Hebbian Plasticity Guides Maturation of Glutamate Receptor Fields In Vivo

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http://dx.doi.org/10.1016/j.celrep.2013.04.003

SUMMARY

Synaptic plasticity shapes the development of functional neural circuits and provides a basis for cellular models of learning and memory. Hebbian plasticity describes an activity-dependent change in synaptic strength that is input-specific and depends on correlated pre- and postsynaptic activity. Although it is recognized that synaptic activity and synapse development are intimately linked, our mechanistic understanding of the coupling is far from complete. Using Channelrhodopsin-2 to evoke activity in vivo, we investigated synaptic plasticity at the glutamatergic Drosophila neuromuscular junction. Remarkably, correlated pre- and postsynaptic stimulation increased postsynaptic sensitivity by promoting synapse-specific recruitment of GluR-IIA-type glutamate receptor subunits into postsynaptic receptor fields. Conversely, GluR-IIA was rapidly removed from synapses whose activity failed to evoke substantial postsynaptic depolarization. Uniting these results with developmental GluR-IIA dynamics provides a comprehensive physiological concept of how Hebbian plasticity guides synaptic maturation and sparse transmitter release controls the stabilization of the molecular composition of individual synapses.

INTRODUCTION

The ability of chemical synapses to change their structural, functional, and molecular properties in an activity-dependent manner has attracted considerable scientific interest over the past few decades (Kandel, 2009). Originally put forward as a theory by Donald O. Hebb (Hebb, 1949), today the term “Hebbian synaptic plasticity” commonly describes a change in synaptic strength that depends on correlated pre- and postsynaptic neuronal activity and acts independently at individual synapses. Thus, Hebbian plasticity represents a powerful synaptic learning rule that provides an attractive subcellular mechanism for models of neuronal network formation, learning, and memory (Abbott and Nelson, 2000). Descriptions of synaptic long-term potentiation (LTP) and long-term depression (LTD) have provided experimental examples of Hebbian plasticity and emphasized the pivotal role of the temporal order of pre- and postsynaptic activity in determining the polarity of synaptic changes (i.e., potentiation or depression; Feldman, 2012).

The excellent genetic accessibility of the glutamatergic Drosophila neuromuscular junction (NMJ) has contributed to its popularity as an experimental system for identifying the molecular mechanisms that govern synaptic function (Bellen et al., 2010). Due to its rapid growth, the larval NMJ exhibits a high degree of developmental synaptic plasticity. Positive and negative feedback loops operate on neuronal structure and presynaptic function in a manner that appears to depend on the duration and site of the activity alteration. Elevated motorneuron activity promotes the growth of neuromuscular boutons (Ataman et al., 2008; Budnik et al., 1990; Sigrist et al., 2003), and both decreased postsynaptic and increased presynaptic excitation facilitate transmitter release from active zones (DiAntonio et al., 1999; Frank et al., 2006; Paradis et al., 2001; Sigrist et al., 2003; Steinert et al., 2006).

The application of in vivo imaging at the Drosophila NMJ has provided valuable information about the developmental maturation of synapses in an intact organism (Rasse et al., 2005; Schmid et al., 2008). During this process, non-NMDA-type ionotropic glutamate receptors (GluRs) assemble as heterotetramers of GluR-II/C/III, IID, and IIE subunits plus either GluR-IIA or GluR-IIB (Featherstone et al., 2005; Marrus et al., 2004; Qin et al., 2005). These two receptor complexes differ markedly in their physiological parameters: GluR-IIB-type receptors desensitize far more rapidly than receptors containing GluR-IIA (DiAntonio et al., 1999). At individual synapses, receptor fields initially grow by incorporating GluR-IIA-type receptors. As the corresponding presynaptic active zone matures, incorporation then shifts toward GluR-IIB, until an even ratio of IIA and IIB is reached at mature synapses (Schmid et al., 2008). A mechanistic understanding of synapse development will require identification of the physiological cues that guide such molecular dynamics. Although at present the identity of these cues remains largely elusive, several lines of evidence indicate that synaptic activity itself is highly influential.

Whereas the total number of receptors is largest opposite active zones that possess a high neurotransmitter release probability, p, (Marrus and DiAntonio, 2004), the relative contribution of GluR-IIA is highest opposite low-p, sites (Schmid et al., 2008). These observations are consistent with subunit dynamics during synapse development and suggest a form of synaptic scaling, i.e., a compensatory effect exerted by the large current-passing...
capacity of GluR-IIA. However, taking chronic changes in neuronal activity into account produces a more complex picture, as sustained increases in neuronal activity, induced either genetically or through elevated locomotion, globally raise synaptic GluR-IIA levels (Sigrist et al., 2000, 2003).

Our goal in this study was to improve our mechanistic understanding of how activity-dependent synaptic plasticity is linked to the development of glutamatergic synapses. To this end, we employed optogenetics at the developing larval Drosophila NMJ. We chose to use Channelrhodopsin-2 (ChR2; Nagel et al., 2003) because this enabled us to induce cellular activity in vivo in an acute, quantifiable manner and direct it solely at pre- or postsynaptic compartments, or at both compartments simultaneously. Focusing on postsynaptic GluRs, our results unveil a Hebbian mode of GluR-IIA incorporation at postsynaptic sites that is counteracted by GluR-IIA removal when synaptic transmission fails to evoke considerable muscle depolarization. We report rapid receptor mobilization rates that were previously undetected by in vivo time-lapse imaging (Rasse et al., 2005; Schmid et al., 2008) and provide a comprehensive physiological picture of how activity-dependent plasticity controls the molecular maturation and stabilization of individual synapses.

RESULTS

Rapid GluR Removal from Synapses

In two-electrode voltage clamp (TEVC) recordings from larval Drosophila NMJs (Figure 1A), we observed a kinetic change of synaptic currents during low-frequency nerve stimulation. Specifically, the decay time constant ($\tau_{\text{decay}}$) of evoked excitatory junctional currents (eEJCs) decreased within 20 min (Figure 1B; $\tau_{\text{decay}}$ at t = 0: 4.99 ± 0.14 ms; t = 20 min: 3.86 ± 0.10; n = 10, p < 0.001 paired t test). The proportion of slowly desensitizing IIA-type GluRs in postsynaptic densities (PSDs) correlates with the length of the $\tau_{\text{decay}}$ of synaptic currents (Schmid et al., 2008).

To examine whether the electrophysiological signature correlated with the molecular composition of PSDs, NMJs were stained against GluR-IIA. Following nerve stimulation, GluR-IIA clusters were significantly smaller in voltage-clamped muscle 6 (M6) compared with its neighboring muscle, M7 (Figure 1C; M7: 0.212 ± 0.030 $\mu$m$^2$; M6: 0.163 ± 0.031 $\mu$m$^2$; n = 6, $p = 0.001$ paired t test). M6 and M7 are innervated by the same motorneurons. Because the membrane potential of only M6 was held constant, these results indicate that preventing postsynaptic depolarization during neurotransmission drove GluR-IIA out of PSDs (there was no difference in the average GluR-IIA cluster size between M6 and M7 on the contralateral, unstimulated side; $p = 0.495$ paired t test, data not shown).

Such rapid GluR mobilization has not previously been observed during the development of this synaptic system in vivo (Rasse et al., 2005; Schmid et al., 2008). We therefore employed ChR2 to obtain a better understanding of the specific role of activity in controlling GluR dynamics in the intact organism.

Quantification of ChR2 Expression and Activity

To attain independent functional control over pre- and postsynaptic compartments, we employed the bipartite Gal4/UAS system (Brand and Perrimon, 1993) for tissue-specific expression of ChR2 (Schroll et al., 2006). Specifically, ChR2 was driven presynaptically in motorneurons (ok6-gal4 > UAS-chop2; “Pre”), postsynaptically in muscles (g7-gal4 > UAS-chop2; “Post”), or in both compartments simultaneously (ok6-gal4 & g7-gal4 > UAS-chop2; “Pre & Post”; Figure 2A). Genetically expressed channelopsin-2 (Chop2) requires addition of its chromophore all-trans-retinal (RAL) to deliver functional ChR2 (Nagel et al., 2003). Raising Drosophila larvae under standard conditions (defined here as 25 °C and 0.1 mM RAL food supplement) yielded the expected distributions of ChR2 immunoreactivity (Figure S1). TEVC recordings were employed to measure photocurrents. Activation of ChR2 in motorneurons (Pre) elicited light-evoked EJCs (EJCs), activation in muscles gave rise to postsynaptic steady-state currents (Post), and combined activation (Pre & Post) produced composite photocurrents (Figure S2).

Activity-Induced Functional Synaptic Plasticity In Vivo

To test for activity-dependent induction of synaptic plasticity, we subjected fully intact, freely moving larvae to light stimulation (peak ~460 nm; Figure S3A), which evoked visible muscle contractions in vivo (Figure 2B). Based on the quantification of ChR2 function (Figure S2), we chose an intermediate irradiance (1.7 mW/mm$^2$ at 460 nm; also for all subsequent experiments), and applied the light according to an established protocol under
standard conditions, though likely with higher light intensity than previously reported (Figure S3B; Movie S1; Ataman et al., 2008). Following the 100-min-long protocol, the larvae were dissected for synaptic structure-function analyses. Importantly, the dependence of functional ChR2 on RAL enabled us to use larvae that were not fed RAL as controls. These larvae were of the same genotype as the respective experimental groups and were also subjected to light stimulation. Hence, the experiments measured effects that were specifically mediated by ChR2 function and could not be attributed to the genetic background or an unspecific influence of light application. Correspondingly, lEJCs could not be triggered when RAL was omitted, and the controls of all three genotypes failed to display light-induced muscle contractions during application of the plasticity protocol (data not shown).

In all three genotypes, neither the NMJ size nor the numbers or average sizes of presynaptic active zones (recognized by the central active zone protein Bruchpilot [BRP]; Wagh et al., 2006) or postsynaptic receptor fields (identified via the universal GluR-IID subunit) were influenced by light stimulation in vivo (Figures S4A–S4C; Table S1). In contrast, electrophysiological recordings revealed pronounced activity-induced functional changes (Figures 2C–2F; Table S2). Solely pre- or postsynaptic or combined activation of ChR2 in vivo led to a significant reduction in the number of vesicles released from active zones per action potential (quantal content; Figure 2D; Pre control: 105 ± 13 vesicles, n = 10 NMJs, Pre RAL: 66 ± 11 vesicles, n = 10, p = 0.021 rank sum test (rs test); post control: 98 ± 6 vesicles, n = 11, post RAL: 68 ± 6 vesicles, n = 11, p = 0.002 t test; Pre & post control: 150 ± 10 vesicles, n = 11 NMJs, Pre & post RAL: 109 ± 13 vesicles, n = 10, p = 0.02 t test). Crucially, a second form of plasticity was induced specifically by combined pre- and postsynaptic ChR2 stimulation. This was manifested in a kinetic change of currents evoked by action potentials (Figures 2C–2F; Table S2; control eEJCs $\tau_{decay}$ 3.87 ± 0.23 ms, n = 11 NMJs, Pre & Post eEJCs $\tau_{decay}$ 5.08 ± 0.32 ms, n = 10, p = 0.006 t test; control minis $\tau_{decay}$ 4.72 ± 0.40 ms, n = 11, $\tau_{decay}$ 4.5 ms, n = 11, $\tau_{decay}$ 7.8 ms, n = 11, $\tau_{decay}$ 10 ms, n = 11)
Pre & Post minis $\tau_{\text{decay}}$ 6.05 ± 0.42 ms, $n = 11$, $p = 0.01$ rs test. The protraction of minis produced a substantially larger quantal charge (~60% increase; Table S2), which in turn gave rise to a normal compound charge transfer (control eEJCs: 483 ± 52 pC, $n = 11$ NMJs; Pre & Post eEJCs: 514 ± 67 pC, $n = 10$; $p = 0.504$ rs test) despite the reduced quantal content. Hence, correlated pre- and postsynaptic activity gave rise to functional plasticity at the level of quantal transmission.

**Connecting Synaptic Structure and Function**

Motivated by the causal link between rapid current decay and low synaptic GluR-IIA levels (Figures 1B and 1C; DiAntonio et al., 1999; Schmid et al., 2008), we examined the receptor subunit composition of Pre & Post PSDs. In agreement with the electrophysiological data, NMJs subjected to correlated pre- and postsynaptic activity displayed more GluR-IIA clusters than the unstimulated controls (Figure 3B; control 481 ± 54 clusters, $n = 25$ NMJs; Pre & Post 742 ± 67 clusters, $n = 21$, $p = 0.002$ rs test). Furthermore, this effect was accompanied by a significant increase in the average size of GluR-IIA accumulations (Figures 3A–3C; control 0.173 ± 0.009 µm², $n = 25$ NMJs; Pre & Post 0.244 ± 0.007 µm², $n = 21$, $p < 0.001$ t test). In contrast, neither the number (control 472 ± 21 clusters, $n = 30$ NMJs; Pre & Post 523 ± 28 clusters, $n = 29$; $p = 0.152$ t test) nor the average size of the GluR-IIB clusters (Figures S4D and S4E; control 0.140 ± 0.003 µm², $n = 30$ NMJs; Pre & Post 0.150 ± 0.005 µm², $n = 29$; $p = 0.063$ t test) were significantly influenced by activity. Thus, combined pre- and postsynaptic stimulation led to a specific increase of GluR-IIA-containing receptors in PSDs.

**Correlative Activity Triggers GluR-IIA Incorporation**

The combined depolarization of motorneuron and muscle evoked more current flow over the postsynaptic membrane than did isolated pre- or postsynaptic activation. Thus, we set out to test whether GluR-IIA-mediated synaptic plasticity was caused merely by stronger stimulation or instead reflected the correlative nature of combined pre- and postsynaptic activation. To this end, we reduced the light pulse duration from 2 s to 15 ms (Figure S5C) while preserving the correlative property of the stimulation (Figures S5A and S5B). Briefly synchronized pre- and postsynaptic activity in vivo did not produce a change in quantal content or current decay (Figure S5D). However, the number of GluR-IIA clusters increased from 576 ± 39 in controls ($n = 25$ NMJs) to 708 ± 51 in Pre & Post larvae ($n = 17$, $p = 0.045$ t test; Figure 3E). Hence, briefly correlated pre- and postsynaptic activity was sufficient to increase GluR-IIA levels in PSDs. Plotting the distribution of GluR-IIA cluster sizes (Figure 3D) illustrates that short pulses led to a uniform increase in the number of clusters (stable mean cluster size), whereas long light pulses produced bigger average clusters (Figure 3B) by generating a greater increase in the number of large clusters (Figure 3C).

**GluR-IIA Incorporation Is Synapse Specific**

In addition to its correlative quality, a defining feature of Hebbian plasticity is its synapse-specific action (Abbott and Nelson, 2000; Hebb, 1949). In order to examine whether GluR-IIA-mediated synaptic plasticity met this criterion at the NMJ, we again expressed ChR2 in motorneurons but this time in combination with a GAL4 line that drives expression only in M12 (Figures 4A and S6). This enabled us to compare activity-induced effects on synapses that experienced either solely presynaptic or combined pre- and postsynaptic stimulation. The analyzed synapses are in close proximity to each other and are formed by both shared and unique motorneurons (Hoang and Chiba, 2001). Hence, this experimental setup provided an ideal internal control for variations arising from differences between individuals. Larvae (Pre & M12-Post) were subjected to the short-pulse protocol in vivo. In control animals, GluR-IIA clusters were significantly smaller in M12 than in the adjacent M13 (M12: 0.195 ± 0.007 µm²; M13: 0.214 ± 0.010 µm²; $n = 18$, $p = 0.011$ paired t test; Figure S5C). While the mean cluster size (control 0.300 ± 0.009 µm²; $n = 18$; $p = 0.010$ rs test) was in line with the standard protocol or with the short pulse protocol (controls in gray; Figure S5S), Inssets show relative change in receptor numbers (Δ).

Figure 3. Correlated Activity Drives Selective Incorporation of GluR-IIA into PSDs

(A) Representative stainings of GluR-IIA clusters (red).

(B) Quantification of GluR-IIA clusters. After ChR2 stimulation (standard protocol, 2 s pulses), GluR-IIA clusters were significantly increased in size and number at Pre & Post NMJs (blue) compared with controls (gray).

(C and D) Mean distributions of GluR-IIA cluster sizes at Pre & Post NMJs (blue) following stimulation with the standard protocol or with the short pulse protocol (controls in gray; Figure S5S). Insets show relative change in receptor numbers (Δ).

(E) Compared with controls, more GluR-IIA clusters were detected at Pre & Post NMJs following brief paired stimulation. Scale bar, 1 µm. Error bars represent SEM.
However, when synapses on M12 repeatedly experienced correlated pre- and postsynaptic stimulation, their size was selectively increased compared with synapses on M13, which had received only presynaptic stimulation and showed no increase in size (M12: 0.226 ± 0.011 μm²; M13: 0.218 ± 0.013 μm²; n = 13, p = 0.415 paired t test; Figure 4B). These results support a synapse-specific mechanism of correlational plasticity that acts locally enough to discriminate between synapses formed on adjacent muscles.

**DISCUSSION**

**Activity-Induced Plasticity**

Repeated light-triggered neurotransmitter release from presynaptic active zones provoked synaptic depression via a decrease in quantal content. Interestingly, muscle depolarization itself also led to a drop in quantal content despite bypassing synapses (Post animals). The latter observation is highly reminiscent of homeostatic communication whereby a retrograde pathway of inverted polarity operates to increase quantal content in response to reduced muscle excitability (DiAntonio et al., 1999; Frank et al., 2006; Paradis et al., 2001). Future studies can now test whether molecular components involved in the homeostatic upregulation of quantal content (Dickman and Davis, 2009) also contribute to its downregulation following postsynaptic ChR2 stimulation.

Pairing pre- and postsynaptic depolarizations repetitively (Pre & Post) triggered a synapse-specific increase in postsynaptic GluR-IIA-type GluRs. Hence, correlated activity initiated a Hebbian form of synaptic plasticity at the Drosophila NMJ. A comparison of the cluster size distributions following brief and long pulses (Figures 3C and 3D) suggests two phases of plasticity. The first phase of activity-induced plasticity (15 ms pulses) promotes an evenly distributed increase in the number of clusters. Therefore, despite an increase in total number, the average size of GluR-IIA clusters is not significantly altered. The next phase (2 s pulses) then leads to an increase mainly in the number of large clusters, and hence the average GluR-IIA cluster size increases (Figure 3B). Neurotransmitter ρ, varies across active zones at the NMJ (Peled and Isacoff, 2011). Because the size of GluR clusters is largest opposite high-ρ, active zones (Marrus and DiAntonio, 2004), it is to be expected that functional

Figure 4. Input-Specific Induction of Hebbian Synaptic Plasticity Completes a Comprehensive Mechanism of Activity-Dependent Receptor Dynamics

(A) Dissected larva expressing EGFP under control of ok6-gal4 & m12-gal4 (arrow indicates CNS, arrowhead points to M12). Double staining against HRP (gray) and ChR2 (blue) at Pre & M12-Post NMJs of M12 and M13 (Figure S6). Scale bars, 1 mm (left) and 20 μm (right).

(B) Comparison of GluR-IIA cluster sizes on M12 and M13 following the short-pulse protocol in control and RAL-fed Pre & M12-Post larvae. Adjacent muscles are connected by a line (gray, individual larvae; black, mean values). GluR-IIA clusters were significantly smaller on M12 than on M13 in controls, but attained an equal size in RAL-fed Pre & M12-Post larvae after the short-pulse protocol. Error bars represent SEM.

(C) Model of activity-dependent GluR-IIA dynamics. Sparse activity occurring only at high-ρ, active zones (high BRP content) induces only weak muscle depolarization and triggers synapse-specific GluR-IIA exit (dotted lines). Synchronized synaptic exocytosis induces strong muscle depolarization and triggers Hebbian GluR-IIA incorporation at all active synapses (arrowheads).
recordings of synaptic currents preferentially sample large receptor fields. For this reason, GluR-IIA incorporation likely remained below the detection threshold in electrophysiological recordings following brief light pulses (Figure S5D).

In view of the unchanged total number of receptor fields (anti-GluR-IID staining) and active zones (Figure S4B), paired stimulation did not appear to give rise to the formation of new synapses. Instead, GluR-IIA was likely incorporated into receptor fields with previously undetectable IIA levels.

**Linking Developmental and Activity-Dependent Synaptic Plasticity**

In vivo imaging suggests that positive feedback initially promotes GluR-IIA incorporation during synapse growth and that GluR-IIA entry is specifically restrained with further maturation, whereas the rate of GluR-IIB recruitment remains constant (Schmid et al., 2008). The physiological signals that guide these synapse-specific molecular dynamics are unknown. We argue that the Hebbian mechanism identified in the present study represents the signal that promotes GluR-IIA entry during synapse development. This is consistent with the observed increase in small clusters following short pulses. Furthermore, in this framework, paired pre- and postsynaptic stimulation would be able to override the inhibition of GluR-IIA incorporation at relatively mature receptor fields (Figure 3C) and thereby restore the “juvenile behavior” of the PSDs.

At the developing *Drosophila* NMJ, receptor field growth is accompanied by BRP-dependent, active zone maturation (Schmid et al., 2008). Correspondingly, large receptor fields are found opposite high-\(p\_\)active zones that are rich in BRP (Marrus and DiAntonio, 2004). Therefore, small, growing receptor fields opposite immature, low-\(p\_\)active zones will tend to be exposed to glutamate only when \(p\_\)is elevated, e.g., during trains of action potentials. Because a large number of other synapses will also be active at these time points, transmitter release will coincide with strong postsynaptic depolarization, leading to Hebbian GluR-IIA incorporation.

A comprehensive model conversely demands a signal to remove GluR-IIA from mature receptor fields in order to describe their diminished rate of IIA incorporation in vivo and to limit receptor-field growth. We reason that such a physiological cue could be provided by sparse (i.e., unsynchronized) transmitter release that preferentially occurs at high-\(p\_\), mature synapses and does not trigger substantial muscle depolarization. This hypothesis is experimentally supported by GluR-IIA removal from synapses when muscle depolarization is prevented during neurotransmission (Figure 1).

Here, we introduce a physiological model (Figure 4C) in which GluR-IIA is increased at simultaneously active synapses via Hebbian plasticity and is decreased at solitarily active synapses. Such solitary activity may be provided by spontaneous transmitter release (i.e., minis). The physiological function of minis has been controversially discussed (Verstreken and Bellen, 2002). Our results suggest that they contribute to “taming the beast” (Abbott and Nelson, 2000); in other words, restraining the extent of Hebbian plasticity. Our model can account for developmental, synapse-specific receptor subunit dynamics, and explains why GluR-IIA levels are higher opposite low-\(p\_\), Ib motorneurons than opposite high-\(p\_\), Is motorneurons (Schmid et al., 2008). This conceptual framework can account for an increase in GluR-IIA following chronic activity elevation (Sigrist et al., 2000, 2003) and is consistent with low synaptic IIA levels in the presence of ambient extracellular glutamate, although, intriguingly, sustained glutamate exposure also affects GluR-IIB (Augustin et al., 2007).

Trains of action potentials are likely the physiological equivalent of paired pre- and postsynaptic depolarization, which simply triggers the Hebbian change more efficiently than solely presynaptic ChR2 stimulation. Notably, rapid GluR-IIA exit can be acutely provoked (Figure 1). This observation is compatible with fast GluR dynamics in mammals, which can operate on a timescale of minutes and well below (Heine et al., 2008). Hence, rapid receptor trafficking also occurs in *Drosophila*, though this probably remains concealed when receptor exit is not explicitly provoked during time-lapse imaging of synaptic development in vivo (Rasse et al., 2005; Schmid et al., 2008).

Perhaps most conspicuously, activity-dependent bidirectional GluR-IIA mobility is reminiscent of subunit-specific AMPA receptor trafficking at mammalian central synapses, which mediates manifold forms of synaptic plasticity (Malinow and Malenka, 2002). Local activity has been shown to drive synapse-specific accumulation of GluR1 AMPA receptors (Ehlers et al., 2007). Whereas high-frequency stimulation triggers LTP and synaptic GluR1 incorporation, low-frequency stimulation triggers LTD and GluR1 removal (Shepherd and Huganir, 2007). Collectively, these considerations support the notion that fundamental mechanisms of synaptic plasticity have been strongly conserved during evolution (Glanzman, 2010).

**EXPERIMENTAL PROCEDURES**

TEVC recordings, stainings, and image analysis were performed essentially as previously described (Schmid et al., 2008). For application of the activity protocol in vivo, freely moving larvae were stimulated with a blue LED. In the figures, the level of significance is marked with asterisks (*\(p \leq 0.05\); **\(p \leq 0.01\); ***\(p \leq 0.001\)). Detailed methods are available in Extended Experimental Procedures.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Extended Experimental Procedures, six figures, two tables, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.04.003.

**LICENSING INFORMATION**

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**ACKNOWLEDGMENTS**

We thank G. Nagel and C. Stangi for supporting the irradiance measurements; E. Buchner, A. DiAntonio, A. Fiala, and S.J. Sigrist for providing fly stocks and reagents; U. Ashery, S. Hallermann, M. Heckmann, and T. Langenhan for scientific discussions; and C. Wirth for technical assistance. This work was supported by grants from the DFG (Emmy Noether Kl 1460/1-1 to R.J.K.) and the GSLS, University of Würzburg (to N.E.).

REFERENCES

EXTENDED EXPERIMENTAL PROCEDURES

Genotypes and RAL Supplementation
All experiments were performed on male third instar larvae. The following genotypes were used: w 1118, ‘Pre’: ok6-gal4/+; UAS-chop2/+; ‘Post’: g7-gal4/+; UAS-chop2/+; ‘Pre & Post’: ok6-gal4/g7-gal4; UAS-chop2/+; ‘Pre & M12-Post’: ok6-gal4/+; m12-gal4/UAS-chop2, to visualize expression (Figure 4A) y/UAS-2xEGFP; ok6-gal4/+; m12-gal4/UAS-CD8::GFP.

RAL was dissolved in pure ethanol yielding a 50 mM stock, which was mixed into the food slurry to give the desired concentration. Controls received the corresponding volume of pure ethanol. Larvae were raised at indicated temperatures in the dark. Standard conditions: 25 °C, 0.1 mM RAL, enhanced expression conditions: 29 °C, 1 mM RAL.

ChR2-Mediated Stimulation
In semi-intact preparations IEJCs were measured by placing dissected larvae under the objective of an upright microscope (Nikon Eclipse FN1). Light from a mercury lamp (Nikon intensilight C-HGFI) passed a GFP band-pass filter (460-500 nm) to stimulate ChR2. Irradiance was adjusted by neutral density filters. IEJCs (‘Pre’, ‘Pre & Post’) and ChR2-mediated currents in the muscle (‘Post’) were quantified by applying 2 s light pulses at 0.2 Hz while the muscle was held in TEVC. ‘Post’ amplitudes were measured at 0.3 s after onset of the light pulse.

For application of the activity protocol in vivo, larvae were washed and transferred to the stimulation arena: a perforated hollow plastic cylinder (1.3 cm diameter, 0.8 cm height) placed on a moist filter paper. Light was applied through the glass cover, using a 3 W LED lamp (LEDxON®, Germany) producing an irradiance of 1.7 mW/mm² at 460 nm homogeneously in the arena. To ensure stimulation at room temperature (19 °C - 23 °C), the arena was cooled using a large water reservoir. Under these conditions, all experimental groups exhibited visible muscle contractions upon illumination. Control groups (not fed RAL) exhibited neither muscle contractions nor electrophysiologically measured IEJCs, even when raised at 29 °C and subjected to highest light intensities.

For electrophysiological recordings, larvae were dissected as soon as the 100 min protocol ended. For stainings, 3-4 controls and 3-4 RAL fed larvae, separated by a septum in the arena, were stimulated simultaneously, dissected and fixed within 1 hr after the end of the protocol.

Semi-Intact Preparation
Semi-intact preparations of late third instar larvae were obtained as follows. Animals were dissected in ice-cold calcium free HL-3 (Stewart et al., 1994) or HL-3.1 to match the recording solution (Feng et al., 2004). HL-3 was used for all stainings. The larva was immobilized, dorsal side up, on a Sylgard® pad with two sharp pins. A cut was made along the dorsal midline and after pinning down the body wall, the internal organs were removed carefully while severing the tracheal connections to the muscles. For measurements of IEJCs the ventral ganglion (VG) was left intact while the rest of the CNS was removed to reduce endogenous input to motorneurons. For measurements of eEJCs the nerves innervating muscles were carefully cut and the entire CNS was removed.

Immunohistochemistry
Staining followed a standard protocol (Schmid et al., 2008). In brief, larvae were fixed for 5 min in –20 °C methanol (for GluR-IIA stainings) or for 10 min in ice-cold 4% paraformaldehyde (in PBS; for all other stainings). Unspecific blocking was blocked with 5% normal goat serum (Jackson Immunoresearch) in PBT (PBS with 0.05% Triton). Primary antibodies were used in the following dilutions: mouse anti-ChR2 (supernatant 1:1, 15E2 mfd Diagnostics in cooperation with the E. Bamberg lab; Kleinlogel et al., 2011), mouse anti-BRP (1:250, nc82, a gift from E. Buchner), mouse anti-GluR-IIA (1:100 or 1:200, 8B4D2 Developmental Studies Hybridoma Bank, University of Iowa), rabbit anti-GluR-IIB (1:2500, a gift from A. DiAntonio), rabbit anti-GluR-IID (1:1000, a gift from S.J. Sigrist). All secondary antibodies were diluted 1:250: Alexa 488 goat anti-rabbit and goat anti-mouse (Invitrogen, USA), Cy3 goat anti-rabbit and goat anti-mouse (Dianova, Germany), Atto 647N goat anti-mouse (Attotec, Germany), directly conjugated goat anti-HRP-Cy3 and goat anti-HRP-Cy5 (Dianova, Germany).

Imaging and Analysis
ChR2 stainings were imaged with a line scanning confocal LSM 5 system (Zeiss) with a 1.25 numerical aperture, 63x oil immersion objective. Confocal stacks were acquired with a voxel size of 100x100x400 nm xyz (for NMJs) and 100x100x1000 nm xyz (for ventral ganglia). Synaptic markers were imaged with a line scanning confocal SP5 system (Leica Microsystems) with a 1.4 numerical aperture, 100x oil immersion objective. Voxel size was set to 75x75x420 nm xyz. To ensure comparability, all larvae from one experiment were stained in the same vial and imaged in the same session. Acquisition alternated between control and RAL fed groups and was performed under equal laser and imaging settings. The mean background intensity was never significantly different between control and RAL fed groups.

Image analysis was carried out in ImageJ (rsbweb.nih.gov/ij/) essentially as previously described (Schmid et al., 2008). In brief, for BRP spots or receptor clusters, the image background was subtracted from maximum projections of confocal stacks before applying a Gaussian blur (0.9 pixel). Images with a mean background intensity > 25 arbitrary units were excluded from the analysis. Images were not normalized. After thresholding and removing non-synaptic staining manually, single spots were detected with the ‘Find
Maxima' command and analyzed via 'Analyze Particles' to deliver information on size and number. To avoid analysis of unspecific staining a minimum size-threshold was applied. The same procedure was followed to determine the area covered by HRP using the 'Measure' command.

Apart from background subtraction and removal of non-synaptic staining all steps were automated. Identical analysis settings were applied for each fluorescent channel in the same experiment. In combination with equal laser power and imaging settings, a genuinely quantitative analysis of synaptic markers was feasible. For muscles 12 and 13, synapses belonging to shared and unique motorneurons were combined in the analysis.

In the figures, example images are maximum projections of confocal stacks, background subtracted, blurred and normalized. GluR-IIA and IIB are shown after thresholding.

**Electrophysiology**

For electrophysiological recordings following the stimulation protocol, HL-3 was used in order to ensure comparability with previous studies (Hallermann et al., 2010; Schmid et al., 2008). The composition of HL-3 (Stewart et al., 1994) was (in mM): 70 NaCl, 5 KCl, 20 MgCl2, 10 NHCO3, 5 D-(-)-trehalose, 115 sucrose, 5 HEPES, CaCl2 1, pH was adjusted to 7.2. HL-3.1 had a lower Magnesium concentration of 5 mM (original publication 4 mM; Feng et al., 2004). For recordings during light application 1.5 mM Ca2+ was used.

Sharp intracellular electrodes with resistances of 9 - 21 MΩ, filled with 3 M KCl were utilized in connection with TEVC amplifiers Axoclamp 900A or AxoClamp 2B (Axon Instruments/Molecular Devices, USA) to make recordings from muscles 6 in abdominal segments 2 and 3 at room temperature (19 – 23 °C). Only measurements from cells with an initial membrane potential ≤ −50 mV and a membrane resistance ≥ 4 MΩ were analyzed. Both minis (−80 mV holding potential; recorded for 90 s) and eEJCs (−60 mV) were low pass filtered at 1 kHz and sampled with 10kHz. Clamp speed was tuned to respond to a voltage step from −60 mV to −70 mV within 0.75 ms for eEJCs and 1.5 ms for minis, IEJCs, and light-induced postsynaptic depolarizations. Holding currents never exceeded ±10 nA.

To trigger eEJCs, 300 µs pulses were applied to the innervating nerve via a suction electrode (diameter approx. 15 µm; filled with extracellular solution), at a voltage (typically 10 - 15 V; Grass S88 stimulator and isolation unit SIU5, Astro-Med, USA) around 1.5 times larger than the threshold necessary to evoke the compound eEJC of both motorneurons.

To assess basal synaptic transmission 15 EJCs evoked at 0.2 Hz were averaged per cell. The quantal content was estimated by dividing the mean eEJC amplitude by the mean mini amplitude measured in the same cell. Here, mini amplitudes were corrected for the more hyperpolarized holding potential (amplitude reduction to 75%; Hallermann et al., 2010). To measure τdecay, the decaying phase of synaptic currents was fitted with a mono-exponential function from 60% of the peak amplitude to the end of the event (Pawlu et al., 2004).

**Statistics**

Comparisons of independent groups were performed with t tests unless the data were not distributed normally or showed a different variability between the two groups. In these cases a Mann-Whitney rank sum test (rs-test) was used. In the figures, the level of significance is marked with asterisks: *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001. To compare adjacent NMJs on M12 and M13 (Figure 4B) or M6 and M7 (Figures 1B and 1C) from the same animals, paired t tests were performed. Data are presented as mean ± standard error of the mean.

**SUPPLEMENTAL REFERENCES**


Figure S1. ChR2 Expression Patterns, Related to Figure 2
Antibody stainings against ChR2 (α-ChR2, blue) and HRP (α-HRP, gray) in three different genetic backgrounds: ‘Pre’, ‘Post’, ‘Pre & Post’. Shown are maximum projections of confocal stacks of NMJs and ventral ganglia (VG). Interestingly, expression of ChR2 in muscles yields a strong signal at the NMJ, possibly due to the large membrane surface provided by the sub-synaptic reticulum. For VG only three optical slices were projected for the sake of clarity. Larvae were raised under standard conditions. Scale bar 30 μm.
Figure S2. Quantification of Light-Evoked Activity at the NMJ, Related to Results

TEVC measurements in semi-intact preparations during light stimulation.

(A) Dependence of lEJC frequencies on irradiance in ‘Pre’ larvae raised under different conditions (red curve shows standard conditions) and recorded in two different hemolymph-like salines (HL-3 and HL-3.1). lEJCs could not be reliably evoked in HL-3, which is attributable to its higher Mg$^{2+}$ ion concentration, resulting in decreased axonal excitability (Feng et al., 2004). Raising animals at elevated temperature (29°C) increases ChR2 expression by enhancing GAL4 activity. Blue bars in frequency diagram indicate conditions of example traces (center) and asterisk denotes the expanded current (right). The rapid rise (right) and bursting pattern (center) of lEJCs illustrate that APs provoked synchronous vesicle exocytosis, comparable to previous work (Pulver et al., 2009). Under standard conditions, postsynaptic depolarization could not be detected when the nerve was severed (data not shown), confirming that APs were initiated in motorneuron cell bodies (Figure S1).

(B and C) Standard conditions.

(B) Steady-state amplitudes of ChR2-mediated postsynaptic currents recorded at different light intensities in ‘Post’ animals. Arrow indicates the transient peak typical of ChR2 (Nagel et al., 2003), arrowheads show minis.

(C) Frequencies of lEJCs are comparable at ‘Pre’ (black) and ‘Pre & Post’ (blue) NMJs. Error bars represent SEM.
Figure S3. Stimulation Conditions In Vivo, Related to Figure 2

(A) Emission spectrum of the stimulation LED, which provided homogeneous 1.7 mW/mm² irradiance at 460 nm in the arena.

(B) Standard 100 min plasticity protocol: five activity blocks (consisting of 2 s light pulses delivered at 0.2 Hz), each lasting 5 min and separated by 15 min rest (Ataman et al., 2008).
Figure S4. Stable Synapse Size and Number following Light-Induced Activity In Vivo, Related to Results

(A) Representative triple stainings from ‘Pre & Post’ (upper panels) and control animals (lower panels), against horseradish peroxidase (α-HRP, gray), a reliable marker of the neuronal membrane, GluR-IID (green), a subunit present in all non-NMDA type glutamate receptors at the NMJ, and Bruchpilot (BRP; magenta), a core active zone protein (Kittel et al., 2006). Arrows indicate enlarged boutons.

(B and C) Quantification of molecular markers. Neither synapse number (B), or size (C; BRP and GluR-IID signals), nor NMJ area (HRP signal) differed significantly between experimental groups (‘Pre’ white, ‘Post’ light blue, ‘Pre & Post’ dark blue) and their respective controls (gray adjacent bars).

(D) Example stainings (α-GluR-IIB, yellow) and (E) data summary illustrate that GluR-IIB cluster size and number was unchanged by ‘Pre & Post’ stimulation. Scale bars 1 μm, HRP panel 20 μm. Error bars represent SEM.

(BRPA) Controls
(Pre) Pre
(Post) Post
(Pre & Post) Pre & Post

(B) Number of Synapses

(BRP) Controls
(Pre) Pre
(Post) Post
(Pre & Post) Pre & Post

(C) Synapse Size (μm²)

(BRP) Controls
(Pre) Pre
(Post) Post
(Pre & Post) Pre & Post

(GluR-IID) Controls
(Pre) Pre
(Post) Post
(Pre & Post) Pre & Post

(HRP) Controls
(Pre) Pre
(Post) Post
(Pre & Post) Pre & Post

(D) Example stainings
α-GluR-IIB

(E) IIB Cluster Size (μm²) and Number of IIB Clusters

(Pre & Post) Pre & Post

(Pre) Pre
(Post) Post

(Pre & Post) Pre & Post
Figure S5. Short Paired Stimulation, Related to Figure 3

(A–D) Larvae were raised under enhanced expression conditions (29°C, 1 mM RAL) to ensure high reliability and temporal precision of IEJC.

(A) α-HRP (gray) and α-ChR2 (blue) costaining reveals ChR2 expression in motorneuron boutons (in contrast to standard conditions; Figure S1) following presynaptic expression (ok6-gal4 > UAS-chop2; a ‘Pre’ NMJ is shown to avoid contamination by the postsynaptic ChR2 signal).

(B) At ‘Pre & Post’ NMJs, a 15 ms, 1.7 mW/mm² light pulse (framed by stimulation artifacts) evoked two IEJC (arrowheads) coinciding with muscle depolarization (TEVC in HL-3.1). Below: quantification of IEJC delays relative to light application and compared to ‘Pre’ NMJs after severing motorneuron axons (latency first: 7.25 ± 0.36 ms, n = 4 NMJs; latency second: 19.53 ± 1.85 ms, n = 3; latency in nerve-cut animals IEJC: 9.88 ± 0.55 ms, n = 4) indicates that the first IEJC was likely initiated in the terminal axon segment while the second was generated in the cell body.

(C and D) Scheme of short pulse protocol (C) and mean quantal content (D; Control 136 ± 13 vesicles, n = 12 NMJs; ‘Pre & Post’ 127 ± 14, n = 12, p = 0.650 t test), eEJC decay (Control 6.54 ± 0.24 ms, n = 12 NMJs; ‘Pre & Post’ 6.74 ± 0.15 ms, n = 12, p = 0.492 t test) and mini decay (Control 7.24 ± 0.22 ms, n = 13 NMJs; ‘Pre & Post’ 7.63 ± 0.15 ms, n = 12, p = 0.201 rs-test) following stimulation at ‘Pre & Post’ (blue) and control NMJs (gray). Scale bar 20 μm. Error bars represent SEM.
Figure S6. M12-Specific Expression of ChR2, Related to Figure 4
Scheme of ChR2 distribution in ‘Pre & M12-Post’ larvae raised under enhanced expression conditions (29°C, 1 mM RAL). Postsynaptic expression was driven by m12-gal4 (Inaki et al., 2010).