the constitutively active BMP receptor in motor neurons (the BMP type I co-receptor UAS-Act-Tkv). It is also possible to overexpress the ligand in muscle, using the UAS-Gbb effector. For loss of function studies in specific cells, there are dominant-negative transgenes that suppress downstream signaling components, such as the transcription factor Mad (UAS-Mad₁), as well as expressible forms of inhibitory proteins that suppress TGF- β signaling (UAS-Dad).

Wg signaling can be suppressed using both classic mutations targeting Wg or its receptor frizzled, as well as a DN form of the frizzled receptor. To bypass early lethality caused by Wg loss of function, hypomorphic or ts alleles are used. Retrograde signaling from synapse to cell body can be disrupted using dominant negative transgenes that target the p150 dynactin component of the dynein-dynactin complex (UAS-GluedDN or UAS- Δ GI) (McCabe et al. 2003; Mathew et al. 2005). This approach has been extensively used to show the need for retrograde growth factor transport to the cell body, but

TABLE 5. Methods to alter growth factor signaling

Effect	Tool (gene symbol)	Comment	Commonly used alleles (allele type: stock #)	Reference(s)
Increased BMP signaling	<i>Daughters against dpp</i> (<i>Dad</i>)	Loss of function of inhibitory Smad	<i>Dad</i> ²⁷¹⁻⁶⁸ ; <i>Dad</i> ^{j1E4}	Sweeney and Davis (2002), McCabe et al. (2004)
	UAS-Gbb	Used for overexpression of BMP ligand		McCabe et al. (2004)
	UAS-Act-Tkv	Constitutively active type I BMP receptor		McCabe et al. (2004), Collins et al. (2006), Wang et al. (2007), O'Connor-Giles et al. (2008)
Decreased BMP signaling	<i>glass bottom boat</i> (<i>gbb</i>)	Secreted BMP ligand	<i>gbb1</i> (null); <i>gbb2</i> (null); <i>gbb3</i> (hypo); <i>gbb4</i> (hypo)	McCabe et al. (2003)
	<i>wishful thinking</i> (<i>wit</i>)	Type II BMP receptor	<i>wit</i> ^{A12} (5173); <i>wit</i> ^{B11} (5174); <i>wit</i> ^{HA1} (hypo); <i>wit</i> ^{HA2} ; <i>wit</i> ^{HA3} ; <i>wit</i> ^{HA4} ; <i>wit</i> ^{HA5} ; <i>wit</i> ^{S126215}	Aberle et al. (2002), Marques et al. (2002)
	UAS-Dad	Used for overexpression of an inhibitory Smad		McCabe et al. (2004), Goold and Davis (2007)
	UAS-Mad1	Mutant Smad that lacks DNA-binding activity		Takaesu et al. (2005)
Decreased Wg signaling	<i>wingless</i> (<i>wg</i>)	Secreted growth factor	<i>wg</i> ^{I-12} (null: 7000); <i>wg</i> ^I (hypo: 2978)	Packard et al. (2002), Ataman et al. (2008)
	<i>frizzled 2</i> (<i>fz2</i>)	wg receptor	<i>fz2</i> ^{C1}	Mathew et al. (2005)
	UAS-DFz2DN	Dominant negative frizzled		Packard et al. (2002), Mathew et al. (2005)
Disrupted retro-grade transport	UAS-GluedDN/ UAS- Δ GI	DN disruption of dynein/dynactin complex		McCabe et al. (2003), Mathew et al. (2005)

must be applied with caution, as it undoubtedly disrupts multiple retrograde signals, as well as potentially affecting orthograde transport as well.

Transcription Factors

Activator Protein 1 (AP-1: Fos/Jun)

The transcription factor AP1, a heterodimer of Fos and Jun, plays a key role in synaptic plasticity at the NMJ. The genetic tools that have been used to manipulate AP-1 function at the NMJ include transgenic lines that allow for expression of wild-type (UAS-Fos, UAS-Jun) or dominant negative (UAS-fbz, UAS-jbz) forms of these transcription factors (Table 6). Overexpression of both Fos and Jun (referred to as UAS-AP1) in neurons increases both the size of the synapse and the strength of transmission; however, expression of either Fos or Jun alone does not produce these effects. Conversely, the expression of either dominant negative construct in neurons leads to a smaller synapse with reduced synaptic strength (Sanyal et al. 2002). Directed expression of AP-1 or dominant negative molecules is an effective means of influencing synaptic plasticity, but little is known about the *in vivo* mechanisms that regulate the activity of these important transcription factors at the NMJ.

cAMP Response Element Binding Protein

Transgenic cAMP response element binding (CREB) protein activator (Creb2a) and CREB blocker (Creb2b) lines have been used to alter CREB activity at the NMJ (Table 6). These transgenic tools are available in either heat shock-driven forms or as UAS lines. Activation or inhibition of CREB using the heat shock-driven lines in an otherwise wild-type background has little effect on the structure or function of the synapse (Davis et al. 1996). Inhibition of CREB in a *dnc* mutant- or AP-1-overexpressing background prevents the increase in synaptic strength but does block the growth of the synapse (Davis et al. 1996; Sanyal et al. 2002). Conversely, CREB activation can increase synaptic strength when the synapse is structurally expanded via reduction in Fas2 levels (Davis et al. 1996).

Environmental Manipulations

Elevated Rearing Temperature

Chronic rearing of wild-type larvae at 29°C or 30°C leads to increased larval locomotion, growth of the synapse, and increased synaptic strength (Sigrist et al. 2003; Zhong and Wu 2004) (Table 7). The temperature-dependent growth and potentiation of the synapse phenocopies the effects of activity-dependent growth in many ways, with reduced levels of Fas2 and increases in postsynaptic GluRs (Schuster et al. 1996b; Sigrist et al. 2000, 2003). The temperature-dependent expansion and strengthening of the synapse is blocked when neural activity is silenced with mutations that affect Na⁺ channels or when cAMP levels are reduced (Sigrist et al. 2003; Zhong and Wu 2004). Rearing temperature

TABLE 6. Methods to alter transcription factor signaling

Effect	Tool (gene symbol)	Comment	Commonly used alleles (allele type: stock #)	Reference(s)
Increased AP-1 activity	UAS-AP1	UAS-fos and UAS-jun together		Sanyal et al. (2002)
Decreased AP-1 activity	UAS-Fbz	Dominant inhibitory form of Fos	(7214; 7215)	Sanyal et al. (2002)
	UAS-Jbz	Dominant inhibitory form of Jun	(7217; 7218)	Sanyal et al. (2002)
Increased CREB activity	Creb2a	Transgenic CREB activator; heat shock or UAS driven		Davis et al. (1996)
Decreased CREB activity	Creb2b	Transgenic CREB repressor; heat shock or UAS driven		Davis et al. (1996), Sanyal et al. (2002)

TABLE 7. Environmental conditions that affect synaptic plasticity

Environmental condition	Effect	References
Elevated rearing temperature	Increased neural excitability and increased locomotor activity	Sigrist et al. (2003), Zhong and Wu (2004), Schuster (2006), Steinert et al. (2006)
Transfer of larvae to moist food-free agar plate	Acute increases in locomotor activity	Sigrist et al. (2003), Schuster (2006), Steinert et al. (2006)

also interacts with hyperexcitability, as shown by the enhanced growth of *Sh* synapses at 25°C when compared with *Sh* animals at 18°C or wild-type animals raised at 25°C (Zhong and Wu 2004). Although increased rearing temperature is a potent inducer of plasticity phenomena at the synapse, elevated temperature is likely to have a broad range of effects on larvae and the molecular mechanisms that underlie temperature-dependent events at the synapse are not well understood. Thus, elevated rearing temperature is often best used as a complementary approach, along with genetic approaches, to manipulate synaptic plasticity at the NMJ.

Acute Increases in Locomotor Activity

The increased locomotor activity of animals raised at 30°C is likely a key component of the temperature-dependent growth and strengthening of the synapse. When larvae are transferred from food slurry to moist, food-free agar plates, they show greatly increased locomotor activity with significant individual variability (Sigrist et al. 2003; Steinert et al. 2006). Larvae that maintain a high level of locomotor activity under these conditions for up to 2 h have enhanced synaptic transmission. This experience-dependent potentiation of the synapse has distinct phases. No changes in synaptic transmission are observed until after a period of ~40 min of fast crawling, at which point increased quantal sizes are observed. After ~90 min of fast crawling, quantal sizes have returned to baseline whereas evoked responses remain elevated, suggesting increased transmitter release (Steinert et al. 2006). Larvae raised on agar plates for 12–18 h have only slightly larger synapses than their vial-raised counterparts (Sigrist et al. 2003). Thus, this method can effectively potentiate synaptic transmission without the potentially pleiotropic effects of elevated temperature. This method is, however, more labor intensive, requiring monitoring the locomotor activity of individual larvae for up to 2 h, and it does not produce detectable synaptic growth.

CONCLUSIONS AND PROSPECTS

At present, the researcher has an impressive array of well-characterized genetic methods to effectively control the function and plasticity of the NMJ. In many cases, the manipulations can be performed both acutely or over longer developmental periods. However, experimental control of the NMJ is only part of the problem facing the investigator: To complement these genetic tools, we need transgenes that report either membrane electrical excitability, levels of synaptic transmission, or the activity of molecules in the downstream signaling cascades. The optical clarity of the larval NMJ suggests that the new tools will likely involve transgenic fluorescent reporters. We therefore look forward to the development of effective, expressible constructs for reporting synaptic function at the single-cell level to complement the impressive genetic tools described in this article.

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