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Michael R. Kreutz
Carlo Sala *Editors*

Synaptic Plasticity

Dynamics, Development and Disease

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Dynamics, Development and Disease

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Preface

Synapses are sites of a specialized cell-cell contact between neuronal cells and represent the major structure involved in chemical neurotransmission in the nervous system. It is widely believed that glutamatergic synapses are important loci for modifying the functional properties of CNS networks, possibly providing the basis for phenomena collectively referred to as "learning and memory". Given their importance, it is not surprising that enormous efforts are being made to understand the formation, structure, function and regulation of glutamatergic synapses. To date, significant progress has been made in our understanding of their ultrastructure, molecular composition, and physiological properties, as well as the principles of how these synapses are initially assembled and "plastically" modified.

The term synaptic plasticity covers many different aspects of use-dependent synaptic modifications and is commonly used in a broader sense describing aspects of synaptic signal transmission as well as structural alterations in the molecular make-up of the synapse related to synaptic signaling events. The capacity of the nervous system to modify its own organization is remarkable; plastic changes can occur as a consequence of many events, including the normal development and maturation of the organism, the acquisition of new skills ('learning') and after brain damage. This response specificity is not always intrinsic to neurons; rather, it can develop as a consequence of interaction with the environment and thus involves information processing and memory storage.

Disturbances of synaptic and neuronal plasticity ultimately result in diseases affecting cognitive functions and it has become increasingly clear during recent years that synaptopathies – disruptions in synaptic structure and function – in many cases are the major determinant of many brain disorders. These diseases and related animal models include Alzheimer's, prion diseases, Down's syndrome, Huntington's or Parkinson's diseases as well as schizophrenia and autism spectrum disorders that almost ultimately result in disturbed plasticity processes. In accord, it is becoming increasingly clear that studies of synaptic plasticity and memory formation are critical for understanding the initial stages of these disorders. At an early stage in most of these diseases no structural damage exists but rather an impairment

of synaptic function. Interventions aimed to preserve or even restore synaptic function will probably delay the onset or might even provide a cure for such disorders. A general strategy to treat these types of diseases can therefore be plausibly based on knowledge, how to maintain the plastic properties of neurons in the adult and aging brain.

Crucial technological advancements have recently accelerated progress in our understanding of synaptic processes, five of them are listed here: (1) Live-cell imaging has provided essential constraints regarding the kinetics (rate constants) and spatial organization of signaling pathways, (2) the development of next generation sequencing allows individual transcriptome profiling, (3) quantitative synaptic proteome profiling of normal and disease brain has established protein interaction networks databases for signaling pathway analysis, (4) optogenetic tools are available to study neuronal function in vivo, (5) and finally, progress in computer simulation and neuroinformatics has been crucial for improving the temporal and spatial scale of synaptic plasticity models, because simulating large spatial structures for long durations with high resolution requires trillions of calculations.

In higher mammals the majority of brain glutamatergic excitatory synapses is found on the principal neuron of the cortex and hippocampus, the pyramidal cell. Pyramidal cells are characterized by a complex dendritic cytoarchitecture harboring approximately 10^4–10^5 synaptic contact sites with other neurons. It is estimated less than 1% of all synaptic contacts of cortical pyramids is concerned with the wiring to subcortical areas, implying that the predominant synapse of the mammalian telencephalon is concerned with input from a closely related neuron in terms of cell lineage, morphology and functional characteristics. This fact is mainly emphasized because our knowledge about synaptic plasticity of this type of synaptic input is still very sparse.

We have focused our attention in this book mostly on postsynaptic molecular mechanisms because a lot more knowledge exists with respect to this side of the synapse especially with respect mechanisms underlining synaptic dysfunction and synaptopathies. The purpose of this book is to summarize this knowledge and to provide insights into the most recent developments in the field including the major technological advancements in recent years. The first part of the book concerns structural plasticity at the pre- and postsynaptic scaffold, the molecular dynamics of the synapse and synapto-dendritic plasticity in development and learning. In the second part the book includes chapters on synapse-to-nucleus communication and synaptic dysfunction and synaptopathies. Finally, we want to particularly thank the authors for their contribution. We are very happy that we could convince leading experts in this field to provide a detailed account of the most exciting recent developments.

Michael R. Kreutz
Carlo Sala

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Part I
Molecular Organization of the Pre- and Postsynaptic Scaffold

Chapter 1
Glutamate Receptors in Synaptic Assembly and Plasticity: Case Studies on Fly NMJs

Ulrich Thomas and Stephan J. Sigrist

Abstract The molecular and cellular mechanisms that control the composition and functionality of ionotropic glutamate receptors may be considered as most important "set screws" for adjusting excitatory transmission in the course of developmental and experience-dependent changes within neural networks. The *Drosophila* larval neuromuscular junction has emerged as one important invertebrate model system to study the formation, maintenance, and plasticity-related remodeling of glutamatergic synapses in vivo. By exploiting the unique genetic accessibility of this organism combined with diverse tools for manipulation and analysis including electrophysiology and state of the art imaging, considerable progress has been made to characterize the role of glutamate receptors during the orchestration of junctional development, synaptic activity, and synaptogenesis. Following an introduction to basic features of this model system, we will mainly focus on conceptually important findings such as the selective impact of glutamate receptor subtypes on the formation of new synapses, the coordination of presynaptic maturation and receptor subtype composition, the role of nonvesicularly released glutamate on the synaptic localization of receptors, or the homeostatic feedback of receptor functionality on presynaptic transmitter release.

Keywords BMP signaling • Development • Glutamate receptors • Neuromuscular junction • Wnt signaling

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1.1 Introduction

Excitatory synapses categorized as "glutamatergic" are no less heterogeneous than the many types of neurons that use glutamate in synaptic transmission. In-depth studies on a variety of glutamatergic synapses from different species are thus required to elucidate both general as well as subcategory-defining principles of synapse assembly, function, and plasticity. L-glutamate is the primary transmitter not only at the vast majority of excitatory synapses in the vertebrate CNS but also at arthropod neuromuscular junctions (NMJs). Concerning the latter, illuminative physiological, pharmacological, and ultrastructural analyses have been performed on NMJs from various crustacean and insect model organisms. Undeniably, *Drosophila* has reached a pole position in this respect, mainly because of its unbeatable genetic accessibility. Preceded by pilot studies from Jan and Jan (1976a, b), thorough electrophysiological and morphological studies on NMJs from both wild-type and excitability mutants paved the road for intensive research on this model system. Various aspects of synapse biology such as pre- and postsynaptic assembly, activity-dependent and homeostatic plasticity, or disease-related synaptic dysfunctions are addressed at the larval NMJ. It in fact turns out that many of the underlying molecular mechanisms can be assorted to well-conserved proteins.

In its main part, this chapter is centered around ionotropic glutamate receptors (GluRs) at NMJs. As for studies on neurotransmitter receptors in other systems, findings on junctional GluRs are multifaceted and therefore allow us to bring up a number of current issues in cellular neurobiology. We start out introducing the system, including a brief overview on various forms of plasticity that regulate its structural and functional properties.

1.2 Basic Morphological and Functional Features of Glutamatergic Nerve Terminals at Larval NMJs

The pattern of motoneuron innervation on *Drosophila* abdominal body wall muscles is well-defined and rather stereotypic across segments (Keshishian et al. 1996). A surprising level of complexity is added by the fact that each muscle is innervated by up to four neurons (Fig. 1.1a), which differ by their nerve terminal morphology and transmissive properties. While a few motoneurons are primarily specializing in the release of neuropeptides or catecholamines on subsets of muscles, all muscles receive input from axon terminals of glutamatergic motoneurons (Jia et al. 1993). Such terminals form arrays of boutons, and each bouton is equipped with several, quite evenly spaced active zones (AZs; Fig. 1.1b–c) (Meinertzhagen et al. 1998; Reiff et al. 2002; Dickman et al. 2006), i.e., presynaptic membrane specializations designated for synaptic vesicle release. AZs are commonly associated with protein-rich, electron-dense cytomatrices, which at *Drosophila* synapses typically occur as T-shaped dense bodies (T-Bars;

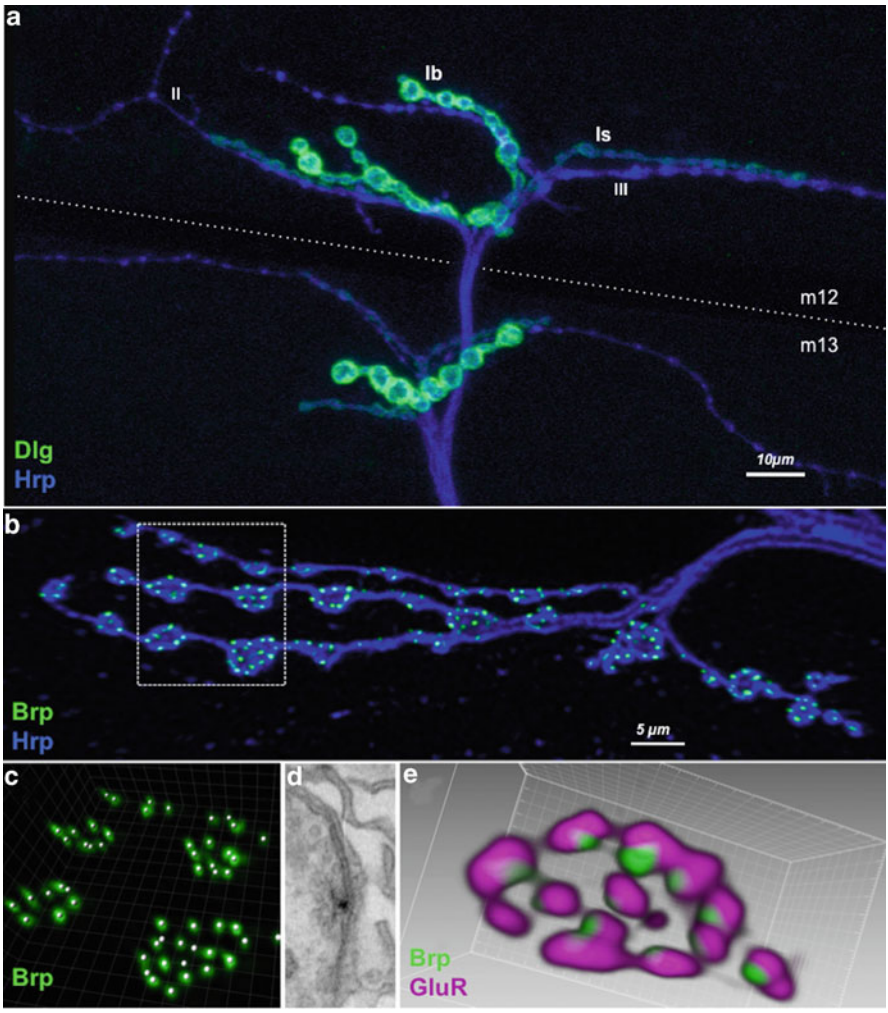

Fig. 1.1 Larval NMJs at various levels of organization. (**a**) Motor nerve terminals on muscle fibers 12 and 13 (*above and below the dotted line*). Type Ib and Is nerve terminals are primarily glutamatergic, whereas type II boutons release octopamine (and glutamate). Peptidergic type III terminals are restricted to muscle 12. A neuronal surface marker (Hrp) was used to visualize all nerve terminals. The scaffold protein Dlg is abundant around type Ib boutons and is also present at type Is boutons, yet at much lower levels. (**b**) Branches of type I nerve terminals on muscle 6, stained for the Hrp-epitope and for the presynaptic marker protein Brp. (**c**) 3D representation of Brp-positive sites corresponding to the delineated region in B. Most Brp-spots correspond to T-bar-shaped dense bodies. (**d**) Electron micrograph displaying a single synaptic contact including an AZ with a T-Bar. Note the clustering of SVs around the T-Bar. (**e**) 3D reconstruction from a confocal stack of a type Ib bouton containing several synaptic contacts that were stained for Brp and the GluR subunit IID

Fig. 1.1d) (Zhai and Bellen 2004; Owald and Sigrist 2009). The presence of a T-Bar indeed indicates a ready for use release machinery and most, though not all AZs of larval NMJs, are decorated by a T-Bar (Atwood et al. 1993; Wichmann and Sigrist 2010).

Each AZ, together with an opposing postsynaptic receptor field (Fig. 1.1e), is referred to as a synaptic contact or simply as a synapse, and a few hundred of these are present at full-grown terminals (Please note, however, that in the literature, the term "synapse" is sometimes used to assign entire NMJs.) Mature wild-type boutons of type 1s motoneurons range in diameter from 1 to 3 μm, thereby harboring 7 AZs on average, whereas boutons of type 1b motoneurons typically are 1–7 μm in diameter and can harbor more than 40 AZs (Johansen et al. 1989; Atwood et al. 1993). Next to this morphological diversification, type 1s and 1b terminals, which are found side by side on many muscles, show meaningful differences regarding synaptic output. The threshold to elicit spiking activity appears higher in type 1s than in type 1b motoneurons, and the former also display a marked delay between stimulation and the appearance of first spikes (Kurdyak et al. 1994; Choi et al. 2004; Schaefer et al. 2010). At the NMJ, however, type 1s terminals evoke stronger postsynaptic responses than type 1b terminals, in agreement with the findings that (1) their AZs display a higher probability to release synaptic vesicles (SVs) upon arrival of an action potential (Atwood et al. 1997) and (2) their SVs are of much greater volume, giving rise to larger quantal size, i.e., postsynaptic current per fusion event (Karunanithi et al. 2002). Type 1b terminals in turn easily facilitate upon repeated stimulation (Kurdyak et al. 1994; Lnenicka and Keshishian 2000), reflecting short-term plasticity in a way typical for synapses with low release probability (Thomson 2000). By analogy to the different types of motoneurons innervating crayfish muscles, type 1b and 1s motor nerve terminals have been referred to as tonic or phasic inputs, respectively (Kurdyak et al. 1994). This dichotomy likely contributes to the versatility of locomotor behaviors (Schaefer et al. 2010). Most remarkably, by using a truly physiological behavioral paradigm, namely, hunger-induced increase in larval locomotion as a means to search of food, Koon et al. (2011) could recently demonstrate cross talk between different motoneuron populations concerning their structural and functional plasticity. Specifically, they showed that starvation-triggered locomotion depends on octopamine release from type II motoneurons, which in turn promotes outgrowth of type I glutamatergic motor nerve terminals, thus revealing a case of metaplasticity at larval NMJs (Sigrist and Andlauer 2011).

1.2.1 Morphogen-Dependent Control of NMJ Development and Plasticity

The formation of NMJs first becomes manifest when filopodia from a motoneuronal growth cone intermingle with filopodia (myopodia) protruding from the respective

target muscle (Ritzenthaler et al. 2000; Ritzenthaler and Chiba 2003). This initial event is followed by the transition of the muscle-attached, flattened axonal growth cone into the bouton-forming motor nerve terminal (Yoshihara et al. 1997). Size matching of the terminals according to the size of their respective target muscles becomes evident shortly after NMJ formation (Nakayama et al. 2006). Challenged by rapid muscle growth during the larval phase, NMJs expand by both elongation and branching of their motor nerve terminals, thereby continuously forming new boutons (Gorczyca et al. 1993; Schuster et al. 1996a; Zito et al. 1999). Concurrently, new synaptic contacts are implemented on both newborn as well as preexisting boutons (Rasse et al. 2005), thus resulting in synaptic strengthening according to muscle size. This process involves retrograde as well as anterograde and autocrine signaling by morphogens, and most notably, it is subject to plasticity. In the following, we briefly elaborate on these aspects.

1.2.1.1 BMP Signaling

A canonical bone morphogenetic protein (BMP) pathway is employed for retrograde control of NMJ growth and function (Marqués and Zhang, 2006). The BMP-7 homolog Glass bottom boat (Gbb) is secreted by muscles to activate presynaptic BMP/TGF-β receptors which then phosphorylate the R-SMAD protein Mothers against decapentaplegic (MAD) (McCabe et al. 2003). Phospho-MAD, upon association with its co-SMAD Medea, shuttles to the nucleus to exert its role as a transcription factor. Recently, expression of the Rac GTPase exchange factor Trio, a regulator of the nerve terminal cytoskeleton, was found to be strongly upregulated by phospho-MAD (Ball et al. 2010). This finding thus provides a mechanistic link between Gbb-triggered synapse-to-nucleus signaling and its growth promoting effect on NMJs (Fuentes-Medel and Budnik 2010). In fact, mutations that block the Gbb pathway cause a substantial reduction in the number of boutons (Aberle et al. 2002; Marqués et al. 2002; McCabe et al. 2003, 2004). Consequently, several factors that attenuate Gbb signaling were identified based on mutant NMJ overgrowth phenotypes. Consistent with the widely observed endocytic control of TGF-β signaling (Chen 2009), most of them have been implicated in the local trafficking of the TGF-β receptors, including their passage through signaling endosomes (Sweeney and Davis 2002; Wang et al. 2007; O'Connor-Giles et al. 2008; Rodal et al. 2008; Kim et al. 2010; see also Bayat et al. 2011 for a review on disease-related aspects of BMP signaling at NMJs). Postsynaptic downregulation of the Gbb signaling pathway involves CIP4, a Cdc42-dependent regulator of membrane-associated actin polymerization, that restricts the release of Gbb, a function that is in turn counteracted by the Cdc42GAP Rich (Nahm et al. 2010a, b). Interestingly, postsynaptic Ca^{2+}/calmodulin-dependent kinase II (CaMKII)-activity, triggered by Ca^{2+}-influx through GluRs, may also act as a negative regulator of retrograde Gbb signaling (Haghighi et al. 2003), and it is therefore tempting to speculate that CaMKII might exert this role by activating CIP4 (Nahm et al. 2010a).

The Gbb signaling pathway has also been implicated in synaptic homeostasis, which is expressed by increased presynaptic glutamate release in response to reduced postsynaptic activity (Haghighi et al. 2003; Goold and Davis 2007). Both decreased muscle membrane excitability (Paradis et al. 2001) and reduced postsynaptic sensitivity to glutamate (Petersen et al. 1997) were initially found to elicit this form of plasticity with little or no effect on junctional growth. Using the glutamate receptor antagonist Philanthotoxin-433 (PhTX) at subblocking concentrations on semi-intact larval body wall muscle preparations, Frank et al. (2006) could demonstrate that the compensatory upregulation of presynaptic release occurs gradually within a few minutes, i.e., on an unexpectedly fast time scale. Interestingly, evoked neuronal activity is not required here, suggesting that integration of relatively few spontaneous release events (measured as miniature excitatory postsynaptic potentials or currents, mEPSPs, or mEPSCs) is sufficient to elicit a retrograde signal that triggers a rapid compensatory upregulation of release. A screen for genetic modifiers has led to the identification of factors acting during the acute phase of synaptic homeostasis including the presynaptic calcium channel cacophony (Cac) and the schizophrenia susceptibility factor dysbindin (Frank et al. 2006, 2009; Dickman and Davis 2009). Gbb signaling, on the other hand, is required to confer a principal, transcription-dependent competence onto motoneurons to express rapid synaptic homeostasis (Goold and Davis 2007). Possibly related to this role of Gbb signaling, some discrete subcellular defects are commonly encountered in mutants that interfere with the pathway. These defects include T-bars that are not properly associated with the presynaptic membrane, local detachment of the pre- and postsynaptic membranes, and decreased stability of axonal and nerve terminal microtubules (Aberle et al. 2002; Marqués et al. 2002; McCabe et al. 2003; Eaton and Davis 2005; Wang et al. 2007). Moreover, the respective mutants display up to 90% reduction in baseline synaptic transmission (Aberle et al. 2002; McCabe et al. 2004). This pleiotropism, further complicated by a regulatory role of Gbb in the expression of the circulating neuromodulatory peptide FMRFamide (Keshishian and Kim 2004), has made it difficult, to distinguish between chronic developmental and more acute, plasticity-related functions of the BMP pathway at the NMJ. Likewise, early requirements for BMP/TGF-β signaling during dendrito- and axonogenesis of vertebrate neurons (Lee-Hoeflich et al. 2004; Podkowa et al. 2010; Yi et al. 2010) might conceal subsequent synaptic functions. Clearly, an identification and functional evaluation of transcriptional targets of the BMP/TGF-β signaling pathway will be relevant here.

1.2.1.2 Wnt Signaling

The prototypic *Drosophila* Wnt morphogen Wingless (Wg) is secreted from motor nerve terminals (Packard et al. 2002; Korkut et al. 2009) and governs NMJ growth and synapse assembly. Evidence for comparable roles of Wnts at mammalian synapses accumulates (Korkut and Budnik 2009; Budnik and Salinas 2011. Downstream of the Wg receptor Frizzled-2 (Fz2), different pathways are employed to

execute Wg instructions on the pre- or postsynaptic side, respectively. Activation of presynaptic Fz2 triggers a cascade which diverts from the canonical Wnt pathway to locally regulate the microtubular cytoskeleton as a prerequisite for proper bouton formation (Miech et al. 2008). Binding of Wg to postsynaptic Fz2 elicits an unconventional synapse-to-nucleus signal that might control the expression of genes involved in postsynaptic differentiation (Mathew et al. 2005; Ataman et al. 2006a; Mosca and Schwarz 2010). Importantly, Wg secretion is upregulated upon acute spaced stimulation and induces profound junctional growth followed by differentiation as reflected by the assembly of new synaptic contacts (Ataman et al. 2008). As this process is dependent on both transcription and translation, it clearly constitutes a mechanism for activity-inducible long-term plasticity.

1.2.2 Activity-Dependent Plasticity

Both the morphological and physiological properties of the growing NMJs are subject to activity-dependent plasticity. Next to mutants with globally altered electrical activity, long-term memory mutants displaying elevated or reduced intracellular cAMP levels, respectively, provide classical examples for this notion (Budnik et al. 1990; Zhong and Wu 1991; Zhong et al. 1992). Chronically elevated cAMP levels due to mutations in the cAMP-phosphodiesterase gene *dunce (dnc)*, for instance, cause junctional overgrowth. In addition, *dnc*-mutant NMJs display increased synaptic strength at low or moderate Ca^{2+}-concentrations, an effect that is due to increased release probability (Zhong and Wu 1991; Zhong et al. 1992). At the same time, short-term plasticity as expressed by posttetanic potentiation and paired-pulse facilitation is impaired in these mutants, likely reflecting changes in the functional status of presynaptic release sites due to chronic changes in cAMP.

Various downstream effectors of cAMP may account for different aspects of the *dnc* phenotype, including ion channels and transcription factors. For instance, cAMP promotes downregulation of the perisynaptic cell adhesion molecule Fasciclin II (FasII) and in parallel activates the cAMP response element binding transcription factor CREB. Both events converge in the observed structural and functional strengthening of NMJs (Schuster et al. 1996b; Davis et al. 1996). The prevailing model for activity-dependent plasticity, in which CREB induces the expression of the immediate early gene products Fos and Jun, however, might not apply here. Instead, Sanyal et al. (2002) could demonstrate that the heterodimeric Fos-Jun transcription factor AP-1 promotes synaptic strengthening as a downstream effector of Jun N-terminal kinase (JNK), thereby in turn inducing CREB expression. Like in *dnc* mutants (Kuromi and Kidokoro 2000), long-term enhancement of synaptic strength by overexpression of AP-1 is likely achieved through persistent mobilization of synaptic vesicles from the reserve pool, possibly involving activation of myosin light chain kinase in the motor nerve terminals (Verstreken et al. 2005; Kim et al. 2009). However, whereas synaptic strengthening in *dnc* mutants involves an increase in the number of synaptic contacts (Renger et al. 2000),

AP-1-induced synaptic strengthening is accompanied by a moderate decrease in the number of synaptic contacts (Kim et al. 2009). In fact, while NMJ growth, the addition of synaptic contacts, and synaptic strengthening occur simultaneously during normal development, they are controlled by divergent pathways as revealed by phenotypes of numerous mutants (e.g., Stewart et al. 1996; Schuster et al. 1996b; Wan et al. 2000; Reiff et al. 2002; Merino et al. 2009).

1.3 Ionotropic Glutamate Receptors at Larval NMJs

The overall synaptic strength of a given NMJ is determined by the number of individual synaptic contacts and by their pre- and postsynaptic properties such as the pool of release-ready vesicles, their individual release probabilities, and postsynaptic responsiveness. As will be described in the following sections, all three determinants are strongly influenced by the composition and availability of synaptic glutamate receptors, which in turn are regulated at various levels.

1.3.1 Subtypes of Junctional GluRs

Approximately 30 putative ionotropic GluR subunits are encoded in the *Drosophila* genome (Littleton and Ganetzky 2000), but only five of them, GluRIIA, GluRIIB, GluRIIC (GluRIII), GluRIID, and GluRIIE, were found to assemble into functional, calcium-permeable receptors at larval NMJs (Schuster et al. 1991; Chang et al. 1994; Petersen et al. 1997; Marrus et al. 2004; Featherstone et al. 2005; Qin et al. 2005). All five are non-NMDA-type receptor subunits, which display significant sequence homologies to vertebrate AMPA- and kainate-type GluR subunits (30–40% identity, 50–60% similarity). Notably, however, fly GluRI, which is even more closely related to AMPA-type receptor subunits, as well as the two fly homologs of NMDA-type receptor subunits have not been detected at NMJs.

Attempts to approach the subunit composition of junctional GluRs by reconstituting them in in vitro systems have failed so far (S.J.S., unpublished). Their stoichiometry was rather inferred from genetic analyses, which point to the existence of two, most likely heterotetrameric receptor subtypes. Apparently, both subtypes contain subunits GluRIIC, IID, and IIE but differ by the incorporation of either GluRIIA or GluRIIB and hence are referred to as A- or B-type receptors, respectively (Fig. 1.2a). Lack of any single of the three default subunits (GluRIIC, IID, IIE) prevents the formation of ionotropic GluRs at body wall muscle NMJs, thus leading to paralysis and late embryonic lethality (DiAntonio et al. 1999; Marrus et al. 2004; Featherstone et al. 2005; Qin et al. 2005). In contrast, mutants lacking either GluRIIA or IIB are viable, and only concomitant loss of both subunits is embryonic lethal. The fact that the presence of just one GluR subtype is sufficient for viability reflects a certain level of redundancy among the two receptor subtypes

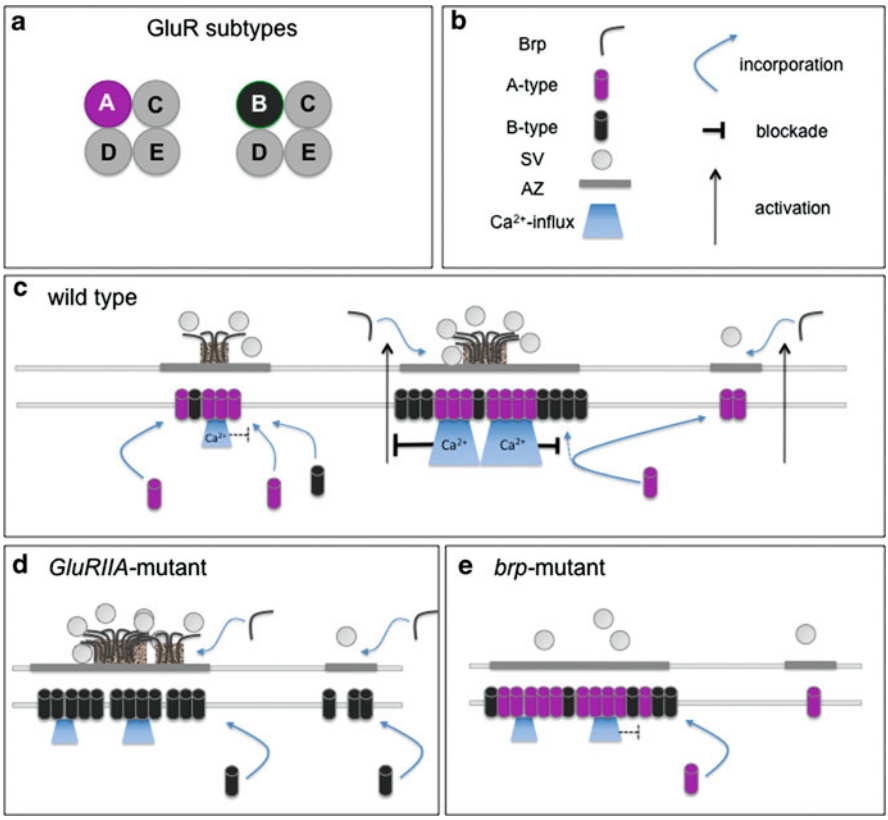

Fig. 1.2 Model for the control of synaptic structure and function by GluRIIA. (**a**) Proposed subunit composition of A- and B-type receptors at NMJs. (**b**) Symbols used in C to E. (**c**) Schematic representation of three synaptic contacts. Mature synapses (*middle*) harbor similar amounts of A- and B-type receptors. Local Ca^{2+}-influx, mainly through A-type receptors, counteracts further incorporation of A-type receptors, which may instead be consumed by premature (*left*) and newborn synapses (*right*). Ca^{2+} may further suppress a retrograde signal that otherwise promotes the strengthening of presynaptic T-Bars by incorporating additional Brp. (**d**) In the absence of GluRIIA, postsynaptic Ca^{2+}-influx is low, thus leading to continued Brp recruitment to AZs, sometimes resulting in AZ profiles with two or more T-Bars. Similar effects can be triggered by acute pharmacological inhibition of GluRs. (**e**) In the absence of Brp, evoked postsynaptic responses are largely diminished. Chronically reduced postsynaptic Ca^{2+}-influx then allows for persistent recruitment of A-type receptors to individual synapses

(Petersen et al. 1997; DiAntonio et al. 1999). In fact, both receptors (1) display virtually identical, high single-channel conductances of approximately 120–150 pS, i.e., approximately five- to tenfold higher than vertebrate AMPA- or kainate receptors (Heckmann and Dudel 1995; Nishikawa and Kidokoro 1995; DiAntonio et al. 1999) and (2) appear similarly abundant, when averaged across all synapses of a larval NMJ (Pawlu et al. 2004; Schmid et al. 2008). B-type receptors, however, desensitize much more rapidly than A-type receptors as revealed by single-channel

analysis on extrajunctional muscle membrane outside-out patches from larvae expressing either receptor subtype alone (DiAntonio et al. 1999). Selective absence of A- or B-type GluRs was accomplished by complementing a deletion of the tandem-arranged *GluRIIA* and *GluRIIB* genes with genomic transgenes encoding either GluRIIA or GluRIIB. In this approach, which precludes unwanted overexpression effects, quantal size was found to be three- to fourfold larger upon complementation by GluRIIA as compared to complementation by GluRIIB (DiAntonio et al. 1999; Schmid et al. 2008). Thus, the postsynaptic sensitivity to glutamate is much higher at synapses enriched for A-type than at synapses enriched for B-type receptors. From this, it follows that the number of synaptic A-type GluRs is the primary determinant of postsynaptic strength and, importantly, for the entry of Ca^{2+} as a second messenger (see below).

1.3.2 Synaptic Clustering of GluRs

The picture of how GluRs get targeted to and clustered at NMJs is far from being complete, especially in terms of the physical interactions involved. Nonetheless, the process has been described in quite some detail, including studies on synaptogenesis during the initial phase of NMJ formation and long-term in vivo imaging of GluR incorporation into synapses, and these studies have revealed important regulatory principles underlying the control of synapse formation.

Transcripts encoding GluR subunits are clearly detectable in muscles before motoneurons and target muscles contact each other (Schuster et al. 1991; Currie et al. 1995; Petersen et al. 1997; Marrus et al. 2004; Qin et al. 2005), implying that innervation does not per se induce GluR expression. In turn, GluRs do not prefigure the postsynaptic area of the presumptive neuromuscular junction, nor are they required to induce presynaptic differentiation (Prokop et al. 1996; Qin et al. 2005; Schmid et al. 2006). Whole-cell patch clamp recordings during focal iontophoresis of glutamate and cell-attached patch clamp recordings at different positions on embryonic muscles revealed that shortly before innervation functional receptors are evenly distributed on the muscle surface (Broadie and Bate 1993a; Nishikawa and Kidokoro 1995). Innervation then induces (1) the recruitment of extrajunctional receptors to the developing receptive fields opposite to AZs (Broadie and Bate 1993b; Saitoe et al. 1997; Chen and Featherstone 2005a) and (2) an increase in receptor expression (Broadie and Bate 1993b). Both effects require at least a minimum of electrical activity within motoneurons (Broadie and Bate 1993c; Saitoe et al. 1997). The mechanism, however, by which neural activity translates into the synaptic clustering of GluRs still remains obscure. Vesicular release of glutamate was ruled out to play a role in this process, as null mutants for the single vesicular glutamate transporter in motoneurons display normal clustering of functional receptors (Daniels et al. 2006). This result is paralleled by findings in Munc13-1, -2 double knockout mice, where postsynaptic differentiation including the clustering of glutamate receptors appears unaffected in the complete absence of

synaptic vesicle release (Varoqueaux et al. 2002). Moreover, a total block of both evoked and spontaneous release did not markedly interfere with synaptic GluR clustering (Schmid et al. 2006), arguing against the previously controversial idea, that the machinery for spontaneous neurotransmitter release might be employed to secrete factor(s) that induce junctional accumulation of GluRs (Saitoe et al. 2001, 2002; Featherstone et al. 2002a, Featherstone and Broadie 2002; Verstreken and Bellen 2002). Signaling pathways across the junctional cleft, that would initiate postsynaptic GluR clustering, thus remain to be identified.

1.3.3 Extracellular Matrix and Cell Adhesion Molecules in Synaptic GluR Clustering

Recently, the presynaptically secreted N-acetyl-glycosaminoglycan-binding glycoprotein termed Mind-the-Gap (Mtg) has been reported to play an important role early in postsynaptic differentiation (Rohrbough et al. 2007; Rushton et al. 2009). A *mtg* null allele was identified in a screen for mutants causing late embryonic lethality associated with severe paralysis. In fact, the junctional recruitment of GluRs and other postsynaptic marker proteins appears strongly diminished, though not completely abrogated in these mutants. Mtg might exert its role by organizing the synaptic cleft matrix as an environment for effective signaling and/or physical trapping of synaptic transmembrane proteins including integrins and the receptor tyrosine kinase Alk (Rushton et al. 2009; Rohrbough and Broadie 2010). While there is no obvious ortholog of Mtg in vertebrates, the crucial involvement of extracellular matrix proteins emerges as a common theme in the clustering of various neurotransmitter receptors (Kummer et al. 2006; Dityatev and Schachner 2006; Wu et al. 2010; see also accompanying article by Frischknecht and Gundelfinger).

By analogy to findings in vertebrates, it appears conceivable that transsynaptic adhesion molecules and synaptic scaffold molecules may play important roles in GluR recruitment and clustering (Gerrow and El-Husseini 2007; Han and Kim 2008). FasII, which is related to vertebrate neural cell adhesion molecule (NCAM), is present at both motor nerve growth cones and their target muscles prior to NMJ formation. Although various aspects of postsynaptic differentiation are impaired by complete loss of FasII, it causes only a moderate decrease in the recruitment of both GluR subtypes (Kohsaka et al. 2007).

The only *Drosophila* neurexin (DNrx1) and at least two out of four neuroligins (DNlg1, DNlg2) were recently shown to be present at NMJs from early onward (Li et al. 2007; Sun et al. 2009; Chen et al. 2010; Banovic et al. 2010; Sun et al. 2011). Loss of function alleles for either protein display discrete phenotypes at NMJs, including altered composition of GluR subtypes (Sun et al. 2011) and accumulation defects of GluRs (Banovic et al. 2010). In *dnlg1* mutants, boutons with differentiated AZs, yet without corresponding accumulation of postsynaptic

GluRs, are frequently observed, and the remaining receptor fields are often enlarged and misshapen (Banovic et al. 2010). Thus, the formation of GluR fields (and other postsynaptic structures) is severely affected by loss of DNlg1. Notably, the conventional concept of presynaptic neurexin primarily forming a functional unit with postsynaptic neuroligin is challenged by the facts that (1) *dnlg1* mutants display stronger physiological and structural phenotypes than *dnrx* mutants (Banovic et al. 2010), (2) *dnrx-dnlg2* double mutants show strongly enhanced phenotypes as compared to the respective single mutants (while *dnrx*- and *dnlg1* mutations are nonadditive), and (3) Dnrx might also be expressed in muscles (Chen et al. 2010).

1.3.4 Cytoskeletal and Scaffold Components

Among the few proteins hitherto shown to co-enrich with GluRs within the discrete, several hundred nanometers wide postsynaptic sites are the p21-activated kinase (PAK), the Rho-type GTPase exchange factor dPix (Parnas et al. 2001), and Dreadlocks (Dock), a functional homolog of human Nck, which can act as an adaptor protein to link receptor tyrosine kinases (or other transmembrane proteins) to effectors of the actin cytoskeleton, including activated Pak (Li et al. 2001). GluRs, in particular GluRIIA, are reduced by about 50% at synapses that lack Pak, dPix, or Dock, leading to a significant decrease in quantal size (Albin and Davis 2004). Genetic analyses further implied that Pak can be activated by both Rac and Cdc42 and acts with Dock in the same pathway to control GluR abundance. The existence and identity of a postsynaptic Dock-binding receptor, however, remains obscure. Moreover, although Pak localizes to postsynaptic sites opposite to embryonic AZs even in the complete absence of GluRs, its further accumulation is strongly impaired when GluRs are expressed at a minimum level ($\leq$5% of normal) required for survival (Schmid et al. 2006). This observation not only reflects interdependency between Pak and GluRs during synaptic maturation but also correlates with an important structural role of GluRIIA in synapse development that was unraveled in the course of that study.

A possible role for the actin cytoskeleton in GluR anchorage was assessed by applying via a patch pipette (1) latrunculin A, which precludes actin polymerization and (2) cofilin, which induces depolymerization of filamentous actin (Chen et al. 2005b). Both drugs were found to reduce synaptic GluRIIA, but not GluRIIB. This subunit-selective effect may be attributable to Coracle (Cor), a *Drosophila* homolog of the mammalian cytocortical protein 4.1, which was shown to physically interact with GluRIIA in vitro (but not with GluRIIB). In fact, synaptic GluRIIA was found severely reduced in the absence of Cor, whereas GluRIIB levels remained normal (Chen et al. 2005b).

A number of well-conserved scaffold molecules have been implicated in NMJ organization and function (Ataman et al. 2006b). In mammalian neurons, members of the family of Dlg-like membrane-associated guanylate kinases (MAGUKs) play a pivotal role in the trafficking, surface expression, and synaptic clustering of both

NMDA- and non-NMDA-type receptors (Funke et al. 2005; Elias and Nicoll 2007). Discs large (Dlg), the prototypic *Drosophila* MAGUK, is highly enriched at the postsynaptic site of larval NMJs; however, it is virtually excluded from the GluR fields and is therefore, like many other NMJ components, considered as a perisynaptic component (Thomas et al. 2010). In conjunction with two other scaffold proteins, Metro and DLin-7, Dlg limits the size of GluR fields (Karunanithi et al. 2002; Mendoza-Topaz et al. 2008; Bachmann et al. 2010), and this role may involve direct interactions with FasII (Stewart et al. 1996; Thomas et al. 1997; Zito et al. 1997). Pre- and postsynaptic spectrin is required for proper junctional recruitment of Dlg (Featherstone et al. 2001), and RNAi-mediated disruption of the postsynaptic spectrin lattice alone causes conspicuous disorganization of junctional Dlg along with a pronounced broadening of synaptic areas, suggesting that Dlg acts downstream of spectrin to execute its role in proper dimensioning of GluR fields (Pielage et al. 2006).

1.3.5 In Vivo Observation of GluRs During Synapse Addition at NMJs

Based on the relative transparency of the larval cuticle, NMJs can be assessed by confocal microscopy on intact animals provided that they express a fluorescently tagged protein that enriches at NMJs (Zito et al. 1999). This approach has been used to analyze the dynamics of GluRs at individual synapses of selected NMJs over a period corresponding to about 20% of larval development. Fluorescently tagged GluRIIA and GluRIIB expressed from "genomic" transgenes comprising all introns and regulatory upstream sequences were found to mimic the respective endogenous GluR subunits in terms of functionality, expression levels, and subcellular distribution (Rasse et al. 2005; Schmid et al. 2008). More than hundred newborn receptor fields per NMJ were detected during the observation period. Within 6–8 h (at 25°C), each of them reaches final size of 0.24 μm^2 on average. Fluorescence recovery after photobleaching (FRAP) and photoactivation analyses revealed that, once arrived at the synapse, GluRs, especially type A, are largely immobile, although local internalization and recycling at individual synapses cannot be ruled out. This is somewhat in contrast to the mobility of a large percentage of synaptic AMPA receptors in cultured mammalian neurons (Newpher and Ehlers 2008; see also accompanying article by Heine). In particular, there is little if any exchange of GluRs between synapses within a given NMJ. Instead, GluRs from all over the muscle contribute to the maturation of growing GluR fields.

Simultaneous quantification of differently fluorescently tagged GluRIIA and GluRIIB showed that the former is prevalent at small receptor fields (Fig. 1.2b–c). With subsequent growth, a clear shift from GluRIIA-dominated immature synapses to a balanced composition at mature synapses became evident. Thereby, a strong negative correlation between presynaptic maturation as indicated by increasing

amounts of the major constituent of T-Bars, i.e., the protein Bruchpilot (Brp; related to vertebrate ELKS/ERC/CAST) (Fouquet et al. 2009), and the ongoing incorporation of GluRIIA was observed (Fig. 1.2c). Consistent with the idea that glutamate-triggered Ca^{2+}-influx through GluRIIA might itself constitute a negative feedback signal, Schmid et al. (2008) could show that blockade of evoked glutamate release leads to unrestricted synaptic recruitment of GluRIIA on the expense of GluRIIB incorporation (Fig. 1.2d). Likewise, in mutants for Brp and mutants for the Brp-binding partner DSyd-1, where the prevention of T-Bar formation causes a severe deficit in evoked release (Wagh et al. 2006; Kittel et al. 2006; Owald et al. 2010), increased accumulation of GluRIIA becomes apparent (Fig. 1.2e).

1.3.6 GluRIIA-Dependent Synapse Formation and Plasticity

In animals raised under standard conditions, loss of GluRIIA causes only a moderate reduction in the number of boutons and synaptic contacts (Reiff et al. 2002; Schmid et al. 2008), but a severe reduction in quantal size. Nonetheless, *GluRIIA* mutant NMJs show normal evoked response when stimulated with single action potentials (Petersen et al. 1997). This is brought about by an increase in presynaptic release (quantal content), which in turn is tightly correlated with ultrastructural adaptations toward more and bigger T-Bars per synapse, most likely due to recruitment of additional Brp protein (Reiff et al. 2002). Recently, Weyhersmüller et al. (2011) showed that paired-pulse ratios are similar in *GluRIIA* mutants and controls, implying that the release probability of individual vesicles remains unchanged in the mutants. At the same time, the number of release-ready vesicles was found to be almost doubled. Moreover, this presynaptic adaptation to loss of GluRIIA was accompanied by a moderate but significant increase in the size of Brp clusters at AZs. Interestingly, an increase in synaptic Brp content was even traceable on a short-term scale after blocking GluRs acutely with PhTX (see above) (Weyhersmüller et al. 2011). Increases in Brp at individual active zones triggered by loss of conductance through postsynaptic GluRIIA may therefore be part of the observed homeostatic response. This view is further substantiated by a study, in which the probability of SV release at individual AZs was determined based on real-time imaging and was found to correlate with the amount of Brp at these sites (Peled and Isacoff 2011). At this point, we refer the reader to recent reviews on plasticity phenomena concerning AZs, T-Bars, and related specializations in other species as substrates for presynaptic modes of plasticity (Owald and Sigrist 2009; Wichmann and Sigrist 2010; Sigrist and Schmitz 2011).

It is conceivable that Ca^{2+}-entry through GluRs, mainly GluRIIA itself, constitutes a crucial determinant for negative feedback onto presynaptic release. In fact, constitutive expression of a CaMKII-inhibiting peptide leads to increased quantal content (Haghighi et al. 2003; Morimoto et al. 2010), and expression of a constitutively activated CaMKII was found to interfere with homoeostatic compensation in one study (Haghighi et al. 2003). In a similar, though not identical

approach, Morimoto et al. (2010) found that active CaMKII downregulates synaptic GluRIIA levels along with proper homeostatic upregulation of synaptic vesicle release. The way CaMKII joins in here thus remains to be specified, e.g., in terms of substrates, that may play a role in retrograde signaling (see also above notes on retrograde BMP signaling). The postsynaptic scaffold protein Dystrophin (Dys) is to be named here, as *dys* mutants display increased presynaptic release despite normal levels of GluRIIA and no change in the number of synaptic contacts, yet paralleled by a more prominent appearance of T-Bars (van der Plas et al. 2006). This phenotypic constellation is consistent with Dys acting downstream of GluRIIA in controlling presynaptic release. The identification of mammalian Dys as a target for phosphorylation by CaMKII is further suggestive, and it is tempting to speculate that Dys mediates negative feedback upon phosphorylation by CaMKII. NMJ localization of Dys depends on the transmembrane protein dystroglycan (Bogdanik et al. 2009), and a genetic modifier screen led to the finding that the RhoGAP crossveinless-c and its target Cdc42 act in concert with Dys (Pilgram et al. 2011).

A second Ca^{2+}-sensor that has been implicated in GluRIIA-dependent retrograde control at NMJs is synaptotagmin 4 (Syt4) (Yoshihara et al. 2005). In contrast to CaMKII, however, Syt4 is required for a positive feedback loop, activated by synapse-specific Ca^{2+}-influx. High-frequency stimulation induces Syt4-dependent postsynaptic vesicle fusion, which is required to induce facilitated presynaptic release (expressed as an increased mEPSP rate) and, presumably, cytoskeletal rearrangements, that in turn lead to NMJ expansion. These responses are dependent on presynaptic protein kinase A (PKA), a major target of cAMP (Yoshihara et al. 2005). Thus, the role of Syt4 may be well related to activity-induced synaptic strengthening, which is mimicked by elevated cAMP levels in *dnc* mutants and which is accompanied by increased numbers of boutons and synaptic contacts. Notably, a concomitant increase in GluRIIA levels was observed (Sigrist et al. 2000) and indeed shown to be limiting in this process (Sigrist et al. 2002). Muscle-specific overexpression of GluRIIA is indeed sufficient to induce junctional overgrowth and a proportional increase in synapse numbers (Sigrist et al. 2002). The in vivo relevance of this regulation was highlighted by the finding that enhanced larval locomotor activity induces qualitatively equivalent changes in a GluRIIA-dependent manner (Sigrist et al. 2003). Strikingly, the amount of GluRIIA within individual synapses does not change during activity-induced synaptic strengthening, consistent with the aforementioned, maturation-related restriction of GluRIIA recruitment to individual synaptic contacts (Schmid et al. 2008). Therefore, limited consumption of GluRIIA at individual synapses warrants the availability of this receptor subtype for the formation of additional synapses. While GluRs get recruited to synapses from all over the muscle during NMJ expansion (Rasse et al. 2005), there is evidence that local synthesis of GluRIIA at NMJs might be employed to meet the requirement for activity-induced formation of synapses and that posttranscriptional control of the translational initiation factor eIF-4E is crucially involved in this regulation (Sigrist et al. 2000, 2002; Reiff et al. 2002;

Menon et al. 2004, 2009). The ultimate proof for such a translational control of GluRIIA, however, is still missing.

It appears conceivable that GluRIIA and GluRIIB compete for default subunits and/or by occupying slots within presumptive receptor field areas. Overexpression of GluRIIB was indeed found to interfere with functional A-type receptor expressivity, thus leading to a decrease in quantal size (DiAntonio et al. 1999; Sigrist et al. 2002). It remains questionable, however, whether competition is relevant within the physiological range of expression. A recent study by Karr et al. (2009) demonstrated that limited availability of default subunits accounts for only a moderate increase in the number of surface-expressed and synaptic GluRs when expression levels of both GluRIIA and GluRIIB were substantially elevated due to a lack in posttranscriptional suppression by microRNAs. Thus, competition at the level of receptor assembly may become effective as a mechanism to adjust synaptic receptor composition when expression levels of GluRIIA and GluRIIB change asymmetrically.

1.3.7 Control of Synaptic GluRs by Ambient Glutamate

Following initial observations which pointed to nonvesicular release of glutamate as a negative regulator of synaptic GluR accumulation (Featherstone et al. 2000, 2002), Augustin et al. (2007) investigated Genderblind (Gb), the xCT subunit of the cystine/glutamate transporter for its impact in this mode of regulation. Reminiscent to obvious expression of its mammalian homolog in border areas of the brain, Gb is expressed in surface glia of the CNS and within a particular glia cell (now termed Gb cell) associated with NMJs. Compared to ambient glutamate concentrations in the mammalian brain, which are normally in a low micromolar range (Featherstone and Shippy 2008), glutamate concentrations in the larval hemolymph are in a millimolar range, yet close to the level required for half-maximal activation of junctional receptors (Heckmann et al. 1996). Loss of Gb causes an ~50% reduction in hemolymph glutamate concentration. Under this condition, the junctional amount of both GluR subtypes was more than doubled, and this effect was further enhanced when glutamate was completely omitted experimentally. Importantly, by using a glutamate antagonist (γ-D-glutamylglycine), which prevents glutamate-dependent desensitization, the effect of reduced glutamate was mimicked, implying that ambient glutamate interferes with synaptic clustering of GluRs via desensitization of the receptors. As pointed out by Featherstone and Shippy (2008), a steady-state desensitization of GluRs offers additional options for regulating synaptic strength, either cell-autonomously by altering desensitization kinetics of GluRs or systemically by altering ambient glutamate levels. The role of xCT in regulating neural functions has recently been further corroborated by demonstrating that it is required for regulating synaptic strength in adult flies and thereby controls courtship behavior (Grosjean et al. 2008).

1.4 Concluding Remarks

The *Drosophila* larval NMJ has proven a highly versatile model to unravel structure–function relationships during synaptogenesis, synaptic transmission, synapse maintenance, and synapse remodeling. Time and again, proteins relevant for these processes emerge from unbiased forward genetic screens for mutants affecting synaptic function and/or structure, a trademark strategy so far largely confined to *Drosophila melanogaster* and the nematode *Caenorhabditis elegans*. Typically, the merit of such screens lies in the shortcut toward the functional characterization of the identified genes or proteins. For instance, fruitful screens were built onto the ability to generate flies in which all photoreceptor cells in the eyes are homozygous for a given mutation, whereas cells of all other tissues remain heterozygous (Stowers and Schwarz 1999). This way, mutations affecting synaptic transmission can be screened for by performing electroretinograms on the easily accessible compound eyes. Once identified, mutants are then often subjected to further analysis at the NMJ. In several instances, factors identified in these or other screens were found to be homologous to hitherto poorly characterized proteins implicated in human neurodegenerative or other neurological disorders (e.g., Zhai et al. 2006, 2008; Dickman and Davis 2009; Kim et al. 2010).

Complementary to forward genetic approaches, well-conserved synaptic proteins such as the GluRs are often directly assessed for their role at NMJs. The genetic toolbox for refined analyses is constantly upgraded, including comprehensive collections of transgenic fly stocks allowing for cell-type-specific RNAi-mediated knockdown of almost every gene (Dietzl et al. 2007; Ni et al. 2009) and the establishment of techniques for generating small deletions (Parks et al. 2004) or even predefined gene targeting by homologous recombination (Gong and Golic 2003). Moreover, by optimizing recombineering techniques, it has now become possible to generate genomic constructs of more than 100 kb, which, for instance, can be used to express fluorescence-tagged synaptic proteins at or near endogenous levels, an advantage that can hardly be overestimated (Venken et al. 2006, 2009; Ejsmont et al. 2009). In fact, the benefit of this approach has been well exemplified by the aforementioned expression of junctional GluRs from conventionally cloned genomic transgenes and their subsequent assessment by life imaging (Rasse et al. 2005; Schmid et al. 2008). Future studies may therefore be expected to include other synaptic proteins in this sort of analysis, thus leading to a more detailed view on the molecular dynamics of glutamatergic synapses.

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