ness, these NPAS2-expressing regions of the brain receive and process stimulatory neuronal activity that entrains the molecular clock in the same manner argued for the retino-SCN pathway (*16*). This entrainment could occur by three interlinked events: (i) generation of extracellular glutamate by active neurotransmission, (ii) stimulation of glycolysis and lactate production by localized glutamate uptake into astrocytes, and (iii) fluctuation of intracellular redox potential by facilitated lactate transport and its consumption as a metabolic fuel in nearby neurons. If correct, this hypothesis may ultimately help to explain how the mammalian forebrain oscillates between states of alertness and tiredness.

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17. For Northern blots (*26*), we prepared a G3PDH probe by reverse transcription polymerase chain reaction (RT-PCR) using mouse brain RNA as a template and the following primers: forward, 5′-ACCACAGTCCATGCCATCAC-3′; reverse, 5′-TCCACCACCCTGTTGCTGTA-3′. The resulting PCR product was cloned into pGEM-T and confirmed by DNA sequence analysis. A partial cDNA clone corresponding to bases 293 to 899 of the LDHA gene (GenBank, accession number BC001829) was used as the probe for LDHA mRNA.
18. GenBank accession Y00309 provided genomic sequence information for the mouse LDHA gene. A ~1.5-kb fragment of the mouse LDHA gene promoter and upstream sequences was amplified with the following primers: forward, 5′-GGAGCTCCCCTGTCCACGAATCCTTCTGG-3′; reverse, 5′-AGATCTGGCCTTAAATGGAAGCTCCGCGC-3′. The PCR product was cloned into the Sac I and Bgl II sites of the pGL3-Basic luciferase reporter plasmid.
19. GenBank accession Y00309 revealed the location of the TATA box of the LDHA gene promoter at nucleotides −20 to −25 relative to the transcription start site. The sequence also contained optimal NPAS2:BMAL1 binding sites at nucleotides −78 to −83 (sequence 5′-CACGTG-3′) and −171 to −176 (sequence 5′-CACGTG-3′).
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22. For EMS assays (*50*), reactions contained recombinant transcription factors (0.3 to 0.5 μg/reaction or, in the case of the bHLH fragments, 100 ng/reaction) and radioactive, double-stranded oligonucleotide probe (200 nM). The sequence of the two strands of the DNA probe were 5′-GGGGCGCCACGTGAGAGG-3′ and 5′-GGCCTCTCACGTGGCGCC-3′. The DNA was radiolabeled by filling in with [α-^{32}P]deoxycytidine triphosphate with the Klenow fragment of DNA polymerase. Solutions of nicotinamide dinucleotides, prepared fresh for each experiment, were solubilized in deionized water, and concentrations were determined by absorbance at 260 nm. For the NAD cofactor response, the binding data were fit with nonlinear regression to variable slope sigmoidal dose-response curves with the PRISM program (GraphPad, San Diego, CA).
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52. We thank N. Grichine for assistance with protein structure analysis; J. Albanesi for help in analysis of equilibrium binding data; J. D. McGarry, K. Uyeda, D. Lane, and R. Estabrook for advice on the biochemistry of NAD redox; R. Bruick and J. Garcia for helpful discussions; L. Huang for synthesis of the BMAL1 bHLH-alone peptide; M. A. Phillips for pHisTev; and M. Brown and J. Goldstein for critical review of the manuscript. J.R. was supported by an NIH predoctoral fellowship (NIH5-T32-GM08-291-12) and S.L.M. by NIH grant 1RO1MH59388 and unrestricted funds provided by an anonymous donor.

14 March 2001; accepted 11 June 2001
Published online 5 July 2001;
10.1126/science.1060698
Include this information when citing this paper.

Absence of Junctional Glutamate Receptor Clusters in *Drosophila* Mutants Lacking Spontaneous Transmitter Release

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Little is known about the functional significance of spontaneous miniature synaptic potentials, which are the result of vesicular exocytosis at nerve terminals. Here, by using *Drosophila* mutants with specific defects in presynaptic function, we found that glutamate receptors clustered normally at neuromuscular junctions of mutants that retained spontaneous transmitter secretion but had lost the ability to release transmitter in response to action potentials. In contrast, receptor clustering was defective in mutants in which both spontaneous and evoked vesicle exocytosis were absent. Thus, spontaneous vesicle exocytosis appears to be tightly linked to the clustering of glutamate receptors during development.

In 1952, Fatt and Katz described miniature end-plate potentials, which provided a basis for the theory of quantal synaptic transmission (*1*). A single miniature end-plate potential arises when a synaptic vesicle fuses spontaneously with the presynaptic membrane and releases a quantum of transmitter (spontaneous vesicle exocytosis). However, little is known about the

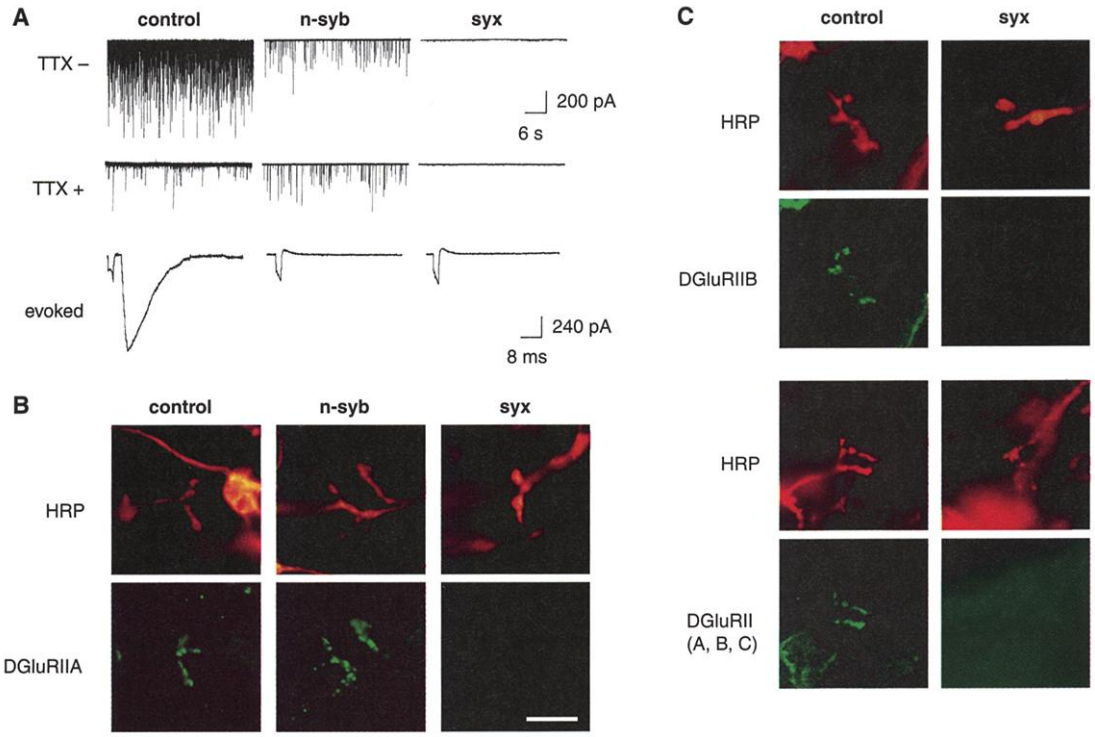

Fig. 1. The correlation between mESCs and GluR clustering. (**A**) Synaptic currents in each column were from the same muscle of embryos or newly hatched larvae. The addition of 1 μM TTX (TTX+) had no substantial effect on synaptic activity in *n-syb* and *syx* embryos. Each trace is representative of recordings from at least six embryos of that genotype; the recordings lasted at least 15 min each. The evoked responses were initiated by microelectrode stimulation of the CNS (evoked). (**B**) GluR localization at embryonic NMJs. Embryos at 22 hours after fertilization were double-stained with anti-HRP (orange) and anti-DGluRIIA (green) (*14*). Immunofluorescence images are of muscle 12 and 13. (**C**) GluR localization at embryonic NMJs stained with antibodies directed against different subunits. DGluRIIB was stained with a transgenic embryo in which the subunit was myc-tagged (*17*) (upper panel). AS5, which recognizes DGluRIIA, DGluRIIB, and DGluRIIC, was used (*18*) (lower panel). Scale bar, in (B) is 10 μm for (B) and (C).

functional importance of this process. Presynaptic and postsynaptic neurotoxins that allow spontaneous vesicle exocytosis to persist have little effect on synaptic development, including postsynaptic accumulation of receptors (*2*, *3*). During the development of *Drosophila* neuromuscular junctions (NMJs), glutamate receptors (GluRs) cluster in the postsynaptic membrane in a manner that depends on nerve-muscle contact (*4*). To investigate the role of spontaneous secretory events in receptor clustering, we used *Drosophila* mutants with distinctive secretory defects. Mutations of *neuronal-synaptobrevin* (*n-syb*) or *cysteine string protein* (*csp*) selectively prevent nerve-evoked exocytosis whereas spontaneous vesicle exocytosis persists (*5*–*7*). In contrast, *syntaxin-1A* (*syx*) or *shibire* (*shi*) mutations eliminate both spontaneous and evoked exocytosis (*8*, *9*), thereby allowing one to distinguish the role of spontaneous secretory events.

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We first characterized neuromuscular transmission in wild-type and mutant *Drosophila* embryos or larvae (*10*–*12*). A typical record of a burst of excitatory synaptic currents (ESCs), which often exceeded 600 pA in amplitude in a newly hatched wild-type larva (control), is shown in Fig. 1A (TTX–). In the presence of tetrodotoxin (TTX), the bursting of ESCs was suppressed, and ESCs seldom exceeded 400 pA (TTX+). A similar suppression of bursting and reduction in the amplitude of ESCs was observed when the ventral nerve cord was removed. Thus, propagated activity in the nervous system triggered multiple vesicle exocytosis and contributed to the ESCs. Concomitantly, the residual events [miniature ESCs (mESCs)] in these wild-type larvae (TTX+) were due to spontaneous vesicle exocytosis.

We first investigated an *n-syb* null mutant (*13*, *14*), in which nerve-evoked ESCs but not mESCs are lost (*5*, *6*). Consistent with these findings, we detected ESCs in *n-syb* embryos but virtually no large-amplitude ESCs characteristic of nerve-evoked ESCs (Fig. 1A). This apparent absence of evoked responses (but persistence of mESCs) was confirmed by the fact that TTX had no effect on the frequency or amplitude of ESCs and that no evoked ESCs were elicited by nerve stimulation (Fig. 1A).

In *syx* mutants, both nerve-evoked and mESCs were undetectable (*8*). In agreement with this phenotype (Fig. 1A), neither nerve-evoked nor mESCs were detected during observations exceeding 15 min each in seven cells. Although we could not completely eliminate the possibility of missing very infrequent occurrences, it is clear that the frequency of mESCs in *syx* embryos is far lower than the frequency of mESCs in *n-syb* embryos. Given the distinct phenotypes of the *n-syb* and *syx* mutants, we examined the distribution of postsynaptic GluRs in these embryos.

Figure 1B shows the distribution of GluRs at embryonic NMJs (*14*). Concomitantly, we stained these preparations with antibody against horseradish peroxidase (anti-HRP), which binds to a neuronal surface antigen (*15*) and reveals the presynaptic terminals. Immunoreactive GluRs formed prominent junctional clusters that closely mirrored the presynaptic elements in wild-type and *n-syb* mutants. Although this finding apparently contrasts with the observation in *para* (Na^+ channel) mutants that neural electrical activity is essential for the clustering of receptors (*16*), it should be noted that the *n-syb* mutation is a more subtle perturbation of this system. Unlike *n-syb*, however, *syx* mutants rarely had discernible junctional GluR clusters, although they invariably formed neuromuscular contacts (*14*). Because the antibody we used recognizes only the DGluRIIA subunit, other subunits might have substituted to form functional receptor channels. To exclude this possibility, we used a transgenic line in which the DGluRIIB subunit was

tagged by myc (*17*). *syx* embryos carrying this myc-tagged GluR exhibited no clustering of receptors. However, receptor clusters were clearly stained in control organisms carrying this myc-tagged receptor (Fig. 1C). As another control, we used the antibody AS5, which recognizes DGluRIIA, DGluRIIB, and DGluRIIC subunits (*18*). The result was indistinguishable from that obtained with the DGluRIIB antibody (Fig. 1C).

These findings raised the possibility that the absence of detectable mESCs in these mutants (Fig. 1A) may have arisen from a deficit of postsynaptic GluRs. Moreover, the lack of receptor clusters could either be a developmental consequence of a lack of vesicle fusions in the nerve terminal, or it could be due to a requirement for syntaxin in the trafficking of GluRs to the postsynaptic membrane. Indeed, *syx* is required for cell viability in *Drosophila* (*19, 20*), and the development of both the neuron and muscle in *syx* embryos is likely to be due to small amounts of maternal Syx (*19*). If this residual Syx is not adequate for the maintenance of normal vesicular traffic to the cell surface, GluRs may not be inserted appropriately in the sarcolemma. To address these issues, we determined whether *syx* mutants responded to applied glutamate and also whether junctional GluR clusters were restored in *syx* mutants by selectively inducing the presynaptic or postsynaptic expression of a *syx* transgene.

The application of L-glutamate by pressure ejection (*14*) onto the junctional region of *syx* muscles indicated that some sensitivity was lost concomitantly with the loss of clusters. The pressure ejection of L-glutamate at the junctional region yielded robust inward currents in wild-type (987 ± 277 pA, mean ± SD, $n = 6$) and *n-syb* mutants (1099 ± 289 pA, $n = 7$), but glutamate-evoked currents were much smaller in *syx* mutants (116 ± 118 pA, $n = 6$) (Fig. 2A). The small response in *syx* mutants contrasts with an earlier report that postsynaptic glutamate responsiveness is at the control level in *syx* mutants (*8*). Although we cannot account for this discrepancy, what is important here is that even with this diminished glutamate sensitivity, we would have detected mESCs in *syx*

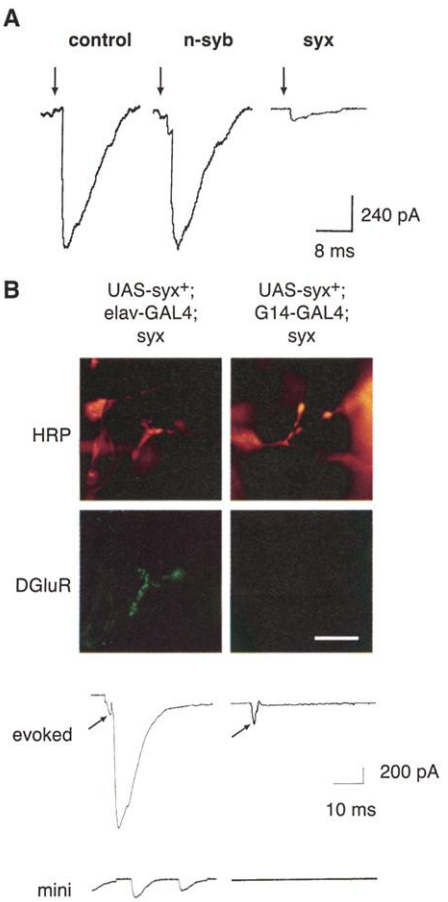

Fig. 2. Functional GluRs are expressed in *syx* mutants. (**A**) The postsynaptic response to pressure ejection of L-glutamate (arrows). (**B**) (Upper panels) Junctional GluR clusters were restored in *syx* mutants by the targeted expression of the syx^+ transgene in neurons [GAL4-responsive upstream activity sequence (*UAS-syx*$^+$*; elav-GAL4; syx)* but not in muscle (*UAS-syx*$^+$*; G14-GAL4; syx)*]. Although they did not hatch, *UAS-syx*$^+$*; elav-GAL4; syx* embryos showed muscle movements that imply functional synaptic transmission. Scale bar, 10 μm. (Lower panels) Nerve-evoked synaptic currents were observed after stimulation at the CNS (arrows) in *UAS-syx*$^+$*; elav-GAL4; syx* embryos but not in *UAS-syx*$^+$*; G14-GAL4; syx* embryos (evoked). mESCs were also observed in the *UAS-syx*$^+$*; elav-GAL4; syx* mutants but not in the *UAS-syx*$^+$*; G14-GAL4; syx* mutants (mini).

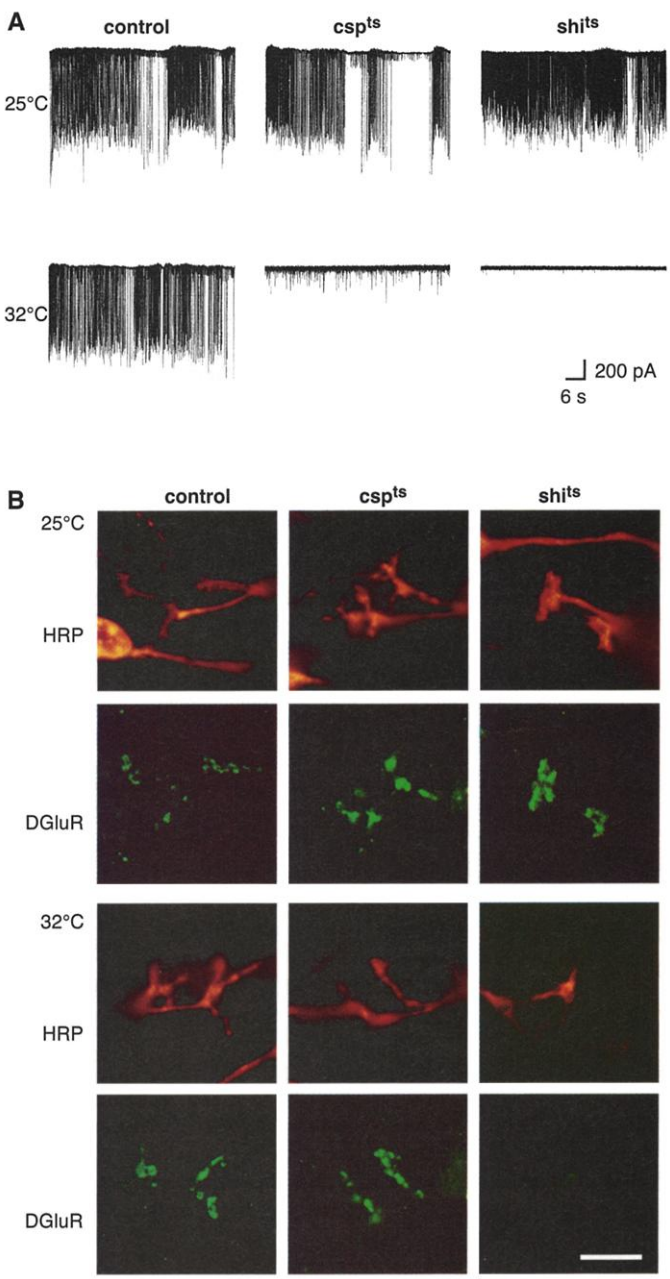

Fig. 3. The correlation between mESCs and GluR clustering. (**A**) Neuromuscular transmission in csp^{ts} and shi^{ts} embryos. In each column, ESCs were recorded from the same muscle at the permissive temperature (25°C) and were recorded again 10 min after the temperature was raised to 32°C. Vigorous ESCs were seen in wild-type embryos at both temperatures. In csp^{ts} mutants, episodic bursting of ESCs was observed as it was in wild-type embryos at 25°C, whereas only mESCs were seen at 32°C. In shi^{ts} mutants, ESCs occurred at 25°C but not at 32°C. (**B**) GluR localization at embryonic NMJs. Embryos were reared at 25°C throughout development (25°C) or shifted to 32°C at 13 hours after fertilization (32°C). Scale bar, 10 μm.

mutants if their terminals were spontaneously releasing transmitter. We could have recognized mESCs as small as 20 pA under the recording conditions we used (*10*). Thus, the complete absence of detectable mESCs could not be attributed to a lack of postsynaptic sensitivity.

The low sensitivity to glutamate in *syx* mutants could reflect the diminished trafficking of GluRs to the surface. Thus, we tested whether the clustering defect was due to a pre- or postsynaptic action of *syx* by the targeted expression of the *syx* transgene with either the neuron-specific embryonic lethal, abnormal vision (*elav*)–yeast transcription activator GAL4 driver or the muscle-specific G14-GAL4 driver (*14*). Neuron-specific expression of the *syx* transgene restored GluR clusters but muscle-specific expression did not (Fig. 2B). Evoked and mESCs were readily observed in transgenic embryos in which neuron-specific expression of the *syx* transgene was restored but not in transgenic embryos in which *syx* transgene was expressed in muscles (Fig. 2B, lower panels). Thus, a comparison of the phenotypes of *n-syb* and *syx* led us to hypothesize that spontaneous secretory events at the NMJ are critical to the formation of GluR clusters.

We used two temperature-sensitive (ts) paralytic mutants to examine further the correlation between spontaneous vesicle exocytosis and GluR clustering. At elevated temperatures, a defect in dynamin in *shi^{ts}* blocks synaptic vesicle recycling and thereby depletes the terminals of synaptic vesicles (*9*). In contrast, *csp^{ts}* mutations appear to interfere with excitation-secretion coupling in the terminal (*7, 21*). Synapses in *shi^{ts}* mutants became completely silent at a nonpermissive temperature (32°C), whereas *csp^{ts}* mutants lost evoked responses while retaining mESCs (*7*) (Fig. 3A). Because of these differences in release properties at the nonpermissive temperature, we compared the distribution of GluRs in these lines. At a permissive temperature (25°C), when release properties were similar among these embryos (Fig. 3A), GluR clustering was comparable for wild-type, *csp^{ts}*, and *shi^{ts}* mutant embryos (Fig. 3B). However, this situation changed when embryos were moved to the nonpermissive temperature at 13 hours after fertilization, which is when nerve-muscle contacts first form (*22, 23*). The development of GluR clusters was not perceptibly altered in wild-type and *csp^{ts}* mutants at 32°C. However, no detectable GluR clusters were observed in *shi^{ts}* mutants (Fig. 3B), as was the case in *syx* mutants. These results again suggest a tight link between spontaneous vesicle exocytosis and GluR clustering.

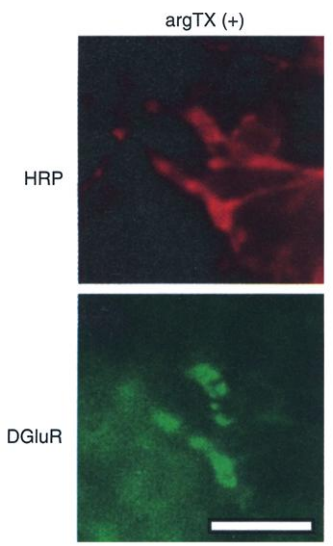

Fig. 4. Argiotoxin (argTX) does not affect receptor clustering. ArgTX was injected into embryos at 12 to 13 hours after fertilization. Embryos were double stained with anti-HRP and anti-DGluR-IIA at 21 to 22 hours after fertilization. Scale bar, 5 μm.

Further insight into the nature of interaction of presynaptic and postsynaptic elements came from the injection of argiotoxin (*14*) into wild-type embryos at concentrations that block all muscle contractile activity. In these embryos, GluRs still clustered postsynaptically (Fig. 4). Thus, it was not the activation of postsynaptic GluRs that directed GluR clustering. Similar findings have been reported in vertebrates, where α-bungarotoxin did not impede the clustering of acetylcholine receptor (AChR) (*3*). As in vertebrates, secretion of molecules, such as agrin for AChRs (*24*), ephrins for *N*-methyl-D-aspartate (NMDA)–type GluRs (*25*), and neuronal activity-regulated pentraxin for α-amino-3-hydroxyl-5-methyl-4-isoxazole propionic acid (AMPA)–type GluRs (*26*), may drive receptor clustering by being released with, or in parallel to, the neurotransmitter at *Drosophila* NMJs.

We documented a positive correlation between ongoing spontaneous vesicle exocytosis and the embryonic development of GluR clusters at *Drosophila* NMJs. Nerve-evoked vesicle exocytosis is not necessary for this process, because although neither *n-syb* nor *csp^{ts}* mutants showed any demonstrable nerve-evoked ESCs, GluRs still clustered. mECSs persisted in both mutants. However, when spontaneous secretory events were absent (as in *shi^{ts}* mutants at the nonpermissive temperature or in *syx*), junctional GluR clusters were exceedingly infrequent. If GluR clustering were solely contingent on the nerve-muscle contact, GluRs should have clustered at the contacts in *shi^{ts}* mutants raised at the nonpermissive temperature and in *syx* mutants. In a recent study of mice lacking an isoform of *munc 18-1*, there was no demonstrable change in AChR clustering, although both spontaneous and evoked neurotransmitter release were absent (*27*). Together with our observations, these data suggest that *munc 18-1* is not involved in the secretion of the agent that induces clustering of neurotransmitter receptors, whereas *syntaxin* is essential for this process.

The absence of clusters in *Drosophila* in *syx* and *shi* mutants implies that spontaneous secretory events are related to GluR clustering and probably to cluster stabilization as well. Moreover, it is the clustering of these receptors, rather than their surface expression, that depends on spontaneous secretion: Functional GluRs were detected in *syx* mutants although they had rarely formed detectable clusters at the synapse. The link between spontaneous vesicle exocytosis and receptor clustering must now be clarified.

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28. We thank A. Nose for the G14-GAL4 enhancer trap line; A. DiAntonio for the myc-tagged GluRIIB line; J. Morr, B. Schmidt, H. Betz, and C. Schuster for the AS5 antibody; and Y. Kubo (Physiology) at Tokyo Medical and Dental University, H. Okado and S. Ohsako at Tokyo Metropolitan Institute for Neuroscience, and A. Grinnell (Physiology) and B. Howard (Biological Chemistry) at the University of California Los Angeles for critical reading of the manuscript. Supported by a grant-in-aid from the Ministry of Education, Science, Sports and Culture of Japan (M.S and Y.K.), the Brain Science Foundation (M.S.), and by NIH (J.A.U).

3 April 2001; accepted 25 May 2001