Development/Plasticity/Repair

Presynaptic Impairment of Synaptic Transmission in *Drosophila* Embryos Lacking Gsα

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Gsα is a subunit of the heterotrimeric G-protein complex, expressed ubiquitously in all types of cells, including neurons. *Drosophila* larvae, which have mutations in the Gsα gene, are lethargic, suggesting an impairment of neuronal functions. In this study, we examined synaptic transmission at the neuromuscular synapse in Gsα-null (dgs^{R60}) embryos shortly before they hatched. At low-frequency nerve stimulation, synaptic transmission in mutant embryos was not very different from that in controls. In contrast, facilitation during tetanic stimulation was minimal in dgs^{R60}, and no post-tetanic potentiation was observed. Miniature synaptic currents (mSCs) were slightly smaller in amplitude and less frequent in dgs^{R60} embryos in normal-K^+ saline. In high-K^+ saline, mSCs with distinctly large amplitude occurred frequently in controls at late embryonic stages, whereas those mSCs were rarely observed in dgs^{R60} embryos, suggesting a developmental defect in the mutant. Using the *Gal4-UAS* expression system, we found that these phenotypes in dgs^{R60} were caused predominantly by lack of Gsα in presynaptic neurons and not in postsynaptic muscles. To test whether Gsα couples presynaptic modulator receptors to adenylyl cyclase (AC), we examined the responses of two known G-protein-coupled receptors in dgs^{R60} embryos. Both metabotropic glutamate and octopamine receptor responses were indistinguishable from those of controls, indicating that these receptors are not linked to AC by Gsα. We therefore suggest that synaptic transmission is compromised in dgs^{R60} embryos because of presynaptic defects in two distinct processes; one is uncoupling between the yet-to-be-known modulator receptor and AC activation, and the other is a defect in synapse formation.

Key words: **Gsα; *Drosophila*; synaptic transmission; metabotropic glutamate receptor; octopamine receptor; neuromuscular junction.**

Introduction

In a variety of cells, extracellular signals induce cellular responses through a family of receptors with seven transmembrane domains (7TMR). Essential components of this cascade are intermediary heterotrimeric G-proteins composed of α, β, and γ subunits. These G-proteins couple the receptors to appropriate intracellular effectors (Morris and Malbon, 1999). This signaling pathway plays a key role in triggering physiological responses to a wide variety of hormones, neurotransmitters, and sensory stimuli. In this scheme, the α subunit is essential for coupling of receptors to appropriate effectors, and many 7TMRs may couple to the same α subunit to mediate cellular responses in different contexts.

The well studied example of this signal transduction pathway involves 7TMR coupling to G-protein complexes containing Gsα. When the receptor is activated, Gsα stimulates adenylyl cyclase (AC), resulting in elevation of cAMP. Because involvement of cAMP in learning and memory has been well documented in a variety of systems (Kandel and Abel, 1995), Gsα may play an important role in neural functions. To study Gsα function, *Drosophila* mutants were isolated (Wolfgang et al., 1990, 1991). A hypomorphic allele, dgs^{B19}, is viable, and neuromuscular transmission has been studied in third instars. At low-frequency stimulation, synaptic transmission was normal. However, synaptic facilitation during tetanus and post-tetanic potentiation (PTP), i.e., short-term plasticity, were not observed (W. J. Wolfgang, C. Clay, J. Parker, R. Delgado, Y. Kidokoro, P. Labarca, and M. Forte, unpublished observation). Similar phenotypes are found in *rutabaga*[1] (rut^1), in which Ca^{2+}–calmodulin-responsive AC is defective (Livingstone et al., 1984; Zhong and Wu, 1991). If modulator receptors, activated by tetanus, are coupled to AC through Gsα, we expect *dgs*-null mutants to manifest similar phenotypes as rut^1.

At neuromuscular synapses of newly hatched *Drosophila* larvae, activation of metabotropic glutamate receptors (mGluRs) facilitates synaptic transmission, which is mediated by the cAMP–PKA cascade. Both a membrane-permeant cAMP analog and forskolin, an activator of AC, mimic the mGluR response, and the response is inhibited by a blocker of AC and greatly reduced in rut^1. It appears that presynaptic mGluRs are positively coupled to AC, possibly through Gs (Zhang et al., 1999). Because there is only one gene for Gsα (Quan and Forte, 1990), Gsα-null mutation provides us an opportunity to test Gs involvement in mGluR responses. Another modulator at crustacean and insect synapses is octopamine (Breen and Atwood, 1983; Klaassen and Kammer, 1985; Hidoh and Fukami, 1987). Activation of octopamine receptors, cloned from *Drosophila* heads, elevates the cAMP level (Han et al., 1998).

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dgs also appears to be involved in synapse formation. Boutons at neuromuscular synapses in *Drosophila* third instars are more numerous in a hyperexcitable double mutant, *ether-á-go-go, Shaker*, than in wild-type (Budnik et al., 1990; Zhong et al., 1992). This phenotype was suppressed by additional mutation in *dgs*, indicating that activity-induced synapse formation is dependent on Gsα activities (Wolfgang, Clay, Parker, Delgado, Kidokoro, Labarca, and Forte, unpublished observation).

We studied neuromuscular synaptic transmission in Gsα-null embryos (dgs^{R60}) using the patch-clamp techniques and found that synaptic transmission is presynaptically impaired and neither mGluRs nor octopamine receptors are coupled through Gsα to AC.

Materials and Methods

Fly stocks. Primarily, embryos (19–21 hr after egg laying, AEL) of the strain dgs^{R60c}/*CyO-GFP* were used for the experiment. Homozygotes, which are embryonic lethal, are called dgs^{R60} in the text. Heterozygotes are called dgs^{R60}/+ and were used as a control. As an additional control, we used a rescued strain of dgs^{R60} with a *Gs27* transgene, designated *Gs27*. This rescue construct contains the entire *dgs* gene, which is located on the X chromosome (*Gs27*; dgs^{R60c}/dgs^{R60c}) (Wolfgang et al., 2001). The following transgenic strains were also used to selectively express a transgene: *GsW24*, in neurons or in muscles; dgs^{R60c}/*CyO-GFP*; *UAS-GsW24*; dgs^{R60c}/*CyO-GFP*; *elav-Gal4*; dgs^{R60c}/*CyO-GFP*; and *MHC82-Gal4*. To compare phenotypes, we also used rut^1 (Livingstone et al., 1984). In the metabotropic glutamate receptor response and octopamine receptor response, the involvement of the cAMP–PKA cascade was confirmed by the use of a mutant, *DC0*, in which a major catalytic subunit of PKA is lacking (Lane and Kalderon, 1993). Because a noncontracting double mutant, *DC0* Mhc^1, was readily available, we used it for this purpose. Mhc^1 by itself does not affect synaptic transmission (Yoshihara et al., 2000).

Electrophysiology. Electrophysiological procedures for voltage-clamping embryonic muscles have been published previously (Nishikawa and Kidokoro, 1995). The abdominal longitudinal muscle 6 was used for physiological recordings. The membrane potential was held at −65 mV except for measurement of glutamate-induced currents, in which case the holding potential was −35 mV to reduce the current amplitude.

Nerve stimulation. A tip of micropipette filled with 4 M potassium acetate and having a resistance of ~10 MΩ was placed in the ventral nerve cord at the site from which motor nerves emerged. Rectangular pulses of ~1 μA and 2 msec in duration were delivered to stimulate motor nerves. The amplitude of nerve-evoked synaptic currents was measured in external saline containing 0.5 mM Ca^{2+}. The initial 10 responses evoked at 0.3 Hz were averaged to assess the mean amplitude. To estimate the failure rate, 40 pulses were delivered at 0.3 Hz in the external solution containing 0.2 mM Ca^{2+}. Subsequently, tetanic stimulation was given at 10 Hz for 10 sec. Finally, the stimulus frequency was returned to 0.3 Hz, and 40 stimuli were delivered to assess PTP.

Recording of miniature synaptic currents in high-K^+ saline. In normal saline with 0.2 mM Ca^{2+}, the frequency of miniature synaptic currents was low, and it was difficult to determine the mean amplitude accurately. To increase the miniature synaptic current (mSC) frequency, high-K^+ saline was used (see below for its ionic composition). The frequency was higher in this solution: ~300 events for each cell were collected within a few minutes for measurement of their amplitudes, and an amplitude histogram was constructed.

The amplitude histograms of mSCs were not normally distributed. Some of them were skewed to larger amplitudes. To quantify the extent of skewness, we calculated the following statistical parameters for each histogram:

$$m_3 = \frac{1}{n-1}\sum_i (x_i - \bar{x})^3$$

$$m_2 = \frac{1}{n-1}\sum (x_i - \bar{x})^2 ,$$

where x_i is the amplitude of the ith mSC, $\bar{x}$ is the mean, and n is the total number of mSCs. Here, m_3 and m_2 are the third and second moments about the mean. Using these parameters, the skewness is defined as follows:

$$\text{Skewness} = \frac{m_3}{(m_2)^{3/2}}.$$

The value of skewness is zero when the amplitude histogram is normally distributed, positive when the distribution is skewed toward larger values (as in Fig. 4*Ea*), and negative when it is skewed toward smaller values.

Application of glutamate to activate ionotropic glutamate receptors. To estimate the total amount of glutamate receptors in the postsynaptic membrane, glutamate-induced currents were measured at muscle 6 in Ca^{2+}-free saline. Glutamate was dissolved in a Ca^{2+}-free bath solution at 1 mM and included in a glass pipette that had a tip diameter of ~2 μm. Glutamate was delivered at the synapse by a pulse (100 msec) of gas pressure of 0.5 kg/cm^2. To prevent leaking of glutamate from the puff pipette, a latex bead with a diameter of 2.5 μm (Polysciences, Warrington, PA) was attached at the tip with steady negative pressure inside the puff pipette. This bead readily flew away with an application of positive pressure for glutamate delivery. A typical glutamate-induced inward current had a rise time (time to rise from 10 to 90% of amplitude) of ~30 msec and started to decline during the puff pulse of 100 msec. The amplitude of glutamate-induced currents was often large, >2 nA, at a holding potential of −65 mV, which caused a problem of voltage clamping with a series resistance. To avoid this problem, the holding potential was reduced to −35 mV. Desensitization of glutamate receptors was severe. The second pulse evoked only 50–75% of the first glutamate-induced inward current amplitude after a 3 min resting period.

It should be noted that this method for glutamate application, although aimed at the synaptic area, also activates extrasynaptic receptors. The precise contribution of extrasynaptic glutamate receptor channels to the glutamate-induced currents is not known but is probably small, because receptors are highly localized at the postsynaptic area (Saitoe et al., 2001).

Application of agonists to activate metabotropic glutamate receptors or octopamine receptors. Glutamate (100 μM) or an agonist of metabotropic glutamate receptor, (*S*)4C3HPG (100 μM) or octopamine (10 μM), was puff-applied for 40 sec in high-K^+ saline containing 0.05 mM Ca^{2+} and 3 μM tetrodotoxin (TTX). The frequency of mSCs was counted every 10 sec. The starting point of the 10-sec period was aligned at the onset of the puff pulse for comparison among records from different cells.

Solutions. Ca^{2+}-free saline had the following ionic composition (in mM): 140 NaCl, 2 KCl, 6 $MgCl_2$, and 5 HEPES-NaOH, at a pH of 7.1. To evoke synaptic currents by nerve stimulation, 0.2 or 0.5 mM $CaCl_2$ was added, and the equivalent amount of $MgCl_2$ was reduced. The ionic composition of high-K^+ saline with 0.1 mM Ca^{2+} was as follows (in mM): 78 NaCl, 62 KCl, 5.9 $MgCl_2$, 0.1 $CaCl_2$, and 5 HEPES-NaOH, at a pH of 7.1. Activation of metabotropic glutamate receptors and octopamine receptors was performed in the following high-K^+ saline (in mM): 78 NaCl, 62 KCl, 5.95 $MgCl_2$, 0.05 $CaCl_2$, and 5 HEPES-NaOH, at a pH of 7.1.

Biochemicals. (*S*)4C3HPG and MCCG-I were purchased from Tocris (Essex, UK), and TTX, octopamine, and glutamate were purchased from Sigma (St Louis, MO).

Statistics. First, parameters were tested for the normal distribution using the Kolmogorov–Smirnov test at a value of $p = 0.05$. When they were found to be normally distributed, we used Student's t test to compare two groups, and to compare more than two groups, we used ANOVA with Scheffé's criteria. When they were not normally distributed, we used the Mann–Whitney test.

Results

Synaptic transmission is subtly impaired in *dgs*-null mutant embryos

Although the *dgs*-null mutations are lethal, morphologically the neuromuscular synapse forms normally in embryos (Wolfgang et al., 2001). We examined synaptic transmission at the neuromuscular synapse in mutant embryos at late embryonic stages (19–21

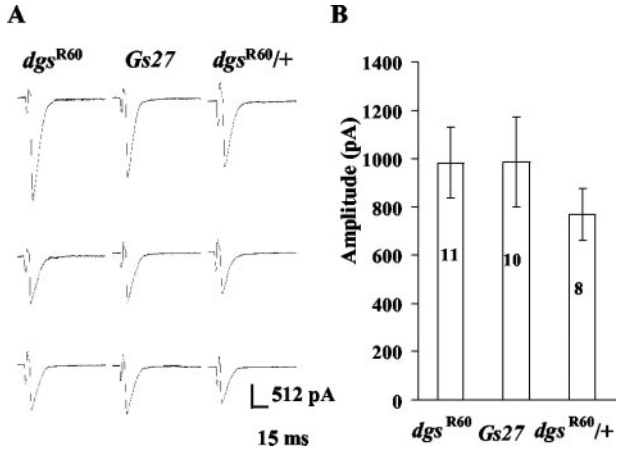

Figure 1. Nerve-evoked synaptic currents in *dgs*^{R60}, *Gs27*, and *dgs*^{R60}/+. External saline contained 0.5 mM Ca^{2+}. *A*, Three sample traces are shown for each strain. The amplitude varied in a large range. *B*, The mean amplitudes are not significantly different among these strains. The bar at the top of each column indicates the SEM, and numbers in columns are the number of cells examined.

hr AEL) using the patch-clamp technique in the whole-cell configuration.

Synaptic transmission in external saline containing 0.5 mM Ca^{2+}

In the external solution containing 0.5 mM Ca^{2+}, nerve stimulation at 0.3 Hz evoked robust synaptic currents in *dgs*^{R60} embryos (*dgs*^{R60}/*dgs*^{R60}). The amplitude of synaptic currents varied widely within one postsynaptic muscle cell and among different cells, and nerve stimulation rarely failed to evoke synaptic currents (Fig. 1*A*, left three traces). As a control, we used embryos of a strain in which a transgene, *Gs27*, was introduced into the background of *dgs*^{R60} (*Gs27*; *dgs*^{R60c}/*dgs*^{R60c}) (Wolfgang et al., 2001) (hereafter, this strain will be called *Gs27*). In these embryos, nerve stimulation also induced robust synaptic currents, rarely failing to evoke them, and the amplitude varied widely (Fig. 1*A*, middle three traces). Similar synaptic transmission was also observed in heterozygotes (*dgs*^{R60}/+) (Fig. 1*A*, right three traces). The mean amplitude, including failures, was calculated by averaging the amplitudes of >10 synaptic currents in each cell of *dgs*^{R60} embryos and was not significantly different from that in *Gs27* or heterozygous embryos (*dgs*^{R60}/+) (Fig. 1*B*). These results are in accord with those in third instar larvae of a hypomorphic allele of *dgs*, *dgs*^{B19} (Wolfgang, Clay, Parker, Delgado, Kidokoro, Labarca, and Forte, unpublished observation).

Synaptic transmission in external saline containing 0.2 mM Ca^{2+}

The large variation of evoked synaptic current amplitudes within a cell and among different cells may prevent detection of subtle impairment of synaptic transmission in *dgs*^{R60} embryos. A part of the variation of synaptic current amplitudes in embryos originates from a large variation of quantal synaptic current amplitudes in embryos (mSCs) (Kidokoro and Nishikawa, 1994). To circumvent this problem, we next measured the quantal content of synaptic currents by the failure method in a lower-external-Ca^{2+} solution, assuming the Poisson statistics for quantal release (Katz, 1969).

In the external solution containing 0.2 mM Ca^{2+}, nerve stimulation at 0.3 Hz often failed to evoke synaptic currents in *dgs*^{R60} embryos (failure rate, 0.88 ± 0.11, mean ± SD, $n = 16$) (Fig. 2A_1, left open column). This failure rate was not different from that in *Gs27* embryos (0.92 ± 0.05, $n = 8$) (Fig. 2B_1, left open column) but was significantly higher than that in heterozygous embryos, *dgs*^{R60}/+ (0.76 ± 0.05, $n = 8$; $p < 0.05$) (Fig. 2C_1, left open column), suggesting that synaptic transmission is slightly impaired in *dgs*^{R60} and *Gs27* embryos.

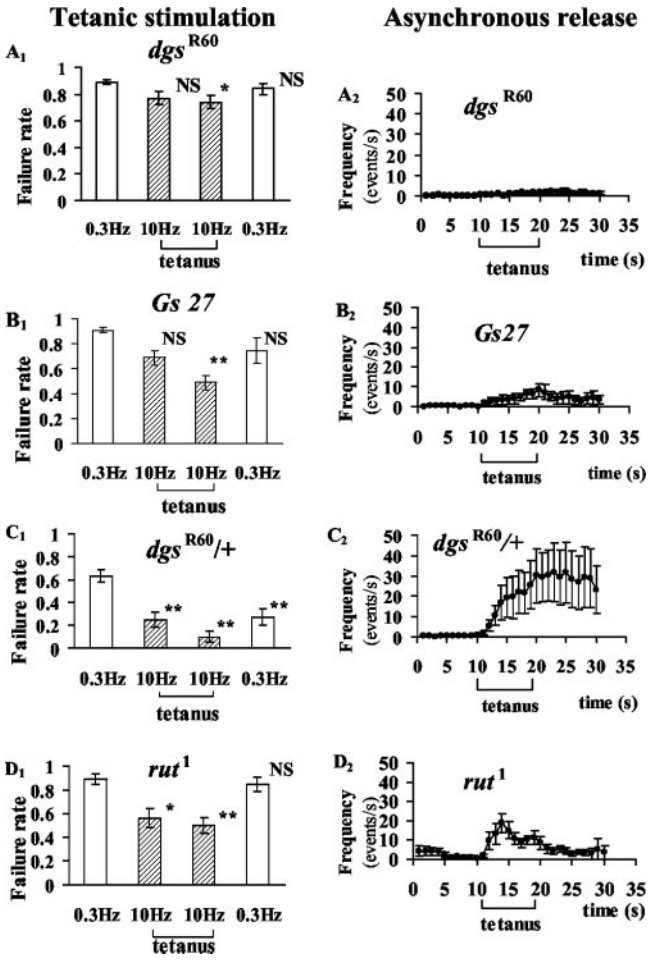

Figure 2. Facilitation during tetanic stimulation and PTP in various strains (tetanic stimulation; A_1–D_1) and asynchronous release of quantal events (asynchronous release; A_2–D_2). At first, the nerve was stimulated 40 times at 0.3 Hz, and the failure rate was determined (left open columns in A_1–D_1). Then stimulation was switched to 10 Hz for 10 sec. The failure rates for the first 50 stimuli (left shaded columns) and those for the last 50 stimuli (right shaded columns) were depicted separately. Finally, the stimulation was switched back to 0.3 Hz (40 stimuli) to assess PTP (right open columns). *A*, *dgs*^{R60}; $n = 12$, where n = number of cells examined. *B*, *Gs27*; $n = 7$. *C*, *dgs*^{R60}/+; $n = 7$. *D*, *rut*^{1}; $n = 6$. Bars at the top of each column in A_1–D_1 and at each data point in A_2–D_2 are the SEM. A single asterisks indicates a statistical difference from the pretetanic failure rate at $p = 0.05$; double asterisks indicate statistical significance at $p = 0.01$. NS, No significance. This series of experiments was performed in normal saline with 0.2 mM Ca^{2+}.

We further examined the synaptic facilitation during tetanus and PTP. When the nerve was stimulated at 10 Hz for 10 sec, facilitation during tetanus in *dgs*^{R60} embryos was not as prominent as in *Gs27* and far less than in *dgs*^{R60}/+ (Fig. 2A_1–C_1, middle two shaded columns). When the failure rate during the last 50 stimuli (right shaded column) was compared with that before tetanus (left open column), it was slightly lower in *dgs*^{R60} embryos, whereas it was much lower in *Gs27* or in *dgs*^{R60}/+ embryos (Fig. 2A_1–C_1). After tetanus, synaptic transmission was potentiated, and consequently the failure rate was reduced for a prolonged period of time in *dgs*^{R60}/+ (Fig. 2C_1, right open column)

but not in *dgs*^{R60} embryos or in *Gs27* embryos (Fig. 2*A*$_1$,*B*$_1$, right open columns). Thus, synaptic transmission in *dgs*^{R60} and *Gs27* embryos appears to be mildly impaired presynaptically. Because the *Gs27* transgene includes the entire *Gsα* gene, it is surprising that synaptic transmission in *Gs27* embryos was not as robust as in *dgs*^{R60}/+. This could be a positional effect of the *Gs27* insertion site on the X chromosome. In addition, although it contains all *Gsα* sequences encoding Gsα protein and flanking 5′ and 3′ genomic regions, it may be that the *Gs27* transgene does not contain all sequences required to precisely mimic the regulation of the endogenous *Gsα* gene *in vivo*.

In a mutant, *rut*^{1}, in which AC is defective (Livingstone et al., 1984), PTP is abolished in third instar larvae (Zhong and Wu, 1991). If Gsα were positively coupling a synaptic modulator receptor with AC, we would expect the phenotype in *dgs*^{R60} embryos to be similar to *rut*^{1}. We thus examined *rut*^{1} embryos in the same protocol and found that facilitation during tetanus was clearly observed but PTP was not (Fig. 2*D*$_1$). Thus, these phenotypes in *rut*^{1} embryos are qualitatively similar to those in *dgs*^{R60}, although quantitatively, facilitation during tetanus was more pronounced in *rut*^{1} (Fig. 2*D*$_1$). Furthermore, the reduced facilitation during tetanus and lack of PTP were also found in third instar *dgs*^{B19} larvae (Wolfgang, Clay, Parker, Delgado, Kidokoro, Labarca, and Forte, unpublished observation) as reported previously in *rut*^{1} third instars (Zhong and Wu, 1991).

During and after tetanus, asynchronous transmitter release was clearly enhanced in the controls (in *Gs27* and *dgs*^{R60}/+) (Fig. 2*B*$_2$,*C*$_2$), whereas that in *dgs*^{R60} embryos was minimal (Fig. 2*A*$_2$). In *rut*^{1}, an enhancement of asynchronous release was clearly observed during tetanus but was much less after tetanus (Fig. 2*D*$_2$) compared with that in *dgs*^{R60}/+ (Fig. 2*C*$_2$). Thus, facilitation of nerve-evoked synaptic transmission and asynchronous release during and after tetanus changed in parallel among different strains, suggesting that the same mechanism, such as an elevation of $[Ca^{2+}]_i$ and/or cAMP in the terminal, underlies both of these phenomena.

Miniature synaptic currents were infrequent and smaller in Gsα-null embryos

In saline with a normal Ca^{2+} concentration (1.5 mM), muscles occasionally contracted and stretched presynaptic nerves, which increased the frequency of mSCs. Thus, it was difficult to accurately estimate the resting mSC frequency. To avoid this problem, mSCs were examined in 0.2 mM Ca^{2+} saline with 3 μM TTX during a 10 min recording period (Fig. 3*A*). The frequency was significantly lower in *dgs*^{R60} than in *Gs27* or in *dgs*^{R60}/+ embryos (Fig. 3*B*). This result again suggests that synaptic transmission in *dgs*^{R60} embryos is presynaptically compromised. In *rut*^{1}, the mean frequency was not different in controls (Fig. 3*B*).

We also noticed that amplitudes of mSCs in *dgs*^{R60} embryos were somewhat smaller compared with those in *Gs27* or in *dgs*^{R60}/+ embryos (Fig. 3*C*). Because the amplitude varied widely in all strains and there were not enough events in each cell during the 10-min observation period, all synaptic events were pooled, and the mean amplitudes were compared. The mean amplitude in *dgs*^{R60} was significantly smaller than that in *Gs27* or in *dgs*^{R60}/+ embryos (Fig. 3*C*). This result suggests that the quantal size of synaptic currents is smaller in *dgs*^{R60} embryos than that in *Gs27* or in *dgs*^{R60}/+. However, because the numbers of events were small and the amplitudes varied widely (Fig. 3*D*), this conclusion should be considered tentative. In *rut*^{1}, the mean amplitude was not different in *dgs*^{R60}/+ or in *Gs27*.

To confirm the smaller amplitude of mSCs in *dgs*^{R60} embryos,

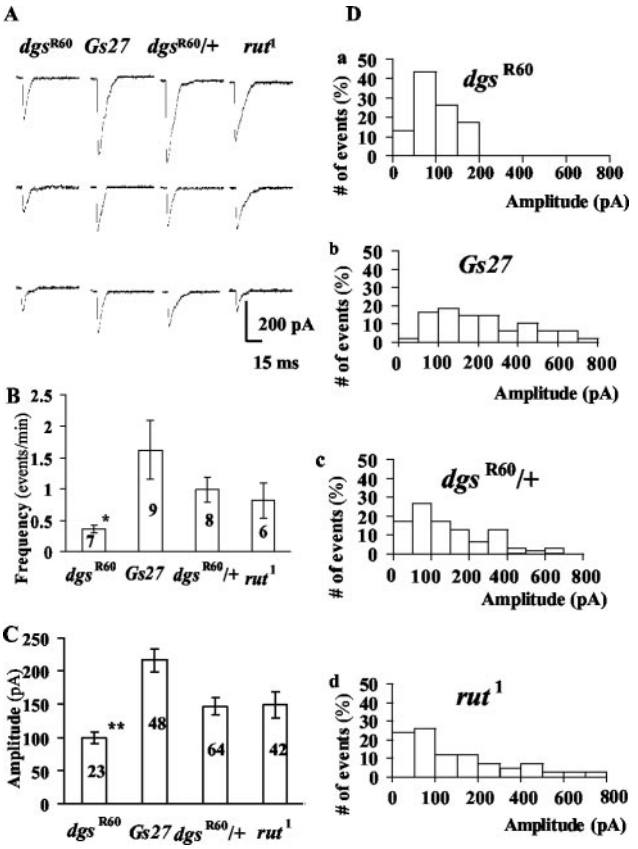

Figure 3. Miniature synaptic currents (mSCs) in normal saline with 0.2 mM Ca^{2+}. *A*, Sample current traces. Three traces are shown for each strain. *B*, The mean frequency of mSCs in each strain. The number in each column is the number of cells examined. The bar at the top of each column is the SEM. Double asterisks indicate a statistical difference at $p = 0.01$ from *Gs27* and *dgs*^{R60}/+. *C*, The mean amplitude of mSCs in each strain. The number in each column is the number of events that were pooled among cells examined. Double asterisks indicate a statistical difference from *Gs27*, *dgs*^{R60}/+, and *rut*^{1}. *D*, Amplitude histograms for each strain. Events from a number of cells recorded in each strain were pooled. The number of events for each strain is the same as shown in *C*.

we next measured mSCs in 62 mM K^+ saline with 0.1 mM Ca^{2+}, in which the mSC frequency was higher and sufficient numbers of events (>300) were collected within 2 min in each cell (Fig. 4). The amplitudes of mSCs were larger in high-K^+ saline in all strains compared with those in normal-K^+ saline (Fig. 4*A*,*B*). This is, at least in part, a result of the higher conductivity of K^+ compared with Na^+ through the *Drosophila* glutamate receptor channel: at the −65 mV membrane potential, K^+ ions carry ~27% more inward currents through glutamate receptor channels than Na^+ ions do when K^+ totally substitutes Na^+ in the external solution (Chang et al., 1994). Under the conditions of the present experiments, we would expect ~12% larger inward currents. Because in normal saline, the mean mSC amplitude in wild-type embryos is ~190 pA at the holding potential of −65 mV (Kidokoro and Nishikawa, 1994; Deitcher et al., 1998), this difference in the channel conductance for K^+ and Na^+ does not fully account for the large mSCs (380–400 pA on average) (Fig. 4*B*) in control strains. An inspection of the amplitude histograms in Figure 4*Eb*,*c* immediately revealed that in high-K^+ saline, there were distinctly more mSCs with large amplitudes, whereas in normal saline the mSC distribution was skewed. Thus, in high-K^+ saline, mSCs with large amplitudes occurred frequently,

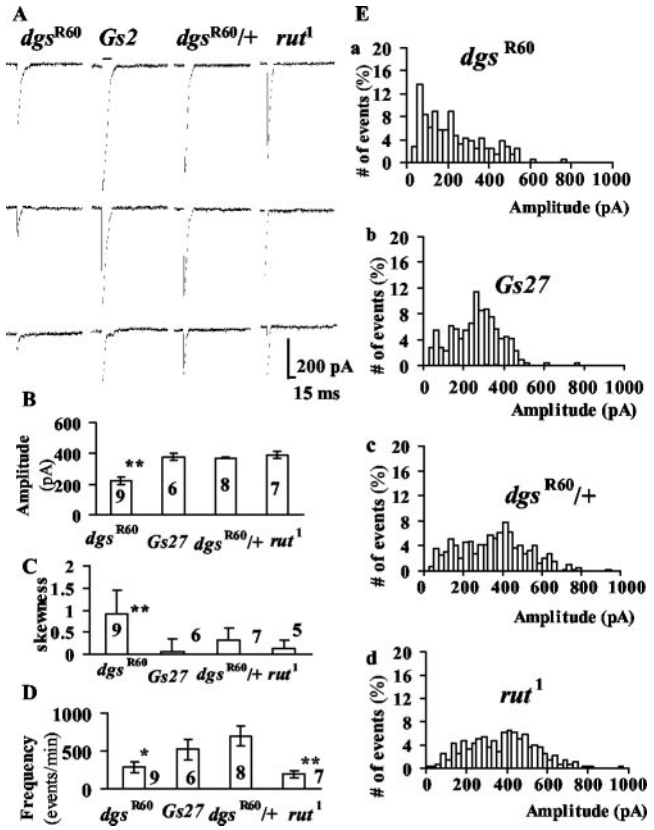

Figure 4. mSCs in high-K^+ saline with 0.1 mM Ca^{2+}. *A*, Sample current traces. Three samples are shown for each strain. *B*, The mean amplitude for each strain. The bar at the top of each column is the SEM. Double asterisks indicate a statistical difference at $p = 0.01$ from *Gs27*, $dgs^{R60}/+$ and rut^1. The number in each column is the number of cells examined. *C*, The skewness of amplitude histogram. The skewness is defined in Materials and Methods. The bar at the top of each column is the SEM. The number is the number of cells examined. Double asterisks indicate a statistical difference at $p = 0.01$ from *Gs27*, $dgs^{R60}/+$, and rut^1. *D*, The frequency of mSCs. The bar at the top of each column is the SEM. The number is the number of cells examined. A single asterisk indicates a statistical difference at $p = 0.05$; double asterisks indicate a statistical difference at $p = 0.01$ from *Gs27* and $dgs^{R60}/+$. *E*, Amplitude histograms from a cell in each strain.

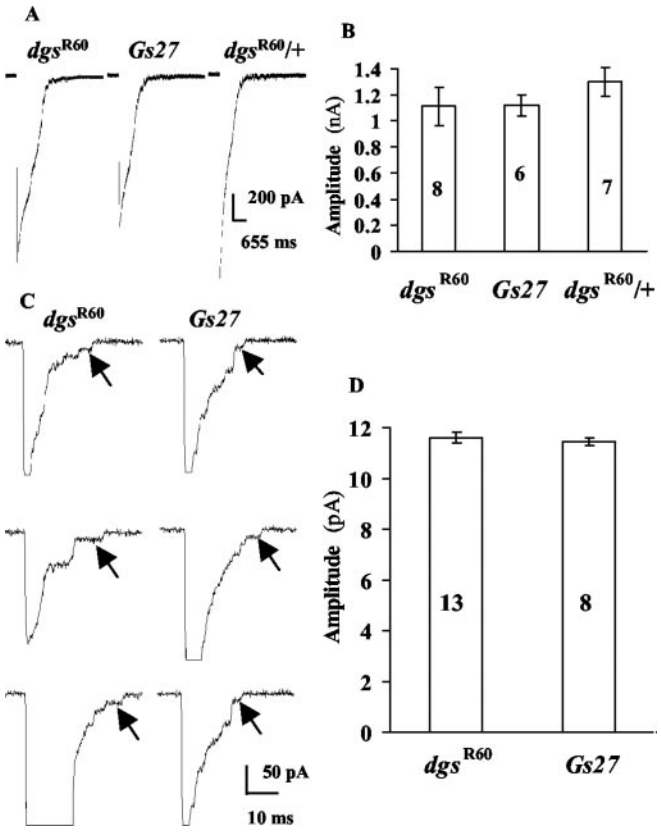

Figure 5. Glutamate-induced currents and single glutamate receptor channels currents. *A*, Glutamate-induced currents. Glutamate (1 mM) was puffed for 100 msec to the synaptic area in Ca^{2+}-free saline. The holding potential was −35 mV. *B*, The mean amplitude of each strain. The bar at the top of each column is the SEM, and the number is the number of cells examined. The holding potential was −35 mV. *C*, Single glutamate receptor channel currents. Three sample synaptic currents are shown in each strain. The peak of synaptic currents is saturated. Spontaneous synaptic currents were recorded in high-K^+ saline with 0.05 mM Ca^{2+}. On the falling phase of synaptic currents, there are distinct steps (arrows) that are most likely because of opening of a single channel in the postsynaptic membrane (Nishikawa and Kidokoro, 1995). The amplitude of those steps was measured in >10 synaptic currents for each cell. *D*, The average of single-channel currents in each strain. The bar at the top of each column is the SEM, and the number is the number of cells examined.

resulting in larger mean amplitudes and less skewed amplitude distributions in controls. These large mSCs are not likely to be a result of coincidental superimposition of multiple mSCs, because the rise times in large mSCs were as short as those in small mSCs (Kidokoro and Nishikawa, 1994). But other explanations for large mSCs are possible (Llano et al., 2000). In contrast, the amplitude distribution of mSCs in dgs^{R60} was skewed toward larger amplitudes, and the majority of them were small (Fig. 4*Ea*). Consequently, the skewness (see Materials and Methods for definition) was significantly larger in dgs^{R60} than in controls (Fig. 4*C*). We will later discuss a possible mechanism for this change in the amplitude distribution in high-K^+ saline in controls and the significance of this phenotype in dgs^{R60}. In rut^1, the amplitude distribution was similar to that in controls (Fig. 4*Ed*).

The frequency of mSCs in high-K^+ saline was lower in dgs^{R60} and rut^1 embryos than in controls (Fig. 4*D*). It should be noted that the mSC frequency in normal saline was not low in rut^1 (Fig. 3*B*). Because the frequency of mSCs was high in high-K^+ saline, the lower mSC frequency in rut^1 is probably reflecting the smaller size of the exo/endo cycling pool (readily releasable pool) (Kuromi and Kidokoro, 2000).

Properties of postsynaptic glutamate receptor channels are not different in dgs^{R60} embryos from those in controls

The smaller amplitude of mSCs in dgs^{R60} embryos could be a result of either presynaptic factors, such as smaller amount of glutamate in vesicles, or postsynaptic factors, such as lower densities of glutamate receptors or smaller conductance of synaptic glutamate receptor channels. To distinguish these possibilities, we next examined the properties of postsynaptic glutamate receptor channels.

Glutamate-induced currents

To assess the total number of glutamate receptors in the postsynaptic membrane, 1 mM glutamate was puff-applied at the neuromuscular synapse in abdominal longitudinal muscle 6. The mean amplitude of glutamate-induced inward currents was not different in dgs^{R60} embryos from that in controls (Fig. 5*A*,*B*), suggesting that the total number of postsynaptic receptors is not the cause for smaller amplitudes of mSCs in dgs^{R60} embryos.

Single-channel current amplitudes

In the falling phase, the synaptic current often changes in steps, revealing underlying single-channel currents. The smallest step amplitude probably corresponds to unitary channel current am-

plitude of the synaptic glutamate receptor channel (Nishikawa and Kidokoro, 1995). To assess the properties of synaptic glutamate receptor channels, we measured the amplitude of each step in dgs^{R60} embryos. The mean amplitude of smallest step amplitudes in dgs^{R60} embryos (11.6 ± 0.8 pA; $n = 13$) was not different from that in *Gs27* (11.4 ± 0.4 pA; $n = 8$) in high-K^+ saline (62 mM K^+) (Fig. 5*C*,*D*). These values are ~10% larger than those observed in normal saline at the same holding potential (10.5 pA for wild-type embryos) (Nishikawa and Kidokoro, 1995), which is most likely to be caused by the higher conductivity of K^+ through the *Drosophila* glutamate receptor channels than Na^+ (it is expected to be ~12% higher in this ionic condition) (Chang et al., 1994). Thus, the unitary glutamate receptor channel current is not different in dgs^{R60} embryos than in *Gs27* or wild-type embryos.

Metabotropic glutamate receptor responses in dgs^{R60} were indistinguishable from those in controls

Activation of metabotropic glutamate receptors (mGluRs) in the presynaptic terminal clearly increases the frequency of mSCs in Ca^{2+}-free saline and enhances synaptic transmission at the neuromuscular synapse of first instar larvae (Zhang et al., 1999). However, in embryos, the effect of an mGluR agonist, (*S*)4C3HPG, on the mSC frequency was somewhat capricious in Ca^{2+}-free saline, and in some cells the effect was not observed. We found that the increase of mSC frequency by glutamate (Fig. 6*A*) or by (*S*)4C3HPG (Fig. 6*C*) was more consistently observed in high-K^+ saline with low Ca^{2+} (0.05 mM) in *Gs27* embryos and was blocked by an antagonist, MCCG-I, indicating that this response is the result of activation of mGluRs (Fig. 6*B*). The response to (*S*)4C3HPG was not observed in *DC0* (Fig. 6*D*), in which a major subunit of PKA is missing (Lane and Kalderon, 1993), suggesting strongly that the cAMP–PKA cascade is involved in this response in accord with previous results in first instars (Zhang et al., 1999).

In dgs^{R60} embryos, puff application of 100 μM (*S*)4C3HPG clearly increased the mSC frequency (Fig. 6*E*). The mGluR response in dgs^{R60} embryos was not different from that in *Gs27* embryos (Fig. 6*C*), indicating that the mGluR response at the embryonic neuromuscular synapse is not mediated by Gsα.

Octopamine receptor responses in dgs^{R60} were indistinguishable from those in controls

The effect of octopamine on synaptic transmission was also examined in high-K^+ saline with low Ca^{2+} (0.05 mM). Puff application of 10 μM octopamine for 40 sec increased the mSC frequency in *G27* embryos (Fig. 7*A*). The dose–response curve is shown in Figure 7*B*, indicating that an apparent K_d is ~10 nM. This value is smaller than that reported for the cloned octopamine receptor (190 nM) (Han et al., 1998), but it is larger than that reported for octopamine response in the crayfish (<1 nM) (Breen and Atwood, 1983). The octopamine response is likely to be mediated by the cAMP–PKA cascade, because the response was not observed in *DC0* (Fig. 7*C*).

In dgs^{R60} embryos, responses similar to those in *Gs27* were observed (Fig. 7*D*), indicating that the octopamine response is not mediated by *Gsα*.

Expression of a Gsα transgene in neurons rescued the synaptic impairment in dgs^{R60}, whereas its expression in postsynaptic muscles did not

In wild-type embryos, Gsα is expressed not only in the presynaptic nerve terminals but also in postsynaptic muscles (Wolfgang et al., 2001). Therefore, the defects in synaptic transmission in dgs^{R60} embryos could be a result of the lack of Gsα in either the presynaptic or postsynaptic cells. To distinguish these two possibilities, we used the *Gal4-UAS* expression system and selectively expressed a Gsα transgene, *GsW24*, in neurons or in muscles in the dgs^{R60} background (Wolfgang et al., 2001). In the transgenic embryos, in which *GsW24* was expressed in neurons, the failure rate of evoked synaptic currents in the external solution containing 0.2 mM Ca^{2+} (Fig. 8*Aa*, left open column) was not significantly different from that in dgs^{R60} (Figs. 2A_1, 8*Aa*), but synaptic facilitation during tetanus was more prominent (Figs. 2A_1, 8*A*, shaded columns). Asynchronous release was observed more frequently in *GsW24*-expressing transgenic embryos than in dgs^{R60} (Figs. 2A_2, 8*Ba*). These phenotypes are similar to those in *Gs27* (Fig. 2B_1,B_2). Furthermore, the amplitude of mSCs in high-K^+ saline was significantly larger in *GsW24*-expressing embryos than

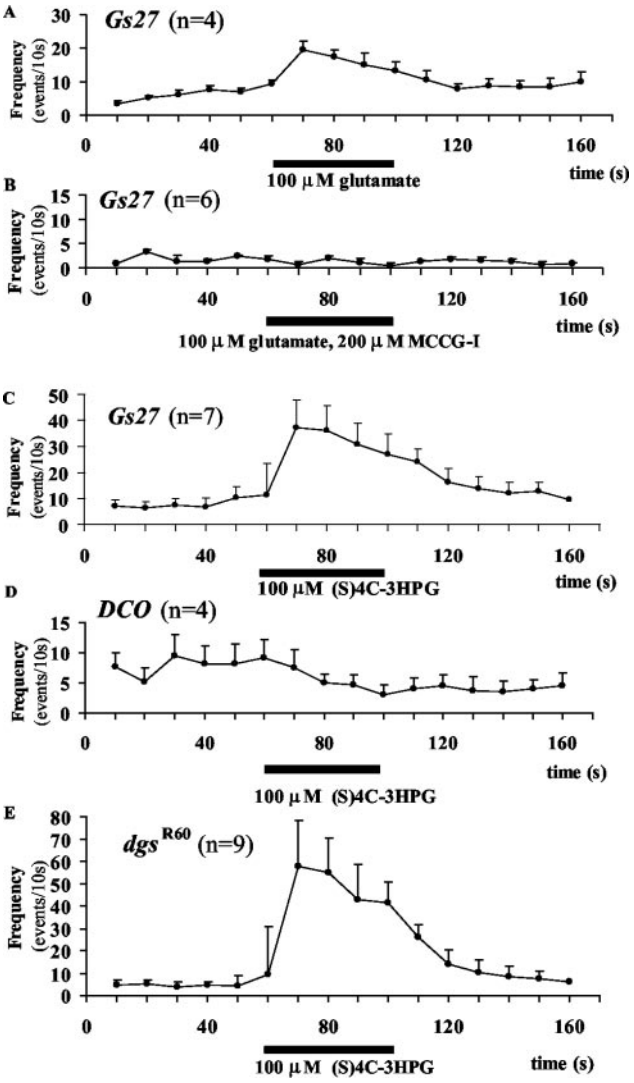

Figure 6. mGluR responses in *Gs27* (*A–C*), in *DC0* (*D*), and in dgs^{R60} embryos (*E*). *A*, Glutamate at 100 μM was puff-applied for 40 sec in high-K^+ saline with 0.05 mM Ca^{2+}, and quantal synaptic events were counted individually every 10 sec. The means of four cells are plotted. Bars attached to each point are the SEM. *B*, A specific mGluR antagonist, MCCG-I, at 200 μM was puff-applied together with 100 μM glutamate. The response was abolished. *C*, A specific mGluR agonist, (*S*)4C3HPG, 100 μM, was puff-applied for 40 sec in high-K^+ saline with 0.05 mM Ca^{2+}. The number of cells examined is seven. *D*, (*S*)4C3HPG at 100 μM was applied in *DC0* embryos. No response was observed. The number of cells examined is four. *E*, The mGluR response evoked with 100 μM (*S*)4C3HPG in dgs^{R60} embryos. The number of cells examined is nine.

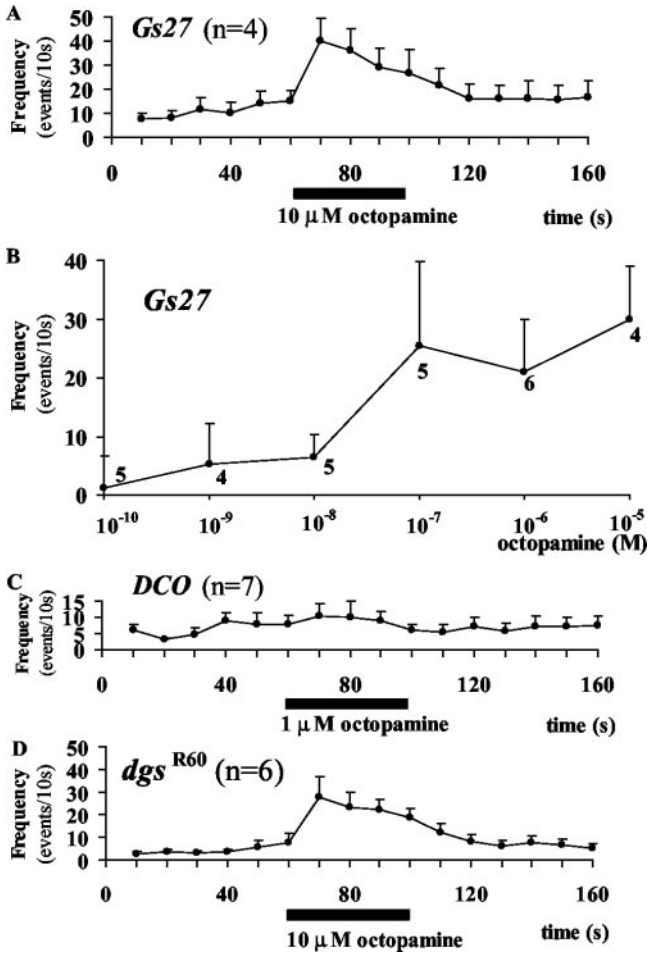

Figure 7. Octopamine receptor responses in *Gs27* (*A, B*), in *DC0* (*C*), and in *dgs*^{R60} embryos (*D*). *A*, Octopamine at 10 μM was puff-applied in high-K^+ saline with 0.05 mM Ca^{2+} for 40 sec, and quantal synaptic events were counted every 10 sec. The means of four cells are plotted, and the bars attached to each point are the SEM. *B*, The dose–response curve for octopamine. Neighboring two data points were connected by a straight line, and apparent K_d was estimated as a octopamine dose that produces the half-maximal response. Bars attached to each point are the SEM, and numbers are the number of cells examined. *C*, Lack of the octopamine receptor responses in *DC0*. The means of seven cells are plotted, and the bars attached to each point are the SEM. *D*, The octopamine receptor response in *dgs*^{R60} embryos. The means of six cells are plotted, and the bars attached to each point are the SEM.

in *dgs*^{R60} and not different from controls (Figs. 4*B*, 8*Ca*). The amplitude histograms were widely spread in *GsW24*-expressing embryos (Fig. 8*Cd*), and the skewness was small (Fig. 8*Cb*), as it was in controls (Fig. 4*C*). The frequency of mSCs was as high as in controls (Fig. 8*Cc*). Altogether, the properties of mSCs in embryos expressing *GsW24* in neurons were similar to those in controls, except that PTP was not as strong as that observed in heterozygous embryos (Fig. 2*C*). In wild-type, Gsα is abundantly expressed in muscles (Wolfgang, Clay, Parker, Delgado, Kidokoro, Labarca, and Forte, unpublished observation). Thus, it is possible that expression of Gsα in muscles is also required for full recovery of functions at the wild-type level. In addition, the positional effect of the *GsW24* insertion site in the chromosome might also contribute to the less complete rescue by *GsW24*.

In contrast, when the transgene, *GsW24*, was expressed in muscles in the *dgs*^{R60} background, none of above-mentioned phenotypes were rescued (Fig. 8*Ab,Bb,Ca–Cc,Ce*).

Together, we conclude that the synaptic impairment in *dgs*^{R60} embryos is the result of lack of Gsα primarily in presynaptic neurons rather than in muscles.

Discussion

Presynaptic defects of synaptic transmission in *dgs*^{R60} embryos

Two distinct sets of phenotypes in synaptic transmission at the neuromuscular synapse in *dgs*^{R60} embryos were revealed, as follows. (1) Slightly smaller quantal content: The failure rate of stimuli to evoke synaptic currents in *dgs*^{R60} embryos was slightly greater in saline containing 0.2 mM Ca^{2+} compared with heterozygotes (Fig. 2), suggesting smaller quantal contents of evoked synaptic currents. This subtle impairment in nerve-evoked synaptic transmission probably correlates with lower frequencies of mSCs (Figs. 3*B*, 4*D*). Synaptic impairment was more clearly demonstrated on stimulation at 10 Hz. In *dgs*^{R60} embryos, we found minimal synaptic facilitation during tetanus and no PTP. Furthermore, asynchronous release of quanta during and after tetanus was much less than in controls. The lack of PTP found in *dgs*^{R60} embryos was similar to that in *rut*^{1} (Fig. 2D_1). At the light-microscopic level, the morphology of neuromuscular synapses in *dgs*^{R60} embryos is not different from that of controls (Wolfgang et al., 2001). Then the defects in *dgs*^{R60} embryos could be in a lower release probability, in a smaller number of release sites, or in a smaller number of release-ready vesicles. (2) Smaller quantal size: Amplitudes of mSCs in *dgs*^{R60} embryos were slightly smaller in normal-K^+ saline than in controls. The difference in mean mSC amplitude was more clearly demonstrated in high-K^+ saline (Fig. 4), in which large mSCs occurred more frequently in controls than in *dgs*^{R60} embryos. Consequently, the amplitude histogram was broadly distributed in controls, whereas it was skewed toward large amplitudes in *dgs*^{R60} (Fig. 4*E*). The frequent occurrence of large mSCs in high-K^+ saline in controls may reflect a developmental process in synapse maturation, which might be defective in *dgs*^{R60} embryos.

These two distinct sets of phenotypes in synaptic transmission in *dgs*^{R60} embryos are both a result of presynaptic defects.

Some phenotypes in *dgs*^{R60} embryos are similar to those in *rut*^{1}, but others are distinctly different

In *rut*^{1}, Ca^{2+}–calmodulin-responsive AC is defective (Livingstone et al., 1984), and mGluR response are markedly reduced. AC coded by *rut* therefore appears to at least partly mediate mGluR responses (Zhang et al., 1999). If Gsα couples a modulator receptor to AC in nerve terminals, we would expect similar phenotypes in *dgs*^{R60} and in *rut*^{1}.

During tetanic stimulation, synaptic transmission was slightly facilitated in *rut*^{1} embryos and in *dgs*^{R60}, but PTP was absent in both mutants (Fig. 2). In third instars of a *dgs* hypomorph, *dgs*^{B19}, both facilitation during tetanus and PTP were absent (Wolfgang, Clay, Parker, Delgado, Kidokoro, Labarca, and Forte, unpublished observation), and in third instars of *rut*^{1}, there was slight facilitation during tetanus but no PTP (Zhong and Wu, 1991). These phenotypes are similar between *rut*^{1} and *dgs*. However, the mean amplitude of mSCs in high-K^+ saline was smaller in *dgs*^{R60} embryos than in *rut*^{1} (Fig. 4*B*). The amplitude histogram was skewed in *dgs*^{R60} embryos (Fig. 4*Ea*), whereas in *rut*^{1} it was more widely distributed (Fig. 4*Ed*) and indistinguishable from controls (Fig. 4*Eb,Ec*). Thus, between the two distinct sets of phenotypes in *dgs*^{R60} embryos, the slightly smaller quantal content is shared with *rut*^{1}, but the smaller quantal size is not. It seems unlikely that the phenotypes in *dgs*^{R60} embryos result entirely from a mechanism similar to that in *rut*^{1}, in which a low level of cAMP pro-

duction during tetanus is probably underlying the lack of PTP (Zhong and Wu, 1991).

Neither mGluRs nor octopamine receptors are coupled to a G-protein containing Gsα

Both mGluR and octopamine receptor responses in *dgs*^{R60} embryos were indistinguishable from those in *Gs27* (Figs. 6, 7), indicating that Gsα does not constitute a G-protein that couples these receptors to AC activation in the presynaptic terminal. At the neuromuscular synapse of first instar larvae, activation of mGluRs with agonists increased the mSC frequency, which was blocked by a specific mGluR antagonist. The effect of mGluR activation was mimicked by forskolin and a membrane-permeant analog of cAMP. Furthermore, an adenylyl cyclase inhibitor blocked the mGluR agonist-induced effects, and in *rut*, the effects were greatly reduced (Zhang et al., 1999). These observations strongly suggest that mGluRs at the presynaptic terminal are coupled to AC, possibly through the Gs-protein. In this study, however, we showed that Gsα is not involved in the mGluR response. Because there is only one gene for Gsα (Quan and Forte, 1990), the coupling between mGluR and AC must be indirect. For example, mGluRs might be coupled to the phospholipase C cascade, activation of which leads to an increase of internal Ca^{2+} and activates Ca^{2+}–calmodulin-responsive AC.

Because there are many G-protein-coupled receptors, our negative findings with two synaptic modulator receptors are not surprising. We found that of two distinct sets of phenotypes in *dgs*^{R60} embryos, one set, slightly smaller quantal content, is similar to that in *rut*[1]. Therefore, it is still possible that an unknown modulator receptor in the presynaptic terminal is coupled to AC through a G-protein containing Gsα.

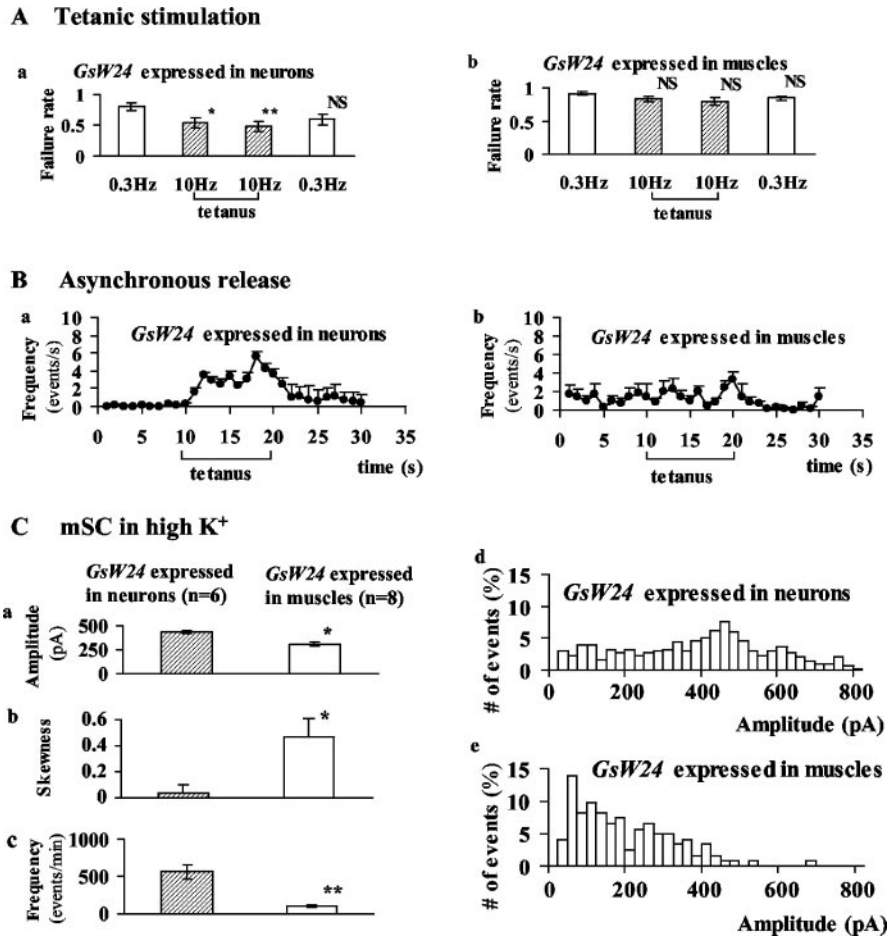

Figure 8. Expression of a Gsα transgene, *GsW24*, in neurons rescued the synaptic impairment in *dgs*^{R60}, whereas its expression in postsynaptic muscles did not. A wild-type transgene, *GsW24*, was expressed with a driver, *elav-Gal4*, in neurons and with another driver, *MHC82-Gal4*, in muscles in the *dgs*^{R60} background. *A*, Facilitation during tetanic stimulation and PTP. At first, the nerve was stimulated 40 times at 0.3 Hz, and the failure rate was determined (left open columns in *Aa, Ab*). Then, the stimulation was switched to 10 Hz for 10 sec. The failure rates for the first 50 stimuli (left shaded columns) and that for the last 50 stimuli (right shaded columns) were depicted separately. Finally, the stimulation was switched back to 0.3 Hz (40 stimuli) to assess PTP (right open columns). When *GsW24* was expressed in neurons (*Aa*), facilitation during tetanus was observed, but when *GsW24* was expressed in muscles (*Ab*), it was not. Bars at the top of each column are the SEM. A single asterisk indicates a statistical difference from the pretetanic failure rate at $p = 0.05$; double asterisks indicate a statistical difference at $p = 0.01$. NS, No significance. The number of cells examined is 10 for *Aa* and 7 for *Ab*. *B*, Asynchronous release of quantal events occurred during this series of stimulations, which increased during and after tetanus as shown in *Ba*, in which *GsW24* was expressed in neurons, but not in *Bb*, in which *GsW24* was expressed in muscles. This series of experiments was performed in normal saline with 0.2 mM Ca^{2+}. The number of cells examined is 10 for *Ba* and 7 for *Bb*. *C*, mSCs in high-K^+ saline with 0.1 mM Ca^{2+}. *Ca*, The amplitude. Left shaded columns are for a strain in which *GsW24* was expressed in neurons ($n = 6$), and right open columns are for a transgenic strain in which *GsW24* was expressed in muscles ($n = 8$). *Cb*, The skewness of amplitude histogram. *Cc*, The frequency of mSCs. The bar at the top of each column is the SEM. The number is the number of cells examined. A single asterisk indicates a statistical difference at $p = 0.05$; double asterisks indicate a statistical difference at $p = 0.01$. *Cd*, Amplitude histogram from a cell in which *GsW24* was expressed in neurons. Similar histograms were observed in six cells in which *GsW24* was expressed in neurons. *Ce*, Amplitude histogram from a cell in which *GsW24* was expressed in muscles. Similar histograms were observed in eight cells in which *GsW24* was expressed in muscles.

Frequent occurrence of large mSCs in high-K^+ saline in control embryos

Unexpectedly, we observed in high-K^+ saline many distinctly large mSCs in *Gs27* and *dgs*^{R60}/+ embryos at the late embryonic stage. The mean amplitude was ~80% larger than in normal saline (compare Figs. 3*C* and 4*B*). The major factor contributing to these large mean amplitudes is frequent occurrence of large synaptic currents (Fig. 4*E*). Large mSCs do occur in normal-K^+ saline, but their frequency is low. In high-K^+ saline, their frequency was elevated disproportionately, resulting in the amplitude histograms with a broader and less-skewed distribution (Fig. 4*C*).

Two peaks in the mSC amplitude distribution have been reported in *Xenopus* nerve–muscle cultures. In younger cultures, the mean amplitude of mSCs is smaller and the amplitude distribution is skewed toward larger amplitudes. The second symmetrical peak in the large-amplitude range appears in older cultures. This change in the amplitude distribution is considered to be a developmental process (Kidokoro, 1984). In *Drosophila*, a similar transition of amplitude histogram from a skewed distribution with a single peak to a distribution with two peaks during development has not been demonstrated (Kidokoro and Nishikawa, 1994). In this study, we observed broader amplitude distributions and sometimes two peaks in control strains in high-K^+ saline.

This could be a change in *Drosophila* embryos corresponding to that observed in *Xenopus* nerve–muscle cultures.

Why do large mSCs occur frequently in high-K^+ saline? Among other possibilities, we favor the following scenario. In rapidly developing embryos, some release sites may be more mature than others. In high-K^+ saline with Ca^{2+}, in which Ca^{2+} levels in the presynaptic nerve terminal are elevated, fusion of vesicles may occur more frequently at those mature release sites than at immature sites. These mature release sites probably face a postsynaptic membrane with a higher receptor density. In dgs^{R60} embryos, however, fewer release sites may be mature and face a postsynaptic membrane with a high receptor density. In addition, those release sites may not be responding to an elevated Ca^{2+} in high-K^+ saline to produce large mSCs, resulting in the smaller mean amplitude with a skewed distribution. In normal saline, these mature release sites may be regulated not to initiate excessive vesicle fusion. Because the mean amplitude of glutamate-induced currents reflecting the total number of receptors was not different between dgs^{R60} embryos and controls (Fig. 5*A*,*B*), these mature release sites with high receptor densities could not be more numerous in controls but must be releasing vesicles more frequently in high-K^+ saline.

In this study, we found the skewed amplitude distributions of mSCs in dgs^{R60} embryos in high-K^+ saline, whereas in controls, amplitude distributions were broader. Because a transition from a skewed amplitude distribution to a broader one has been observed during synapse formation (Kidokoro, 1984), the skewed amplitude distributions in dgs^{R60} embryos could be an indication of immature synapses, suggesting the involvement of Gsα in synapse formation. An observation pointing to the involvement of Gsα in synapse formation was also made in third instar larvae of a hypomorphic mutant, dgs^{B19}. The numbers of boutons and branches of presynaptic terminals at the neuromuscular synapse were smaller in dgs^{B19} third instars than in controls. These phenotypes were not observed in second instars of dgs^{B19}. This finding suggests that during the period of rapid muscle expansion and synapse formation in third instars, activation of Gsα is required (Wolfgang, Clay, Parker, Delgado, Kidokoro, Labarca, and Forte, unpublished observation). The presynaptic defects in dgs^{R60} embryos may be related to a similar process during early synapse formation.

The effects of the null-mutation in *dgs* on synaptic transmission were observed in two aspects. One could be because of uncoupling between the as-yet-unknown modulator receptor and AC activation. This phenotype is similar to that in rut^1. The other is probably a defect in synapse formation. Mature release sites with high receptor densities may not be well developed in dgs^{R60} embryos. To pinpoint the process in which Gsα is involved, it is necessary to further examine synaptic transmission at early stages of development.

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