Differential Regulation of Active Zone Density during Long-Term Strengthening of Drosophila Neuromuscular Junctions

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In this study we established a transgenic Ca\(^{2+}\) imaging technique in Drosophila that enabled us to target the Ca\(^{2+}\) sensor protein yellow Cameleon-2 specifically to larval neurons. This noninvasive method allowed us to measure evoked Ca\(^{2+}\) signals in presynaptic terminals of larval neuromuscular junctions (NMJs). We combined transgenic Ca\(^{2+}\) imaging with electrophysiological recordings and morphological examinations of larval NMJs to analyze the mechanisms underlying persistently enhanced evoked vesicle release in two independent mutants. We show that persistent strengthening of junctional vesicle release relies on the recruitment of additional active zones, the spacing of which correlated with the evoked presynaptic Ca\(^{2+}\) dynamics of individual presynaptic terminals. Knock-out mutants of the postsynaptic glutamate receptor (GluR) subunit DGlur-IIA, which showed a reduced quantal size, developed NMJs with a smaller number of presynaptic boutons but a strong compensatory increase in the density of active zones. This resulted in an increased evoked vesicle release on single action potentials and larger evoked Ca\(^{2+}\) signals within individual boutons; however, the transmission of higher frequency stimuli was strongly depressed. A second mutant (pabp\(^{970}/+\)), which showed enhanced evoked vesicle release triggered by elevated subsynaptic protein synthesis, developed NMJs with an increased number of presynaptic boutons and active zones; however, the density of active zones was maintained at a value typical for wild-type animals. This resulted in wild-type evoked Ca\(^{2+}\) signals but persistently strengthened junctional signal transmission. These data suggest that the consolidation of strengthened signal transmission relies not only on the recruitment of active zones but also on their equal distribution in newly grown boutons.

Key words: transgenic Ca\(^{2+}\) imaging; Cameleon-2; presynaptic Ca\(^{2+}\); long-term strengthening; active zone density; consolidation; synaptic protein synthesis; glutamate receptor; neuromuscular junction; Drosophila

During the rapid growth of developing Drosophila larvae, the body wall muscle cells experience a continuous drop in input resistance (Lnenicka and Keshishian, 2000). To maintain an efficient depolarization of growing muscle cells at all times of development, larval neuromuscular junctions (NMJs) undergo proportional morphological and functional changes that result in enhanced release of presynaptic vesicles and a concomitant increase of excitatory junctional current (EJC) amplitudes. A closely related compensatory mechanism maintains evoked muscle depolarization even in mutants with genetically reduced postsynaptic excitability (Petersen et al., 1997; Davis et al., 1998; DiAntonio et al., 1999). These data have suggested that the level of muscle depolarization is monitored continuously and regulated homeostatically to ensure efficient muscle contraction at all times of larval development (Davis and Goodman, 1998; Paradis et al., 2001).

Besides this developmental control of muscle depolarization, enhanced EJCs (eEJCs) can be further enhanced by the genetic manipulation of neuronal activity and cellular events downstream of neuronal activity, such as increased cellular cAMP levels (Budnik et al., 1990; Schuster et al., 1996; Cheung et al., 1999), elevated CREB- and API-dependent transcription (Davis et al., 1996; Sanay et al., 2002), increased subsynaptic protein synthesis (Sigrist et al., 2000), and altered subunit composition of postsynaptic glutamate receptors (GluRs) (Sigrist et al., 2002). All examples of this adaptive form of junctional strengthening appeared to also rely on an increased evoked release of presynaptic vesicles.

In this study we set out to assess the cellular mechanisms that are involved in the control of persistently strengthened evoked vesicle release at developing NMJs. We first established a new, noninvasive, presynaptic Ca\(^{2+}\) imaging approach in Drosophila, which was based on the targeted transgenic expression of the Ca\(^{2+}\) reporter yellow Cameleon-2 (yCam2) (Miyawaki et al., 1997) in larval neurons and thus in presynaptic terminals of NMJs. We then undertook a detailed analysis of the presynaptic Ca\(^{2+}\) dynamics, junctional signal transmission, and endplate morphology of wild-type and mutant animals that represented both above-mentioned forms of junctional strengthening. These included the knock-out mutant of the postsynaptic glutamate receptor subunit gene dglurIIA, which showed strongly reduced quantal sizes but almost normal eEJC amplitudes attributable to a compensatory increase in presynaptic vesicle release (Petersen et al., 1997; DiAntonio et al., 1999). We also analyzed a mutant with genetically elevated subsynaptic protein synthesis [poly(A)-binding protein (pabp)], which showed unaltered quantal size but strongly increased eEJCs caused by enhanced evoked vesicle release (Sigrist et al., 2000). Our results from both mutants provide evidence that a persistently enhanced evoked release of
presynaptic vesicles is achieved primarily by recruiting additional active zones. In dglurIIA-ko mutants, these additional active zones were distributed in a smaller number of boutons, resulting in an increased active zone density, larger evoked presynaptic Ca\(^{2+}\) signals, and rescued junctional transmission of single stimuli but considerably depressed transmission of repetitive signals. In contrast, in pabp\(^{773/+}\) mutants, the additional active zones were equally distributed in newly grown boutons resulting in a similar active zone density and evoked Ca\(^{2+}\) signals as in wild-type animals and an improved junctional reliability after repetitive stimulation.

**MATERIALS AND METHODS**

**Genetics.** The glutamate receptor alleles dglurIIA\(^{2\theta}\) and df(2R)Pabp have been described in Petersen et al. (1997), and the alleles of the pol(y)-binding gene (pabp\(^{773/+}\), pabp\(^{773}\), and df(2R)Pabp) have been described in Sigrist et al. (2000). Transgenic UAS-yCam2 flies were generated in all yCam2-DNA-hybrids (Sawaki et al., 1997) into the pUAST vector and P-element-mediated transformation of Drosophila (Berkeley Drosophila Genome Project). For all experiments we used the line P\(^{w}\)/UAS-yCam2\(^{2\overline{2}}\);6i+ as yCam2 donor. yCam2 expression was driven in all larval neurons using the Gal4-driver lines (Brand and Perrimon, 1993) elav-Gal4, and C155-Gal4 (generous gift of C. S. Goodman, University of California at Berkeley, Berkeley, CA). The following male genotypes have been analyzed: wt: wild type. Elav-Cam: C155/Y;6i+/+; C155–yCam2: C155Gal4/Y;6i+/+. Elav-yCam2pabp: +; YclavGal4/pabp\(^{773/+}\); P2–6i+/. Elav-Cam/yCam2: C155/Y; dglurIIA\(^{2\theta}\)/df(2L)clh4;P2–6i+.

**Larval culture.** All larvae were raised under tightly controlled culture conditions (high-density culture, 65% humidity) at 28°C. All larvae were raised under tightly controlled culture conditions (high-density culture, 65% humidity) at 28°C. Larval culture. Larval culture. Larval culture. Larval culture.

**Electron microscopy.** Larvae were filleted and processed for ultrastructural analysis as described (70 min fixation in ice-cold 4% paraformaldehyde/PBS) (Sigrist et al., 2002). Ultrathin sections from random positions of 4–16 neuromuscular branches (muscle 6/7, abdominal segment A2) of two animals per genotype were analyzed at 21,000-fold enlargement with a CM10 electron microscope (Philips). The mean number of dense areas (synaptic profiles) and dense bodies (profiles of T-bars) in sections per branch was scored. The perimeter and area of bouton profiles were measured. From these raw data we calculated the ratio of synaptic profiles with or without T-bars, the mean perimeter/area by which bouton profiles are represented in sections, and the relation of the number of T-bar profiles in a bouton profile to its volume (area x thickness of a section 87 nm) or its perimeter.

**RESULTS**

**Targeted transgenic expression of the Ca\(^{2+}\) sensor yCam2 in Drosophila neurons.** To study presynaptic Ca\(^{2+}\) dynamics in single boutons of NMJs, we developed for Drosophila an alternative approach to the so far successfully applied Ca\(^{2+}\) imaging in this system that was based on synthetic Ca\(^{2+}\) sensors (Karunanithi et al., 1997; Umbach et al., 1998a,b; Dawson-Scully et al., 2000; Bronk et al., 2001). This new approach relies on the targeted transgenic expression of the Ca\(^{2+}\) sensor protein yellow cameleon-2 (Miyawaki et al., 1997; Kerr et al., 2000), which offers access to cells and cellular compartments that are difficult or impossible to approach using injection or general loading of synthetic dyes (see Discussion). To bypass such limiting factors we created transgenic flies with a P-element transformation vector containing the yellow Cameleon-2 (yCam2) cDNA (Miyawaki et al., 1997) downstream of an array of UAS sites. This allowed the targeted expression of yCam2 in all cells expressing the yeast transcriptional enhancer Gal4 (Brand and Perrimon, 1993). In this study, we used two Gal4-driver lines (elav-Gal4 and C155-Gal4) that both led to a reliable and strong expression of yCam2 in all larval neurons. yCam2 highlighted the entire larval brain, the motor nerves (Fig. 1A), and junctional nerve terminals at body wall muscles in the larval periphery (Fig. 1B). It is important to note that this transgenic expression of yCam2 was specific for neuronal structures, such as...
boutons at NMJs, and that fluorescence signals were absent from muscles or other non-neuronal tissues (Figs. 2, 3).

After Ca\(^{2+}\) binding, yCam2 undergoes a conformational change, which brings its two chromophores ECFP and EYFP into close proximity (Miyawaki et al., 1997). This greatly increases the probability of fluorescence resonance energy transfer (FRET) from excited ECFP to EYFP, resulting in an increased EYFP (530 nm) emission and a corresponding decrease of the ECFP (485 nm) emission. The FRET-based radiometric nature of this Ca\(^{2+}\) indicator makes it suitable for imaging in whole animal preparations, because it reduces the susceptibility to motion artefacts and greatly increases the accuracy of the measured fluorescence changes (Fan et al., 1999; Kerr et al., 2000). We therefore recorded the emission intensities of ECFP and EYFP (Fig. 1C), either in consecutive stimulation trials to analyze the basic yCam2 properties (Fig. 2) or simultaneously and thus more accurately to analyze synaptic parameters in various genotypes (Figs. 3, 6).

**yCam2 is a reliable Ca\(^{2+}\) sensor in Drosophila neurons**

A considerable problem of presynaptic Ca\(^{2+}\) imaging at NMJs was that the postsynaptic muscle cell starts to contract, in particular during high-frequency (HF) stimulation. To prevent muscle contraction during nerve stimulation, we treated the larval preparations with bath-applied glutamate to desensitize postsynaptic glutamate receptors. To further suppress excitation–contraction coupling, we used thapsigargin to deplete the intracellular Ca\(^{2+}\) stores (Thastrup et al., 1990). These treatments effectively eliminated muscle contraction even during bursts of high-frequency stimulation. In addition, thapsigargin blocks the function of presynaptic Ca\(^{2+}\) stores at Drosophila NMJs (Kuromi and Kidokoro, 2002) and thus reveals the dynamics of evoked presynaptic Ca\(^{2+}\) influx without the functional contribution of intracellular stores.

Using this preparation and the described setups (see Materials and Methods), we observed opposing dynamics of ECFP and EYFP emission intensities in presynaptic terminals of larval NMJs during periods of nerve stimulation (Fig. 2B,C), demonstrating that the shown EYFP emission alterations in Figure 2, D and E, are caused by FRET. Spike train-evoked fluorescence changes displayed homogeneous time courses and peak amplitudes for all analyzed functional areas (Fig. 2A, blue circles, 2C, thin lines) independent of their size and location within the NMJ (see below). Evoked yCam2 fluorescence changes faithfully reflected alterations in extra cellular Ca\(^{2+}\) concentrations (Fig. 2D) and presynaptic frequency of action potentials (Fig. 2E). Simultaneous measurements of both emission wavelengths (Fig. 3A,B) greatly improved the signal-to-noise ratio and allowed us to reliably quantify yCam2 fluorescence changes on a single bouton level (Fig. 3B,C). We therefore applied simultaneous dual-emission imaging to analyze and compare presynaptic Ca\(^{2+}\) dynamics among several animals and genotypes (see Fig. 6). These experiments were performed at stimulation frequencies (40 Hz) and an extra cellular Ca\(^{2+}\) concentration (1 mm) that evoked fluorescence changes well within the observed dynamic range of yCam2 (Fig. 2). Taken together, these results demonstrate that the Ga4-mediated expression of yCam2 in Drosophila neurons represents a noninvasive and sensitive tool to monitor spike train-evoked alterations of presynaptic Ca\(^{2+}\) at junctional boutons.

It is interesting to note that the monitored evoked fluorescence...
alterations of yCam2 showed no correlation to the size of the analyzed ROIs ($R < 0.1$; see Materials and Methods), indicating that the bouton size has no detectable effect on the evoked Ca\textsuperscript{2+} signal. This finding also suggested that the arborizations of the two innervating motoneurons of muscle 6/7, which can be anatomically and physiologically differentiated in large type Ib boutons (2–5 µm diameter) and smaller type Ib boutons (1–3 µm) (Atwood et al., 1993; Kurdyak et al., 1994), show similar evoked Ca\textsuperscript{2+} dynamics. A similar observation has been made previously in this system using the synthetic Ca\textsuperscript{2+}-sensitive dye fluo-3 AM in conjunction with confocal microscopy (Karunanithi et al., 1997); the authors suggested that factors other than differences in pre-synaptic Ca\textsuperscript{2+} entry may govern the physiological differences between both bouton types.

**Expression of yCam2 does not affect the physiological and morphological development of wild-type and mutant NMJs**

The Ca\textsuperscript{2+}-binding module of yCam2 is derived from the calcium-binding protein calmodulin (Miyawaki et al., 1997) and binds Ca\textsuperscript{2+} ions effectively over a broad range of physiological concentrations. This Ca\textsuperscript{2+}-buffering capacity of yCam2 raised the concern that strong transgenic overexpression of yCam2 could act as an additional intracellular Ca\textsuperscript{2+} buffer that may affect the physiology and development of yCam2-expressing cells and animals. We therefore examined the physiological and morphological development of NMJs with and without neuronal yCam2 expression. First we analyzed the basal junctional signal transmission by measuring the postsynaptic input resistance (Fig. 4C) and mEJCs and eEJCs (Fig. 4A,B). From these data we calculated the junctional quantal content (mean eEJC divided by mean mEJC) (Fig. 4C) as an estimate of the number of vesicles that are released in response to a presynaptic action potential. In addition we quantified the number of presynaptic boutons per NMJ (Fig. 5A,B) and examined the relationship between the number of boutons (NMJ size) and junctional quantal content (Fig. 5C). All of the above parameters showed no detectable difference between wild-type animals expressing yCam2 (elav-Cam) and those that did not (Fig. 4B,C, wild type). The decreased input resistance of muscles in C155-Cam animals and the associated slight increase in mEJC and eEJC amplitudes were likely caused by the somewhat larger muscle size of animals harboring the C155-chromosome (our unpublished observations); however, these alterations did not affect the quantal content of these NMJs (Fig. 4C). Thus, in accordance with a similar analysis performed in Caenorhabditis elegans (Kerr et al., 2000), strong yCam2 expression does not interfere with the cellular physiology or development of Drosophila NMJs.

We extended this analysis to two additional genotypes, both of which showed a similarly increased evoked release of pre-synaptic vesicles: animals with elevated subsynaptic translation (pabp\textsuperscript{p970/+}) (Sigrist et al., 2000) and animals with a postsynaptic defect attributable to the loss of the gene encoding the glutamate receptor subunit DGlur-IIA (dglurIIA\textsuperscript{A4D9}/df(2L)clh4) (Petersen et al., 1997). We found that the transgenic...
expression of yCam2 did not again affect the junctional phenotypes known from both mutants; pabp<sup>yCam2/+</sup> larvae that expressed yCam2 in all neurons (elav-Campabp) showed a typical strengthening of junctional signal transmission (Sigrist et al., 2000) as measured by larger eEJC amplitudes and unaltered mEJCs (Fig. 4B), resulting in a significantly increased junctional quanta content (Fig. 4C). elav-Campabp NMJs had also significantly more boutons compared with yCam2-expressing controls (elav-Cam) (Fig. 5B), whereas the muscle size and the muscle R<sub>p</sub> remained unaltered (Fig. 4C). Likewise, as reported previously for the DGluR-IIA-knock-out mutant alone (Petersen et al., 1997; DiAntonio et al., 1999), C155-CamdglurIIA animals showed a reduction of the average quanta size (mEJCs) and normal eEJC amplitudes (Fig. 4B), resulting in a similarly increased junctional quanta content (150%) as seen at elav-Campabp NMJs (Fig. 4C). Under the larval rearing conditions used in this study (see Materials and Methods), C155-CamdglurIIA animals and DGlur-IIA-knock-out mutants developed significantly smaller NMJs on muscles with unaltered R<sub>p</sub> than all other genotypes examined in this study (Fig. 5A,B). Thus, it appears that the yCam2 expression in all neurons does not interfere with the physiological or morphological development of even mutant NMJs and therefore allows a detailed analysis of presynaptic Ca<sup>2+</sup> dynamics in such mutant animals.

It is important to note that the junctional size and strength of elav-Campabp larvae and wild-type animals showed a characteristic proportional relationship (Fig. 5C), which has been described in several recent studies with independent genotypes (Cheung et al., 1999; Sigrist et al., 2000, 2002; Sanyal et al., 2002). NMJs with genetically strengthened signal transmission and unaltered muscle input resistance (R<sub>p</sub>) are proportionally larger than control NMJs (Sigrist et al., 2000). C155-CamdglurIIA animals, which apparently compensate for their postsynaptic defects by releasing more vesicles, represent an exception to this structure–function relationship (Fig. 5C, filled circle): during stimulation, these mutants release an increased number of vesicles from a significantly smaller NMJ. These obvious differences allowed us to address the question of how Drosophila NMJs may mediate persistently enhanced presynaptic vesicle release in different genotypes.

**Larger evoked Ca<sup>2+</sup> signals in presynaptic boutons of C155-CamdglurIIA NMJs compared with elav-Campabp and wild-type animals**

Because evoked vesicle release relies on stimulus-induced changes in presynaptic Ca<sup>2+</sup> influx (Mallart, 1993; Tank et al., 1995; Feller et al., 1996; Sinha et al., 1997; Catterall, 1998; Zucker, 1989, 1999), we hypothesized that the enhanced vesicle release in the above two mutants could be caused by an increased Ca<sup>2+</sup> influx at preexisting active zones (Mallart, 1993) or by an increased sensitivity of the presynaptic release machinery to Ca<sup>2+</sup> (Dawson-Scully et al., 2000). Alternatively, a larger number of released vesicles could also arise from a larger total number of active release sites per NMJ. Given that the mutants showed a similarly enhanced evoked vesicle release compared with wild-type NMJs but large differences in junctional bouton numbers, we attempted to differentiate between these possibilities by examining the spike train-evoked presynaptic Ca<sup>2+</sup> dynamics in these animals (Fig. 6).

Simultaneous EYFP/ECFP emission ratios (ΔR/R) of randomly chosen boutons showed an identical baseline before stimulation in all analyzed genotypes (Fig. 6), suggesting that there were no substantial differences in resting Ca<sup>2+</sup> levels of presynaptic terminals among all examined animals. During stimulation (Fig. 6, black lines), the mean ratio changes observed at boutons of elav-Cam and elav-Campabp animals were indistinguishable (Fig. 6A), indicating that stimulus-induced presynaptic Ca<sup>2+</sup> changes were similar in both genotypes. This observation demonstrates that the enhanced junctional signal transmission measured at elav-Campabp NMJs does not rely on a persistent increase in presynaptic Ca<sup>2+</sup> dynamics. Because all analyzed junctional boutons showed similar Ca<sup>2+</sup> signals during stimulation, it rather appeared that the larger number of released presynaptic vesicles seen in this mutant (Fig. 4C) could be attributable to the larger number of similarly contributing boutons (125% NMJ size) (Fig. 5C, triangle). This interpretation is consistent with the above-mentioned relationship of evoked junctional vesicle release and NMJ size, and it implies that long-term strength-


Enhancement of signal transmission at larval NMJs may be mediated primarily by the addition of active zones that are distributed in newly grown junctional boutons.

In contrast, yCam2 imaging in boutons of C155-CamdgurIIA animals revealed that ∆R/R values increased significantly during nerve stimulation compared with controls (127%) or other genotypes (Fig. 6). Given that the imaged presynaptic terminals of mutant and control animals showed no detectable differences in resting Ca²⁺ levels before and after stimulation, these data suggest that spike train-evoked Ca²⁺ influx into junctional boutons is strongly enhanced in C155-CamdgurIIA animals. This result indicates that either the evoked Ca²⁺ influx at given release sites is enhanced or that the number of responsive release sites within each bouton is increased. In addition it demonstrates for the first time that the compensation for the defective postsynaptic glutamate receptor function, which leads to an increased evoked vesicle release (Petersen et al., 1997; DiAntonio et al., 1999), is likely mediated by a retrograde signaling mechanism that results in an elevation of evoked Ca²⁺ influx into junctional boutons.

As mentioned above, the DGlur-IIA-knock-out mutant represents an exception to the structure–function relationship seen in several other genotypes (Cheung et al., 1999; Sigrist et al., 2000, 2002; Sanyal et al., 2002) because this mutant releases more vesicles (150% junctional quantal content) from a reduced number of boutons (80% NMJ size) (Fig. 5C, circle). The increased presynaptic Ca²⁺ influx per bouton is therefore compatible with the above idea that elevated evoked vesicle release may be mediated by an increased number of active zones, which in this genotype would be packed into a smaller number of boutons. Alternatively, because presynaptic vesicle release increases roughly with the third power of presynaptic Ca²⁺ (Tank et al., 1995; Feller et al., 1996), the elevated junctional quantal content of this mutant also may be linked to an upregulation of presynaptic Ca²⁺ dynamics at preexisting active zones. To differentiate between these possibilities, we performed an ultrastructural examination of NMJs of the genotypes used here.

Increased number of active zones at NMJs with enhanced vesicle release

A large body of evidence has suggested that NMJs of Drosophila larvae harbor a heterogeneous set of synapses that differ in their probability of presynaptic vesicle release (Atwood et al., 1993; Wojtowicz et al., 1994; Atwood and Wojtowicz, 1999). Synapses with a high probability of vesicle release appear to harbor presynaptic T-shaped dense bodies (“T-bars”) and were therefore termed “active zones” (Cooper et al., 1995, 1996), whereas T-bar-free synapses lacked the characteristic clusters of docked synaptic vesicles and are thought to represent sites of low vesicle release probability (Atwood et al., 1993; Wojtowicz et al., 1994; Atwood and Wojtowicz, 1999). To assess whether the increased evoked vesicle release seen in the above mutants is associated with such ultrastructural alterations of the synaptic morphology, we examined ultrathin sections of boutons from wild-type animals, DGlur-IIA-ko mutants (dgurIIA1;ldf(2L)clh4), and the pabp-mutant pabpP970/+;df(2R)Pcl7, which showed for all so far examined junctional aspects stronger phenotypes than the here used allele pabpP970/+ (Sigrist et al., 2000). From a random set of ultrathin sections of 4–16 type Ib bouton branches per genotype (see Materials and Methods), we scored the number of synaptic profiles (Fig. 7A, electron-dense area between arrowheads) per section, the presence or absence of presynaptic T-bar profiles (Fig. 7A, arrows), and the presynaptic terminal area per section (area of the vesicle-filled lumen of type Ib boutons) (Fig. 7B, Table 1).

Our analysis revealed that the number and density of presynaptic T-bar profiles were significantly increased in sections of
**Table 1. Summary of ultrastructural analysis**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>++</th>
<th>pabp^{pos}/Df(2R)Pc7b</th>
<th>DGluR-IIA-ko</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of branches analyzed</td>
<td>4</td>
<td>5</td>
<td>16</td>
</tr>
<tr>
<td>Number of scored ultrathin sections (~87 nm)</td>
<td>244</td>
<td>243</td>
<td>520</td>
</tr>
<tr>
<td>Covered length of NMJ (μm)</td>
<td>21.2</td>
<td>21.1</td>
<td>45.2</td>
</tr>
<tr>
<td>Number of scored synaptic profiles</td>
<td>628</td>
<td>561</td>
<td>872</td>
</tr>
<tr>
<td>Number of scored T-bar profiles</td>
<td>126</td>
<td>185</td>
<td>468</td>
</tr>
<tr>
<td>Mean terminal area (μm²)/section</td>
<td>0.8 ± 0.3</td>
<td>0.9 ± 0.3</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>Number of synaptic profiles/section</td>
<td>2.6 ± 0.9</td>
<td>2.3 ± 0.7</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td>Number of T-bar profiles/section</td>
<td>0.5 ± 0.4</td>
<td>0.8 ± 0.5</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>Number of synaptic profiles/terminal area</td>
<td>3.1 ± 0.6</td>
<td>2.6 ± 0.2</td>
<td>3.7 ± 0.3</td>
</tr>
<tr>
<td>Number of T-bar profiles/terminal area</td>
<td>0.6 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>Number of T-bar profiles/synaptic profile</td>
<td>0.21 ± 0.02</td>
<td>0.25 ± 0.05</td>
<td>0.53 ± 0.03</td>
</tr>
<tr>
<td>Relative density of synapses (%)</td>
<td>100 ± 19</td>
<td>84 ± 6</td>
<td>119 ± 10</td>
</tr>
<tr>
<td>Relative density of active zones (%)</td>
<td>100 ± 21</td>
<td>108 ± 23</td>
<td>310 ± 24</td>
</tr>
</tbody>
</table>

Summary of scored and extrapolated data obtained from randomly sampled ultrathin sections of type Ib boutons at larval NMJs of *Drosophila* (abdominal segment 2; see Materials and Methods). Data were collected and treated separately for each identified type Ib branch. Values represent branch means ± SEM. The relative densities of synapses and T-bars are equivalent to the numbers of synaptic profiles and T-bar profiles per terminal area, respectively, in which the wild-type (+/+) values are set to 100%.

**DGluR-IIA-ko** mutants compared with wild-type or pabp-mutant animals, both of which showed similar values in both aspects (Fig. 7C, Table 1). The **DGluR-IIA-ko** phenotypes were attributable to a significant increase in the number of sectioned T-bar profiles, a simultaneous reduction in the average presynaptic terminal size, and a concomitant reduction in the number of synaptic profiles (Fig. 7B, bars on right). These data demonstrate that the number of presynaptic T-bars is subject to retrograde regulation and suggest that the persistently enhanced evoked vesicle release seen in **DGluR-IIA-ko** animals is caused by an increased number of active zones with high vesicle release probability. In addition, the increased density of active zones was associated with a significantly enhanced evoked Ca²⁺ signal in these boutons, suggesting that T-bar harboring and vesicle-loaded synapses are the sites of spike train-evoked Ca²⁺ influx (Umbach et al., 1998a). We therefore conclude that the increased evoked vesicle release in **DGluR-IIA-ko** mutants relies on an increase in the number of active zones, which because of a junctional growth restriction are distributed at a higher density per bouton and therefore result in enhanced evoked Ca²⁺ signals. This interpretation is consistent with our ultrastructural, morphological, and physiological results obtained from pabp mutants. These mutants also showed an enhanced evoked vesicle release (Fig. 4) from NMJs with an increased total number of junctional boutons (Fig. 5). However, individual boutons harbored a wild-type density of active zones (Fig. 7) and thus resulted in evoked Ca²⁺ signals per bouton that were typical for wild-type animals (Fig. 6).

From these data we conclude that the persistently enhanced presynaptic vesicle release seen in the here-described mutants does not rely on a long-lasting increase in presynaptic Ca²⁺ dynamics at individual sites of vesicle release. Instead, it appears that the most important parameter is the recruitment of additional active zones. These either are packed into preexisting boutons (**DGluR-IIA-ko** mutants) or distributed in newly grown boutons, presumably to maintain a preferred density of active zones (wild-type animals and pabp mutants). The latter interpretation is consistent with results from a previous study performed in *Drosophila* and Sarcophaga (Meinertzhagen et al., 1998) that showed that active zones maintain a typical distance from each other, presumably to ensure unrestricted access to perisynaptic space.

**Figure 8.** Comparison of junctional signal transmission of the indicated genotypes during high-frequency stimulation. A. Representative average traces of eEJP recordings during repetitive stimulation at 7 Hz (9 stimuli). B. NMJs of elav-Camelpabp animals (▲) faithfully transmitted presynaptic stimuli at frequencies of up to 20 Hz. At this frequency, wild-type NMJs (●) showed considerable depression of postsynaptic potentials. C155-CamelpalglurIIA animals (●) showed strong depression of postsynaptic potentials at all examined stimulation frequencies. Data are given as means ± SEM.

**Faithful high-frequency signal transmission at larger NMJs**

The results described so far suggest that active zones and boutons represent functional compartments of NMJs that can be differentially regulated to persistently strengthen evoked vesicle release. To further assess the functional importance of junctional growth, synapse spacing, and presynaptic Ca²⁺, we analyzed eEJP amplitudes during repetitive synaptic signal transmission, a stimulus protocol that better resembled the neuronal activity pattern used for larval locomotion (Cattaert and Birman, 2001).

eEJP recordings during HF spike trains revealed that NMJs of wild-type and elav-Camelpabp animals performed faithful signal transmission with almost constant eEJP amplitudes at stimulation frequencies of up to 10 Hz (Fig. 8B). The larger NMJs of elav-Camelpabp animals, which also harbored a larger total number of active zones, continued to transmit without significant additional depression at higher stimulation frequencies (20 Hz),...
whereas wild-type NMJs showed a considerable depression of signal transmission. This form of short-term depression during HF stimulation has been analyzed extensively in this preparation (Adelsberger et al., 1997; Kuromi and Kidokoro, 1999) and in other preparations (DelCastillo and Katz, 1954; Trussell et al., 1993), and two factors have been proposed to be primarily responsible for this phenomenon: depletion of the readily releasable vesicle pool during HF stimulation (Kuromi and Kidokoro, 1999; Harata et al., 2001) and desensitization of postsynaptic neurotransmitter receptors (Trussell et al., 1993; Adelsberger et al., 1997; Heckmann and Dudel, 1997). Our above findings suggest that one or both of these factors became limiting during 20 Hz spike trains of wild-type NMJs, whereas NMJs of elav-Campapb animals continued to show almost constant eEJP amplitudes. It therefore appears that the addition of active zones and their distribution in additionally grown boutons ensures faithful signal transmission at high neuronal activity rates.

Interestingly, although DGluR-IIA-ko mutants release enough presynaptic vesicles on single action potentials to compensate almost completely for the postsynaptic defect of the DGluR-IIA-ko mutant (Fig. 4) (Petersen et al., 1997), this compensation failed to support efficient signal transmission of higher frequency stimuli. Instead, in all tested HF stimulation patterns, these animals showed a strong depression of postsynaptic responses that tended to saturate at ~60% of the initial eEJP amplitude (Fig. 8B, filled circles). A possible explanation for this enhanced depression of C155-CamdglttIIA NMJs lies in the fact that postsynaptic receptors show a strongly reduced time constant of desensitization in the absence of the DGluR-IIA subunit (Di-Antonio et al., 1999). This could lead to a reduced availability of responsive receptors. In addition, the high density of active zones in this mutant and the strongly increased evoked Ca$^{2+}$ influx into presynaptic boutons together could result in presynaptic Ca$^{2+}$-dependent effects (Zucker, 1989, 1999; Mallart, 1993; Dittman et al., 2000), including a fast depletion of the readily releasable vesicle pool during HF stimulation (Zucker, 1989, 1999; Mallart, 1993; Dittman et al., 2001; Truong et al., 2001), the expression of which can be directed precisely to target tissues, individual cells, and subcellular compartments (Miyawaki et al., 1999; Griesbeck et al., 2001). In this study we used yellow Cameleon-2 (Miyawaki et al., 1997) to establish transgenic Ca$^{2+}$ imaging in Drosophila and to analyze presynaptic function at larval NMJs.

Our experiments with transgenic yCam2 imaging produced results that compared well with a recent series of conventional Ca$^{2+}$ imaging studies. Both approaches faithfully detected stimulation-evoked Ca$^{2+}$ dynamics in boutons of Drosophila NMJs that depended on extracellular Ca$^{2+}$ concentration and stimulus frequency (Fig. 2D,E) (Karunanithi et al., 1997; Umbach et al., 1998a,b; Bronk et al., 2001; Dawson-Scully et al., 2000). The spike frequency and burst duration used to elicit plateauing deviations from resting fluorescence levels were also similar (5–40 Hz for 2–5 sec) (Figs. 2, 3, 7) (Karunanithi et al., 1997; Dawson-Scully et al., 2000; Bronk et al., 2001). In accordance with Karunanithi et al. (1997), the yCam2 fluorescence fluctuations showed no correlation to the size of the analyzed boutons (Fig. 2C2) despite the pronounced differences in synaptic transmission of the larger type Ib boutons and the smaller type Ia boutons (Kuryday et al., 1994; Lnenicka and Keshishian, 2000). One difference between these conventional Ca$^{2+}$ imaging experiments and our study was that we used thapsigargin to block intracellular Ca$^{2+}$ store function (Thastrup et al., 1990), a treatment that thus specifically reveals the evoked Ca$^{2+}$ influx from extracellular space (Kuromi and Kidokoro, 2002). Despite this, the Ca$^{2+}$ imaging data were mostly comparable, suggesting that evoked presynaptic Ca$^{2+}$ signals at larval NMJs originate primarily from Ca$^{2+}$ influx.

Synthetic dyes generally resulted in much higher brightness levels, faster responses, and much higher evoked fluorescence changes compared with yCam2 [synthetic dyes: 250% $\Delta F/F$ (Karunanithi et al., 1997; Dawson-Scully et al., 2000; Bronk et al., 2001); yCam2: 40% $\Delta R/R$ (Fig. 3C)]. Therefore synthetic indicators appear to be a superior tool for detecting small Ca$^{2+}$ signals in this preparation. This apparent disadvantage of using yCam2 was overcome, however, by the high specificity of the transgenic yCam2 expression in conjunction with simultaneous ratiometric measurements. Fluorescence alterations were never detected in the subsynaptic reticulum or the surrounding muscle (Figs. 2, 3), indicating that all fluorescence signals originated in presynaptic boutons and were not contaminated by postsynaptic Ca$^{2+}$ influx or muscular Ca$^{2+}$ release. This feature allowed the use of standard fluorescence microscopy and CCD cameras (Fig. 1B) and may explain the high reproducibility of the recorded yCam2 fluorescence changes (Figs. 2C, 6).

On the basis of these data obtained from the larval Drosophila NMJ, results from C. elegans (Kerr et al., 2000), and recent experiments using aequorin in mushroom body neurons of Drosophila (Rosay et al., 2001), it appears likely that the transgenic expression of recently developed genetically encoded reporter molecules will greatly facilitate future imaging studies in cells and cellular compartments that have been inaccessible so far.
Regulation of active zone number, density, and presynaptic Ca\(^{2+}\) dynamics during junctional development and strengthening

In this study we addressed the question of how NMJs of *Drosophila* larvae achieve the continuous enhancement of evoked vesicle release seen throughout their development and during activity-dependent strengthening. Using wild-type animals and two independent mutants that genetically represent both phases of junctional strengthening, we found that Ca\(^{2+}\)-dependent presynaptic mechanisms, which are known to result in fast and reversible modifications of presynaptic vesicle release (Mallart, 1993; Kamiya and Zucker, 1994; Zucker, 1989, 1999; Dittman et al., 2000), may provide only a minor or transient contribution to enhanced vesicle release during the development and long-term strengthening of junctional signal transmission (Fig. 6). Instead, a persistent enhancement of vesicle release relies primarily on the recruitment of active zones (Fig. 7). This conclusion was further supported by our yCam2-based Ca\(^{2+}\) imaging results, which together with our ultrastructural data and previous observations (Lnenicka and Keshishian, 2000; Harlow et al., 2001) revealed that evoked presynaptic Ca\(^{2+}\) signals correlate with the density of active zones. Our data therefore suggest that enhanced vesicle release is realized by a differential regulation of active zone density in different genotypes: NMJs of *dgglurIIA-ko* mutants compensate for their postsynaptic defect by packing more active zones into preexisting boutons. This leads to a functional compensation, which approaches homeostasis of evoked junctional signal transmission compared with wild type (Petersen et al., 1997) presumably to ensure muscle contraction and animal survival. In contrast, enhanced junctional signal transmission as seen in elav-Camparb animals is mediated by distributing added active zones into newly grown boutons. This leads to homeostasis of active zone density compared with wild-type controls and therefore may reflect the cellular basis of strengthened junctional signal transmission at *Drosophila* NMJs (Fig. 9).

Previous ultrastructural observations from other *Drosophila* mutants and larvae of the flesh fly *Sarcophaga bullata* have already suggested that the density of active zones is tightly regulated (Meinertzhagen et al., 1998; Sigrist et al., 2002), presumably to ensure that individual synapses have sufficient access to, e.g., reserve pool vesicles, vesicle recycling machinery, efficient Ca\(^{2+}\)-buffering systems, or neurotransmitter uptake mechanisms (Kuroi and Kidokoro, 2002). Our data from wild-type and elav-Camparb animals show a similar active zone density and evoked Ca\(^{2+}\) signals per bouton and thus suggest that individual boutons represent functional compartments that are likely to be maintained constant during junctional development and its strengthening. This seems to guarantee uncompromised signal transmission on a single bouton level. From these observations a model emerged that predicts that additional active zones need to be distributed in newly grown boutons (Fig. 9). This would explain the increasing number of genotypes that show a strict relationship between bouton number and transmission strength (Cheung et al., 1999; Sigrist et al., 2000, 2002; Sanyal et al., 2002). Intriguingly, in several other systems the recruitment of active synapses (Isaac et al., 1995; Liao et al., 1995; Bolshakov et al., 1997; Malenka and Nicoll, 1997; Feldman et al., 1999; Ponceur and Malinow, 2001) as well as local morphological alterations of synaptic compartments (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999) have been observed and are thought to represent long-lasting changes in the strength of synaptic communication.

On the basis of the above considerations, it appears surprising that *DGluR-IIA-ko* mutants pack the additional active zones in a smaller number of presynaptic boutons. This results in an increased density of active zones (Fig. 7), a larger stimulation-evoked Ca\(^{2+}\) entry per bouton (Fig. 6A), an enhanced evoked vesicle release, and a wild-type muscle depolarization on single action potentials (Fig. 4). These phenotypes show that mutants with impaired postsynaptic glutamate receptor function are capable of efficiently triggering the recruitment of active zones to compensate for their postsynaptic defect. However, it fails to induce the proportional outgrowth of new boutons that can be observed at wild-type NMJs and several other genotypes. Indeed, a recent analysis of the role of DGluR-IIA subunits in junctional development revealed that the increased expression of DGluR-IIA is sufficient to induce bouton outgrowth (Sigrist et al., 2002). Although it is currently not clear why *DGluR-IIA-ko* mutants accumulate active zones at such an unusual density (Fig. 7), it appears that this mechanism alone is not sufficient to ensure uncompromised repetitive signal transmission (Fig. 8). Although the latter may be attributable to increased postsynaptic desensitization in...
this mutant (Heckmann et al., 1996; Adelsberger et al., 1997; Heckmann and Dudel, 1997; DiAntonio et al., 1999), presynaptic factors like the depletion of the readily releasable vesicle pool (Dohrn and Stevens, 1999; Kuromi and Kidokoro, 1999) also appear likely to contribute to this observation. It is therefore tempting to speculate that this mutant is trapped in a transient phase of junctional strengthening (Fig. 9).

According to such a model (Fig. 9), a postsynaptic sensor (Paradis et al., 2001) would trigger signals that control the recruitment of active zones. The transiently increased density of active zones would trigger a second signal that instructs the resetting of active zone density by distributing them into newly grown boutons. Intriguingly, the chronically hyperactive mutant eng. Sh represents a precedence for this scenario because it shows, presumably because of the continuous hyperactivity stimulus, an increased density of T-bar-harboring active zones (Jia et al., 1993) in an already increased number of junctional boutons (Budnik et al., 1999). These findings provide further evidence for the suggestion that developmental processes and activity-dependent phenomena may use closely related mechanisms (Goodman and Shatz, 1993; O'Leary, 1994).

REFERENCES


