# Rules of Nonallelic Noncomplementation at the Synapse in *Caenorhabditis elegans*

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ABSTRACT

Nonallelic noncomplementation occurs when recessive mutations in two different loci fail to complement one another, in other words, the double heterozygote exhibits a phenotype. We observed that mutations in the genes encoding the physically interacting synaptic proteins UNC-13 and syntaxin/UNC-64 failed to complement one another in the nematode *Caenorhabditis elegans*. Noncomplementation was not observed between null alleles of these genes and thus this genetic interaction does not occur with a simple decrease in dosage at the two loci. However, noncomplementation was observed if at least one gene encoded a partially functional gene product. Thus, this genetic interaction requires a poisonous gene product to sensitize the genetic background. Nonallelic noncomplementation was not limited to interacting proteins: Although the strongest effects were observed between loci encoding gene products that bind to one another, interactions were also observed between proteins that do not directly interact but are members of the same complex. We also observed noncomplementation between genes that function at distant points in the same pathway, implying that physical interactions are not required for nonallelic noncomplementation. Finally, we observed that mutations in genes that function in different processes such as neurotransmitter synthesis or synaptic development complement one another. Thus, this genetic interaction is specific for genes acting in the same pathway, that is, for genes acting in synaptic vesicle trafficking.

THE failure of two recessive alleles to complement one another for a specific phenotype indicates that the mutations are alleles of the same gene (Benzer 1955). However, there are instances in which alleles of two distinct genes do not complement one another even though a wild-type copy of each gene is present. This phenomenon is called nonallelic noncomplementation (also known as unlinked noncomplementation, second-site noncomplementation, extragenic noncomplementation, and transheterozygous noncomplementation). Examples of this phenomenon have been reported in a diverse number of biological processes such as cuticle development (Kusch and Edgar 1986), transcriptional regulation (Rine and Herskowitz 1987; Rancourt *et al.* 1995), cytoskeleton-based cell behavior (Stearns and Botstein 1988; Hays *et al.* 1989; Welch *et al.* 1993; Wang and Bretscher 1997; Halsell and Kiehart 1998), neuron outgrowth (Kidd *et al.* 1999), and oogenesis (Jackson and Berg 1999). In the cases where the gene products are known, the noncomplementing loci frequently encode products that interact physically (Rine and Herskowitz 1987; Stearns and Botstein 1988; Hays *et al.* 1989; Vinh *et al.* 1993; Welch *et al.* 1993; Hill *et al.* 1995; Rancourt *et al.* 1995; Kidd *et al.* 1999; Mounkes and Fuller 1999). Therefore nonallelic noncomplementation between mutations of two different loci is often interpreted as signifying a physical interaction between the products of the genes.

Two models have been proposed to explain nonallelic noncomplementation: the dosage model and the poison model (Stearns and Botstein 1988; Fuller *et al.* 1989). In the dosage model, a decrease in dosage at a single locus does not affect the process; however, a simultaneous decrease at a second locus proves to be detrimental. For example, null mutations in *slit*, a ligand required for proper neuronal migration during development in *Drosophila melanogaster*, fail to complement null alleles of *robo*, a receptor for Slit protein (Kidd *et al.* 1999).

In the poison model, an altered gene product must bind and impair the protein complex with which it is normally associated. Although this first defect does not produce a visible phenotype, a second mutation in another member of the protein complex reveals a visible defect. Such interactions have been observed between α- and β-tubulin genes. For example, in Drosophila and yeast, altered α-tubulins act as poisons by either sequestering β-tubulin or disrupting the polymerization of the microtubule (Stearns and Botstein 1988; Fuller *et al.* 1989; Hays *et al.* 1989).

Proper communication between neurons relies on the regulated fusion of synaptic vesicles with the active zone, a specialized region of the plasma membrane, and the subsequent release of neurotransmitter. For this

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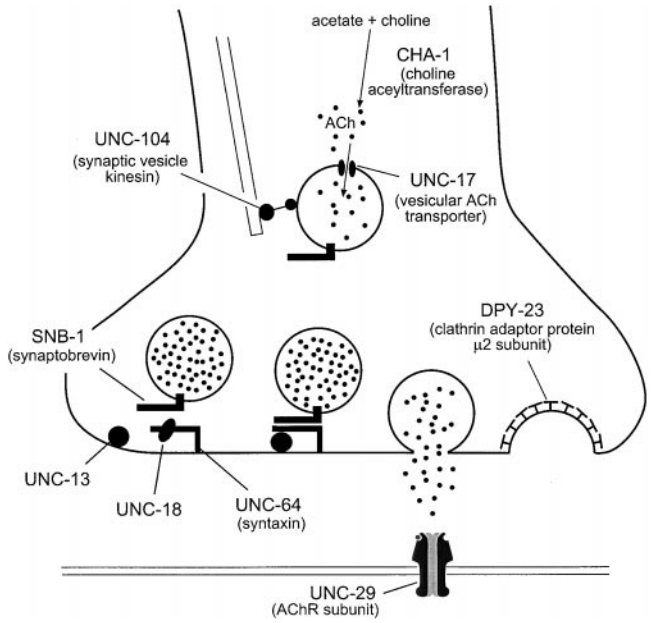


FIGURE 1.—The synaptic vesicle cycle. The proteins used in this study are indicated. ACh, acetylcholine.

process to occur, first, synaptic vesicles and associated proteins must be transported to the synapse from the cell body. Second, the vesicles must be filled with neurotransmitter. Third, the mature synaptic vesicle must be docked and primed so that the vesicle can rapidly fuse with the plasma membrane when the neuron is depolarized. Finally, the vesicle and its associated proteins must be recovered from the plasma membrane through endocytosis to maintain a releasable pool of synaptic vesicles (Figure 1). Two proteins required for the exocytosis step are UNC-13 and syntaxin. UNC-13 is a diacylglycerol-binding protein with multiple C2 $Ca^{2+}$-binding domains (MARUYAMA and BRENNER 1991; AHMED *et al.* 1992). UNC-13 proteins are required for exocytosis in *Caenorhabditis elegans*, mammals, and Drosophila (BROSE *et al.* 1995; BETZ *et al.* 1998; ARAVAMUDAN *et al.* 1999; AUGUSTIN *et al.* 1999; RICHMOND *et al.* 1999). Syntaxin is a member of the SNARE complex that is required for synaptic vesicle fusion with the plasma membrane (SOLLNER *et al.* 1993; HAYASHI *et al.* 1994; SCHULZE *et al.* 1995). UNC-13 and syntaxin physically interact (BETZ *et al.* 1997; AUGUSTIN *et al.* 1999; SASSA *et al.* 1999). It is thought that this interaction mediates a priming step in which the synaptic vesicle becomes fusion competent (BROSE *et al.* 1995; BETZ *et al.* 1998; ARAVAMUDAN *et al.* 1999; AUGUSTIN *et al.* 1999; RICHMOND *et al.* 1999).

During our studies of neurotransmission in the nematode *C. elegans*, we observed nonallelic noncomplementation between mutations in UNC-13 and syntaxin. These proteins are encoded by the genes *unc-13* and *unc-64*, respectively (MARUYAMA and BRENNER 1991; OGAWA *et al.* 1998; SAIFEE *et al.* 1998). Homozygous *unc-13(n2813)* and *unc-64(e246)* mutations cause animals to be uncoordinated. These mutations are recessive and heterozygotes are wild type in behavior. Surprisingly, worms doubly heterozygous for these alleles are uncoordinated (genotype *unc-13(n2813)/+*; *unc-64(e246)/+*) and thus exhibit nonallelic noncomplementation. We used this interaction as a starting point to determine the rules of nonallelic noncomplementation at the synapse. In these experiments, we used a drug assay to quantify the effects of mutations on synaptic function. Sensitivity to Aldicarb, an inhibitor of acetylcholinesterase, is a measure of the amount of acetylcholine released into the synaptic cleft. First, we used null and hypomorphic alleles of both *unc-13* and *unc-64* to distinguish between the dosage and poison models for this interaction. Second, we determined the boundaries of this interaction. Specifically, we determined whether nonallelic noncomplementation could be observed between proteins that physically interact, between proteins that do not interact but are found together in a larger complex, between proteins that simply function in the same pathway and are not in the same complex, and between proteins that are not in the same pathway.

## MATERIALS AND METHODS

**Strains:** N2 var. Bristol was used as the wild-type strain. Worms were cultured and maintained as previously described (BRENNER 1974). The following mutations were used in this study:

Linkage group (LG) I: *unc-13(e51); unc-13(n2813); unc-29 (e1072)*
LG II: *unc-104(e1265)*
LG III: *unc-64(js115)*, *unc-64(e246)*, *bli-5(e518)*
LG IV: *cha-1(p1152)*, *unc-17(e245); unc-17(ox51)*
LG V: *snb-1(md247), snb-1(js124); dpy-11(e224)*
LG X: *unc-18(e81)*, *dpy-23(e840); syd-2(ju37)*

**Generation of single and double heterozygotes:** To generate heterozygous worms for a single mutation, L4 hermaphrodites homozygous for the relevant genotype were mated to wild-type males, except in the cases of *unc-64(js115)* and *snb-1 (js124)*. Since these mutations are homozygous lethal, they are maintained as heterozygotes balanced by *bli-5(e518)* and *dpy-11(e224)* mutations, respectively.

To generate worms heterozygous for two mutations, heterozygous males of one genotype were mated with homozygous L4 hermaphrodites of the second genotype except in the case of *unc-64(js115)*. In this case, *unc-64(js115) +/+ bli-5(e518)* males were crossed to homozygous strains to generate the double heterozygote.

**Drug resistance assay:** Aldicarb (2-methyl-2-[methylthio]-propionaldehyde *O*-[methylcarbamoyl]oxime; Chem Services, West Chester, PA) was solubilized in acetone and then diluted to a working stock solution of 17.5 mM in M9. Standard NGM worm plates were treated with Aldicarb to a final concentration of 0.7 mM and allowed to dry at room temperature. For each experiment, animals that have been adults for <2 days were picked to pretreated plates and scored for resistance after 12 hr of exposure to the drug. Worms were considered resistant if they could move on their own or respond when prodded by a platinum wire. In some experiments, the worms were scored for movement every 4 hr (±30 min) over a 16-hr period.

In other experiments the worms were scored in the 12th hour of exposure.

**Statistical analysis:** Since all of the double heterozygotes were generated by mating heterozygous males with homozygous hermaphrodites, the progeny from any given cross are of two types and thus the relevant genotype is not observable directly. The resistance exhibited by the worms that result from each cross is the average resistance of the two genotypes produced by segregation. To analyze the levels of drug resistance of the relevant genotype in any cross we adopted a maximum-likelihood framework (see the APPENDIX) in which we were able to estimate the resistance of the double heterozygote when given direct measurements of the background genotype. For most cases, including the crosses involving *unc-64(js115)*, the genotype of resistant animals was confirmed by picking resistant animals and observing the genotypes of the progeny. Furthermore, our framework allowed us to compare hypotheses about the relationships between the resistances of different genotypes. In addition, the framework allowed us to test whether or not the level of resistance exhibited by the double heterozygote could be an additive effect of each of the single heterozygotes. The outcomes of these hypothesis tests were used as a basis for distinguishing significance in the level of drug resistance among the double heterozygote genotype and each single heterozygote genotype. We report the maximum-likelihood estimates with the corresponding 2-unit support boundaries in parentheses [*i.e.*, 4% (2–12%)]. The 2-unit support boundaries are often used in likelihood analysis in lieu of confidence limits (EDWARDS 1992) and are approximately the same as the upper and lower 95% confidence intervals for population proportions (SOKAL and ROHLF 1987).

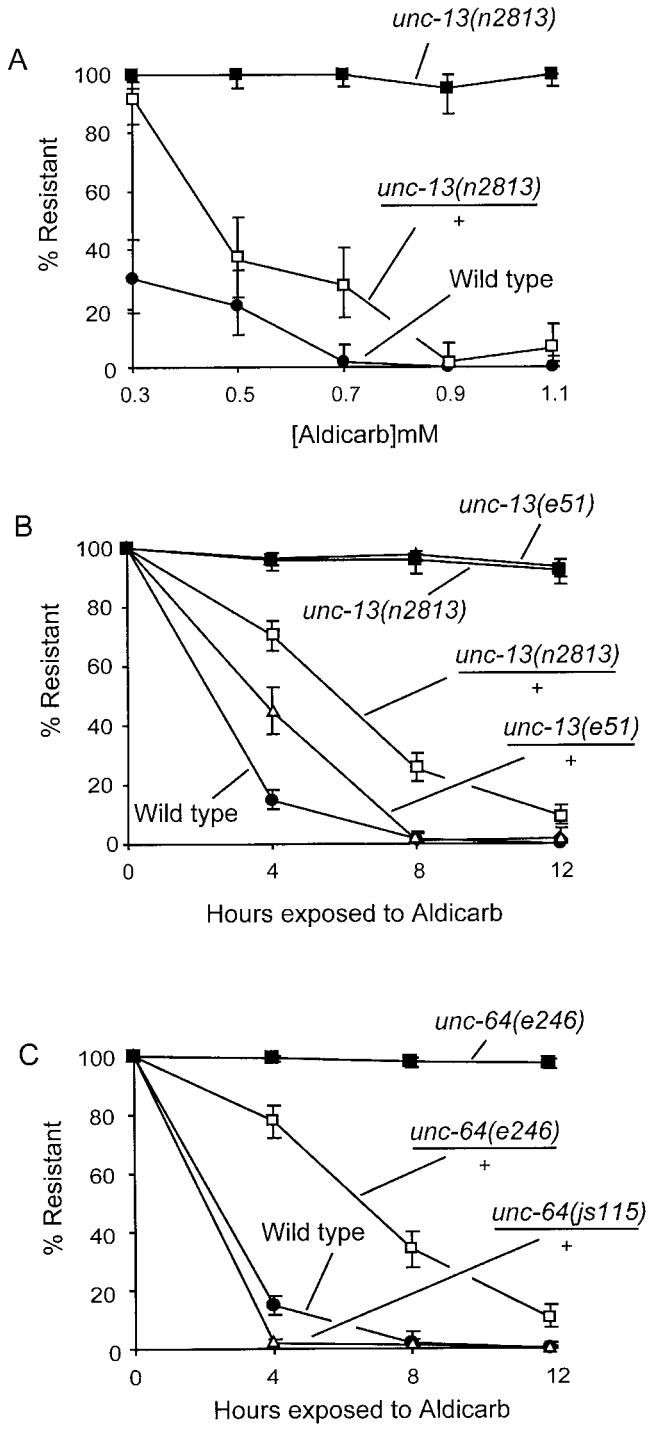


FIGURE 2.—Resistance to Aldicarb reveals a poisonous effect of hypomorphic *unc-13* and *unc-64* synaptic function alleles. (A) Resistance to Aldicarb at different concentrations. Worms were treated with different concentrations of the drug (0.3, 0.5, 0.7, 0.9, and 1.1 mM) and scored for resistance after 8 hr of exposure. (●) Wild type, $n = 3(19)$; (■) *unc-13(n2813)*, $n = 3(15)$; and (□) *unc-13(n2813)/+*, $n = 3(18)$. (B) Adult worms were treated with 0.7 mM Aldicarb and assayed for resistance after 4, 8, and 12 hr of exposure. (●) Wild type, $n = 3(20)$; (■) *unc-13(n2813)*, $n = 3(16)$; (□) *unc-13(n2813)/+*, $n = 3(18)$; (▲) *unc-13(e51)*, $n = 6(52)$; and (△) *unc-13(e51)/+*, $n = 5(30)$. (C) Adult worms were treated with 0.7 mM Aldicarb and assayed for resistance after 4, 8, and 12 hr of exposure. (●) Wild type, $n = 3(20)$; (■) *unc-64(e246)*, $n = 9(32)$; (□) *unc-64(e246)/+*, $n = 6(39)$; and (△) *unc-64(js115)/+*, $n = 9(20)$. $n = 3(19)$ indicates that three plates, each representing one experiment, were counted with an average number of 19 animals per plate. The error bars represent the maximum-likelihood analog of 95% confidence intervals.

## RESULTS

**A quantitative assay:** In the process of making double mutants we observed that worms doubly heterozygous for *unc-13(n2813)* and *unc-64(e246)* (genotype *unc-13/+; unc-64/+*) were uncoordinated. Thus, these mutations exhibited nonallelic noncomplementation. Further characterization of this phenomenon required a quantitative assay. Since the severity of an uncoordinated phenotype is a subjective measure, we chose to use a pharmacological assay to quantify allelic interactions. The inhibitor of cholinesterase, Aldicarb, blocks the breakdown of acetylcholine and causes acetylcholine to accumulate in the synaptic cleft. In wild-type worms, this accumulation results in constitutive depolarization and hypercontraction of the muscle, which leads to paralysis and eventually to death. Mutations that disrupt the release of acetylcholine are resistant to Aldicarb (BRENNER 1974; RAND and RUSSELL 1985; HOSONO *et al.* 1989;

Miller *et al.* 1996). Therefore we used sensitivity to Aldicarb as a measure of synaptic activity.

To maximize the sensitivity of our assay, we identified conditions under which wild-type worms were fully sensitive to Aldicarb, but *unc-13(n2813)* worms were fully resistant. The lowest concentration of drug at which all wild-type animals were paralyzed was 0.7 mm Aldicarb (Figure 2A). *unc-13(n2813)* animals were completely resistant to all concentrations of the drug tested. Thus, this concentration provided the greatest sensitivity to changes in response to Aldicarb and was consistent with results obtained in other studies (Jorgensen *et al.* 1995).

To determine the time of exposure that provided the highest sensitivity to changes in response to Aldicarb, we performed a time course of resistance. When exposed to 0.7 mm Aldicarb, wild-type worms quickly succumbed to the effects of the drug and by 12 hr of exposure almost all the animals were paralyzed (Figure 2B). By contrast, homozygous *unc-13(n2813)* worms remained resistant at 12 hr (Figure 2B) and exhibited only a slight decrease in resistance at 16 hr (data not shown). Since a 12-hr exposure to 0.7 mm Aldicarb provided a satisfactory level of distinction between wild-type and mutant worms we used these parameters to assess synaptic efficacy among all double heterozygotes in this study.

Resistance to Aldicarb provided a more sensitive means of measuring synaptic efficacy than a behavioral analysis. Strong *unc-13* mutants, such as the nonsense mutant *unc-13(e51),* are severely paralyzed as homozygotes. The *unc-13(e51)* mutation is an early stop in the long transcript encoding the synaptically localized isoform of UNC-13 (the short transcript encodes the axonally localized isoform; Nurrish *et al.* 1999; Kohn *et al.* 2000). By contrast, worms homozygous for the weak *unc-13(n2813)* mutation move forward quite well and exhibit only a jerky phenotype when prodded to move backward. The *unc-13(n2813)* mutation is a missense mutation in the C-terminal region of the UNC-13 protein that is common to all splice forms (Kohn *et al.* 2000). However, *unc-13(n2813)* mutants exhibit almost the same level of drug resistance as *unc-13(e51)* mutants, 92% (88–96%) and 93% (90–96%), respectively (Figure 2B). So although *unc-13(n2813)* homozygotes are often difficult to distinguish from wild-type worms on the basis of their behavioral phenotype, they can easily be identified on the basis of drug resistance. Therefore a small decrease in neurotransmitter release can translate into a significant level of resistance to Aldicarb.

**Poison alleles:** To test the dosage and poison models, we needed null and poison alleles of the *unc-13* and *unc-64* loci. The *unc-13(e51)* and *unc-13(n2813)* alleles are recessive; specifically, heterozygotes exhibit a wild-type behavioral phenotype. Surprisingly, we detected a poisonous effect of the weak allele, *n2813*, but not the nonsense allele, *e51*, in our Aldicarb sensitivity experiments. *unc-13(e51)/+* heterozygotes were as sensitive to Aldicarb as wild-type worms at 8 and 12 hr of exposure (Figure 2B). By contrast, *unc-13(n2813)* heterozygotes were weakly resistant to Aldicarb, particularly at intermediate concentrations (Figure 2A) or at short times of drug exposure (Figure 2B). These data indicated that the *unc-13(n2813)* allele has a weakly poisonous effect on synaptic transmission.

Similarly, we detected a poisonous effect of the weak allele of *unc-64*/syntaxin, *e246*, but not the null allele, *js115*, in our drug sensitivity assay. The *unc-64(e246)* mutation is a missense change in the SNARE domain of the UNC-64 protein. The *unc-64(js115)* null lesion is an early stop. As with the *unc-13* null heterozygotes, *unc-64(js115)/+* heterozygotes were as sensitive to Aldicarb as wild-type worms at all times of exposure to the drug (Figure 2C). By contrast, worms heterozygous for the weak allele [*unc-64(e246)/+*] were weakly resistant to Aldicarb at 8–12 hr of exposure (Figure 2C). Thus, like the *unc-13(n2813)* allele, the *unc-64(e246)* allele exhibited a weakly poisonous effect on synaptic transmission in a heterozygote. We used these null and poison alleles of *unc-13* and *unc-64* to test the dosage and poison models for nonallelic noncomplementation.

**Dosage *vs.* poison:** We did not observe resistance to Aldicarb in heterozygous strains for null alleles of *unc-13* or *unc-64* (Figure 2, B and C). Assuming that there is no compensation for the lowered levels of gene product, then a 50% reduction in the concentrations of either of these proteins does not impair neurotransmission. However, in the dosage model of nonallelic noncomplementation, the detrimental effect is observed only when both loci are decreased at the same time. Therefore, to test the dosage model, we constructed worms that had a reduction in gene dosage at both loci simultaneously; that is, we constructed double heterozygotes [*unc-13(e51)/+; unc-64(js115)/+*] and measured the level of drug resistance exhibited by these worms. The double heterozygotes were not resistant to Aldicarb [1% (0–6%); Figure 3A].

Alternatively, in the poison model, nonallelic noncomplementation is observed only if altered gene products impair the protein complexes with which they are normally associated. To test the poison model we constructed worms that were doubly heterozygous for the poison alleles of *unc-13* and *unc-64* [genotype unc-*13(n2813)/+; unc-64(e246)/+*]. The double heterozygote exhibited a significant increase in the level of Aldicarb resistance compared to either heterozygote alone [42% (35–50%) for *unc-13(n2813)/+*; *unc-64(e246)/+*, 4% (2–7%) for *unc-13(n2813)/+*, and 8% (5–11%) for *unc-64(e246)/+*; Figure 3B]. This result suggests that nonallelic noncomplementation between these loci obeys the poison model. In addition, the level of resistance exhibited by the double heterozygote is not due simply to an additive effect of each single heterozygote (Table 1, appendix).

To test whether a single poison gene product can

**TABLE 1**

**Adjusted negative log-likelihood scores of the two models for complementation**

| Double heterozygote $p_3$ | Heterozygote $p_1$ | Heterozygote $p_2$ | Model 1: additive | Model 2: synergistic |
|---|---|---|---|---|
| *unc-13(e51)/+; unc-64(js115)/+* | *unc-13(e51)/+* | *unc-64(js115)/+* | 14.98 | 15.92 |
| *unc-13(n2813)/+; unc-64(e246)/+* | *unc-13(n2813)/+* | *unc-64(e246)/+* | 118.78 | 97.76 |
| *unc-13(n2813)/+; unc-64(js115)/+* | *unc-13(n2813)/+* | *unc-64(js115)/+* | 117.78 | 86.04 |
| *unc-13(e51)/+; unc-64(e246)/+* | *unc-13(e51)/+* | *unc-64(e246)/+* | 41.43 | 40.72 |
| *unc-64(e246)/+; snb-1(md247)/+* | *unc-64(e246)/+* | *snb-1(md247)/+* | 47.53 | 40.26 |
| *unc-13(n2813)/+; snb-1(md247)/+* | *unc-13(n2813)/+* | *snb-1(md247)/+* | 50.18 | 48.52 |
| *unc-13(n2813)/+; unc-18(e81)/+* | *unc-13(n2813)/+* | *unc-18(e81)/+* | 60.01 | 54.16 |
| *unc-13(n2813)/+; dpy-23(e840)/+* | *unc-13(n2813)/+* | *dpy-23(e840)/+* | 48.57 | 39.22 |
| *unc-13(n2813)/+; unc-104(e1265)/+* | *unc-13(n2813)/+* | *unc-104(e1265)/+* | 50.11 | 47.56 |
| *unc-13(n2813)/+; cha-1(pr1152)/+* | *unc-13(n2813)/+* | *cha-1(pr1152)/+* | 30.08 | 31.07 |
| *unc-13(n2813)/+; unc-17(e245)/+* | *unc-13(n2813)/+* | *unc-17(e245)/+* | 30.26 | 30.63 |
| *unc-13(n2813)/+; unc-29(e1072)/+* | *unc-13(n2813)/+* | *unc-29(e1072)/+* | 45.17 | 33.31 |
| *unc-13(n2813)/+; syd-2(ju37)/+* | *unc-13(n2813)/+* | *syd-2(ju37)/+* | 27.90 | 28.91 |

The additive model plays the role of a null model, as it has only two parameters, while the synergistic model has three. We compare the likelihood scores of the two models, adjusted for the number of parameters by the Akaike information criterion (AIC). This penalizes the higher parameter model, because an increase in parameters will always bring a better fit. When the synergistic model has a better AIC than the additive model, we reject additivity. The underlined score represents the accepted model.

sensitize the strain to further mutations, we constructed worms heterozygous for a poison allele at one locus and a null allele at the other locus. In these cases, we still observed nonallelic noncomplementation (Figure 3, C and D). In addition, noncomplementation occurred regardless of which locus encoded the poison allele or the null allele. The level of Aldicarb resistance exhibited by worms heterozygous for the *unc-13(n2813)* poison allele and the *unc-64(js115)* null allele [genotype *unc-13(n2813)/+*; *unc-64(js115)/+*] was 56% (43–69%; Figure 3C). Conversely, the level of Aldicarb resistance exhibited by worms heterozygous for the *unc-13(e51)* nonsense allele and the *unc-64(e246)* poison allele was 17% [10–24%; genotype *unc-13(e51)/+; unc-64(e246)/+*]. This is significantly different than the level of resistance exhibited by the single heterozygotes alone and is not simply an additive effect of the single heterozygotes (Table 1, APPENDIX). These data imply that the presence of one altered synaptic component is sufficient to sensitize synaptic transmission to the levels of interacting gene products.

**Noncomplementation among interacting loci:** Biochemical studies have demonstrated that syntaxin binds to the integral synaptic vesicle membrane protein synaptobrevin and plasma membrane protein SNAP-25 to form a SNARE complex (SOLLNER *et al.* 1993). The crystal structure of the SNARE complex suggests that these three proteins are intertwined when the synaptic vesicle is positioned at the active zone (SUTTON *et al.* 1998). To determine whether other proteins that interact with syntaxin exhibit nonallelic noncomplementation, we tested interactions between the *unc-64*/syntaxin and the *C. elegans* homolog of synaptobrevin, *snb-1*. We tested noncomplementation of *unc-64* using the *snb-1* hypomorphic allele, *md247* (NONET *et al.* 1998). We observed that the double heterozygote exhibited a marked increase in Aldicarb resistance, [48% (29–71%) for *unc-64(e246)/+; snb-1(md247)/+*; Figure 4A] compared to the resistance of either heterozygote alone [8% (5–11%) for *unc-64(e246)/+* and 4% (2–7%) for *snb-1 (md247)/+*]. This result suggests that nonallelic noncomplementation is a more general phenomenon among synaptic genes and is not specific to the *unc-13(n2813)* mutation.

**Noncomplementation among proteins of the same complex:** Nonallelic noncomplementation is often interpreted to signify a direct physical interaction between the products of the loci involved. To test whether nonallelic noncomplementation requires a direct physical association between the noncomplementing gene products, we constructed double heterozygotes between *unc-13(n2813)* and mutations in proteins that are not known to bind to UNC-13. Since SNB-1/synaptobrevin associates only indirectly with UNC-13 as part of a complex with UNC-64/syntaxin (BETZ *et al.* 1997), we tested whether *unc-13(n2813)* would complement *snb-1*. Double heterozygotes between *unc-13(n2813)* and the *snb-1(md247)* weak allele exhibited a level of drug resistance that is significantly different from either heterozygote alone [19% (11–28%) for *unc-13(n2813)/+; snb-1(md247)/+*, 4% (2–7%) for *unc-13(n2813)/+*, and 4% (2–7%) for *snb-1(md247)/+*; Figure 4B]. Therefore nonallelic noncomplementation at the synapse does not require a direct physical interaction between the gene products. However, as observed between *unc-13* and *unc-64*, there was no interaction between null alleles of these loci.

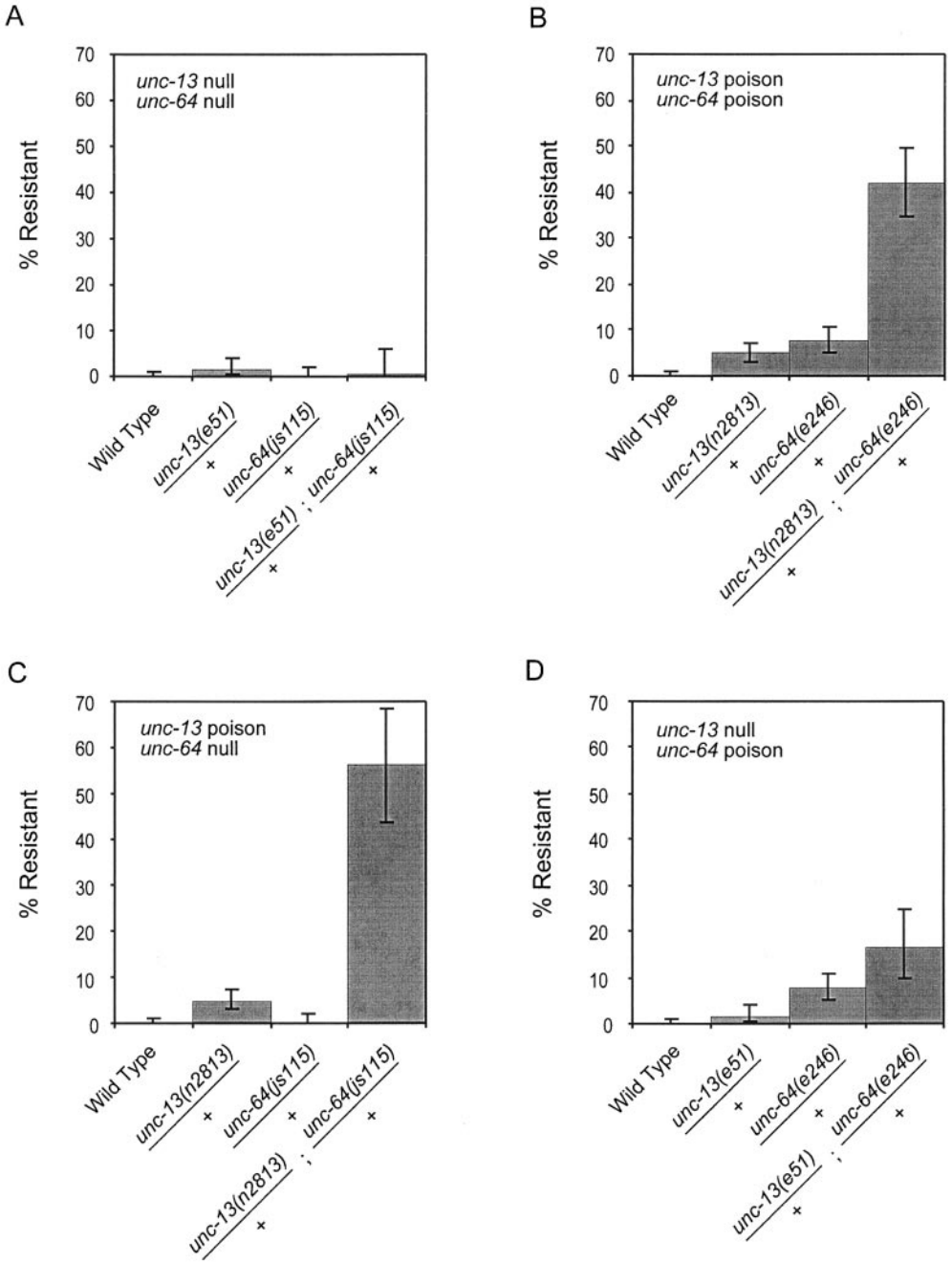


FIGURE 3.—Noncomplementation between UNC-13 and syntaxin requires the presence of a poison allele. Worms singly or doubly heterozygous for *unc-13* and *unc-64* were constructed and tested for resistance to 0.7 mM Aldicarb after 12 hr of exposure and compared to the wild type [first column of each graph, $n = 20(30)$]. (A) Synaptic function is not dosage sensitive. *unc-13(e51)/+*, $n = 7(34)$; *unc-64(js115)/+*, $n = 12(18)$; and *unc-13(e51)/+; unc-64(js115)/+*, $n = 5(27)$. (B) The two hypomorphic alleles exhibit nonallelic noncomplementation. *unc-13(n2813)/+*, $n = 13(32)$; *unc-64(e246)*, $n = 9(33)$; and *unc-13(n2813)/+; unc-64(e246)/+*, $n = 9(33)$. (C) The hypomorphic *unc-13* allele reveals dosage sensitivity to *unc-64* gene product levels. *unc-13(n2813)/+*, $n = 12(30)$; *unc-64(js115)/+*, $n = 12(18)$; and *unc-13(n2813)/+; unc-64(js115)/+*, $n = 8(29)$. (D) The *unc-64* hypomorphic allele reveals dosage sensitivity to *unc-13* gene product levels. *unc-13(e51)/+*, $n = 7(34)$; *unc-64(e246)*, $n = 9(33)$; and *unc-13(e51)/+; unc-64(e246)/+*, $n = 7(29)$.

Specifically, *unc-13(e51)* fully complements the null allele *snb-1(js124)* (data not shown).

Furthermore, we tested whether *unc-13* exhibited nonallelic noncomplementation with *unc-18*. UNC-18 also binds to UNC-64/syntaxin (HATA *et al.* 1993), but does not bind when UNC-13 or synaptobrevin is bound (PEVSNER *et al.* 1994; SASSA *et al.* 1999). We observed that the double heterozygote exhibited more resistance than either heterozygote alone [19% (12–27%) for *unc-13 (n2813)/+; unc-18(e81)/+*, 4% (2–7%) for *unc-13 (n2813)/+*, and 1% (0–2%) for *unc-18(e81)/+*; Figure 4C]. Since *unc-18* heterozygotes (*unc-18(e81)/+*) are wild type for Aldicarb sensitivity (Figure 4C), the *e81* mutation does not appear to have a poisonous effect. Furthermore, *unc-18(e81)* is likely to be a null allele since it is a nonsense mutation and since there is no immunoreactivity in *unc-18(e81)* homozygous worms (GENGYO-ANDO *et al.* 1993). Thus, *unc-13(n2813)* can sensitize synaptic function to decreases in the dosage of proteins that are not components of the SNARE complex.

**Boundaries of nonallelic noncomplementation:** So far our results have demonstrated that a poison allele of *unc-13* can sensitize synaptic transmission to the dosage of components required for synaptic vesicle exocytosis. The availability of gene products at the synaptic terminal

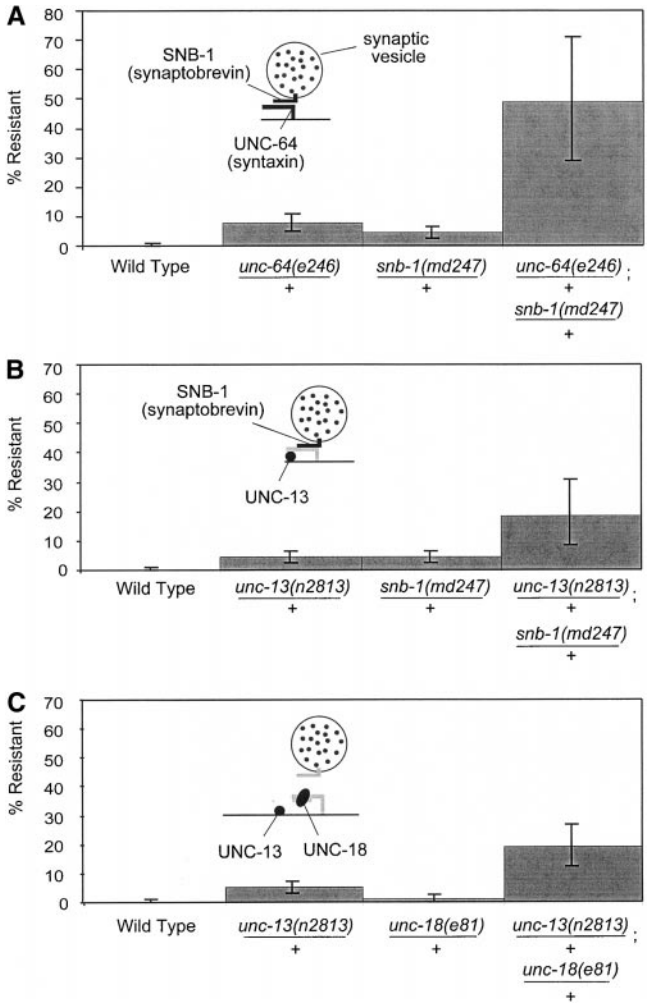


FIGURE 4.—Nonallelic noncomplementation occurs between genes that encode components of the same complex. (A) Syntaxin and synaptobrevin mutations exhibit nonallelic noncomplementation. *unc-64(e246)/+*, $n = 9(46)$; *snb-1 (md247)/+*, $n = 7(27)$; and *unc-64(e246)/+; snb-1(md247)/+*, $n = 4(17)$. (B) UNC-13 and synaptobrevin mutations exhibit nonallelic noncomplementation. *unc-13(n2813)/+*, $n = 12(30)$; *snb-1(md247)/+*, $n = 7(27)$; and *unc-13(n2813)/+; snb-1 (md247)/+*, $n = 5(27)$. (C) UNC-13 and UNC-18 mutations exhibit nonallelic noncomplementation. *unc-13(n2813)/+*, $n = 12(30)$; *unc-18(e81)/+*, $n = 9(28)$; and *unc-13(n2813)/+; unc-18(e81)/+*, $n = 9(30)$. Insets depict gene products at the synapse. In all graphs, wild-type $n = 20(30)$ and resistance was measured after 12 hr of exposure to 0.7 mM Aldicarb.

requires transport from two routes: via kinesin-mediated transport from the cell body and via endocytosis from the terminal itself. Therefore, to explore the ability of a poison allele to reveal dosage sensitivity in the transport machinery, we tested genetic interactions between UNC-13 and synaptic vesicle kinesin and between UNC-13 and the clathrin adaptor complex, proteins that control vesicle trafficking at the synapse.

*unc-104* encodes a kinesin motor protein that transports synaptic vesicles and proteins to the terminal (HALL and HEDGECOCK 1991; OTSUKA *et al.* 1991). There is no evidence to support a direct interaction between UNC-13 and UNC-104 proteins, nor is it suspected that these products participate in the same complex. Worms homozygous for the weak allele, *unc-104(e1265)*, are nearly paralyzed, whereas heterozygotes behave like the wild type. In the presence of Aldicarb, *unc-104/+* single heterozygotes also behave like wild-type animals. However, double heterozygotes exhibited a significantly increased level of Aldicarb resistance compared to either heterozygote [19% (11–28%) for *unc-13(n2813)/+; unc-104(e1265)/+*, 2% (0–5%) for *unc-104(e1265)/+*; Figure 5A]. This result suggests that a poison allele in the fusion machinery can reveal perturbations in trafficking components.

Synaptic gene products are recycled from the plasma membrane via clathrin-mediated endocytosis. Clathrin assembly on the plasma membrane requires the AP-2 adaptor complex. The μ-subunit of the *C. elegans* AP-2 complex is encoded by *dpy-23* (P. BAUM and G. GARRIGA, personal communication). Worms homozygous for the null allele *dpy-23(e840)* are severely uncoordinated and exhibited an intermediate level of resistance to Aldicarb [55% (46–63%), data not shown]. Although *dpy-23* heterozygotes were not drug resistant, double heterozygotes were markedly resistant [42% (29–58%) for *unc-13(n2813)/+; dpy-23(e840)/+*, 4% (2–7%) for *unc-13 (n2813)/+*, and 0% (0–3%) for *dpy-23(e840)/+*; Figure 5B]. This level of resistance is similar to the level of drug resistance exhibited by *dpy-23(e840)* homozygotes. Thus, the poison mutation of *unc-13* can sensitize synaptic function to decreased levels of components required for endocytosis.

**Mutations in distant pathways:** To determine if *unc-13(n2813)* could sensitize neurotransmission to mutations that do not affect the exocytotic machinery, we analyzed interactions with mutations affecting the levels of neurotransmitter in synaptic vesicles. *cha-1* encodes choline acetyltransferase, the biosynthetic enzyme for acetylcholine (RAND and RUSSELL 1984). Null mutations in *cha-1* are lethal whereas a hypomorphic mutation [*cha-1(p1152)*] is viable and paralyzed as homozygotes. Even though the *cha-1* heterozygote has decreased levels of choline acetyltransferase activity (RAND and RUSSELL 1984), heterozygous worms exhibited wild-type levels of Aldicarb resistance [1% (0–4%) for *cha-1(p1152)/+*; Figure 6A]. Furthermore, the double heterozygote was barely distinguishable from the *unc-13* heterozygote alone and any increase in the level of resistance of the double heterozygote could be attributed to an additive effect of both mutations [5% (0–14%) for *unc-13 (n2813)/+; cha-1(p1152)/+* and 4% (2–7%) for *unc-13 (n2813)/+*; Figure 6A, APPENDIX]. Therefore, a poison UNC-13 protein does not sensitize synaptic function to deficits in neurotransmitter supply.

Furthermore, we analyzed genetic interactions between *unc-13(n2813)* and *unc-17*. *unc-17* encodes the vesicular acetylcholine transporter (VAChT) that is re-

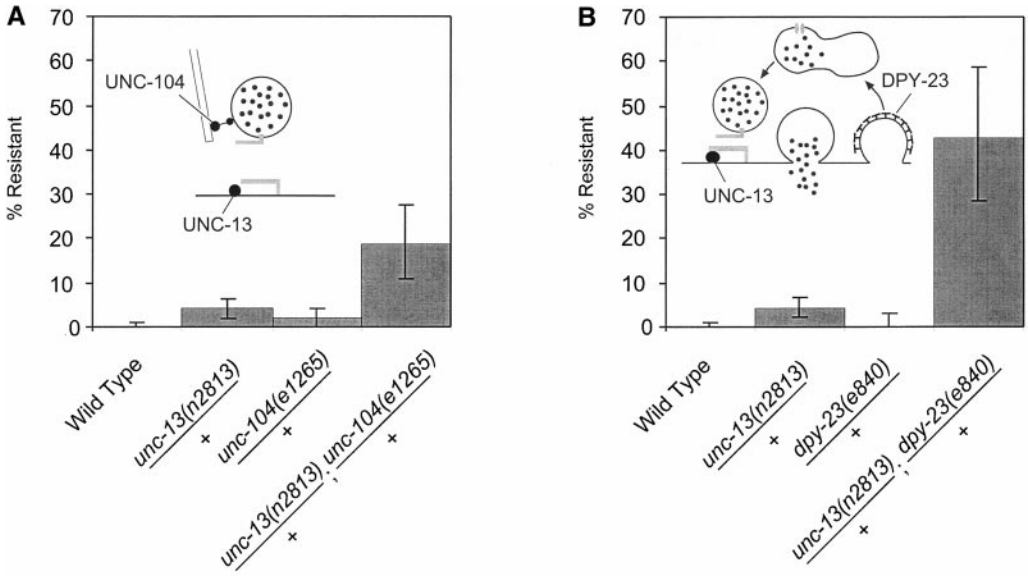


FIGURE 5.—Nonallelic noncomplementation occurs between *unc-13(n2813)* and genes involved in synaptic vesicle trafficking. (A) UNC-13 and synaptic vesicle kinesin mutations exhibit nonallelic noncomplementation. *unc-13 (n2813)/+*, $n = 6(39)$; *unc-104(e1265)/+*, $n = 6(38)$; and *unc-13 (n2813)/+; unc-104(e1265)/+*, $n = 6(38)$. (B) UNC-13 and clathrin adaptor complex mutations exhibit nonallelic noncomplementation. *unc-13 (n2813)/+*, $n = 6(39)$; *dpy-23(e840)/+*, $n = 2(31)$; and *unc-13(n2813)/+; dpy-23(e840)*, $n = 6(22)$. In all graphs, wild-type, $n = 10(32)$ and resistance was measured after 12 hr of exposure to 0.7 mM Aldicarb.

quired for loading synaptic vesicles with acetylcholine (ALFONSO *et al.* 1993). Worms homozygous for the null allele, *ox51*, are lethal, whereas worms homozygous for the weak allele, *e245*, are severely uncoordinated and coiled. *unc-17(e245)* heterozygotes exhibited a small amount of resistance. However, the double heterozygote did not exhibit a level of Aldicarb resistance that was significantly different from the single heterozygotes [3% (0–6%) for *unc-13(n2813)/+; unc-17(e245)/+*, 4% (2–7%) for *unc-13(n2813)/+*, and 1% (0–5%) for *unc-17(e245)/+*; Figure 6B]. Similar results were obtained for the lethal allele, *ox51*, of *unc-17* (data not shown). Thus, a poison UNC-13 protein does not significantly enhance defects in the loading of acetylcholine into synaptic vesicles.

Neurotransmitter from the synaptic vesicle is released into the synaptic cleft and diffuses to receptors on the postsynaptic cell. We observed that *unc-13(n2813)/+* does not complement a mutation in the acetycholine receptor subunit encoded by the *unc-29* gene [26% (19–34%) for *unc-13(n2813)/+; unc-29(e1072)/+;* 1% (0–5%) for *unc-29(e1072)/+*, and 4% (2–7%) for *unc-13(n2813)/+*; Figure 7]. Therefore, perturbations in *unc-13* enhance reductions in the postsynaptic receptors that bind acetylcholine but not reductions in the machinery that loads the vesicle with acetylcholine. These

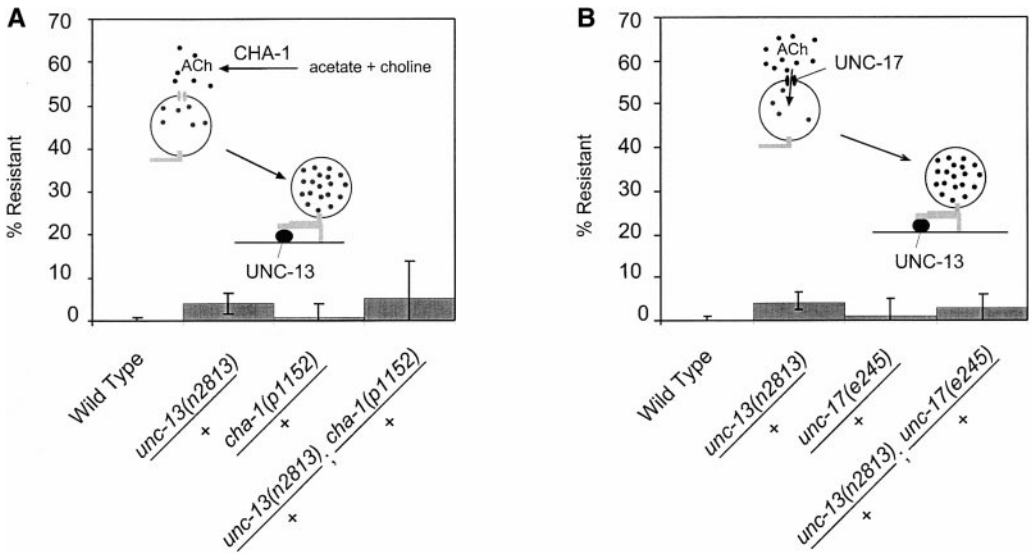


FIGURE 6.—Nonallelic noncomplementation does not occur between distant members of the synaptic vesicle pathway. (A) *unc-13* complements a mutation of the biosynthetic enzyme for acetylcholine. *unc-13(n2813)/+*, $n = 6(39)$; *cha-1(pr1152)/+*, $n = 4(29)$; *unc-13(n2813)/+; cha-1(pr1152)/+*, $n = 5(25)$. (B) *unc*-13 complements a mutation of the acetylcholine vesicular transporter. *unc-13(n2813)/+*, $n = 6(39)$; *unc-17(e245)/+*, $n = 4(22)$; and *unc-13(n2813)/+; unc-17(e245)/+*, $n = 8(31)$. For both graphs, wild-type, $n = 10(32)$; and resistance was measured after 12 hr of exposure to 0.7 mM Aldicarb.

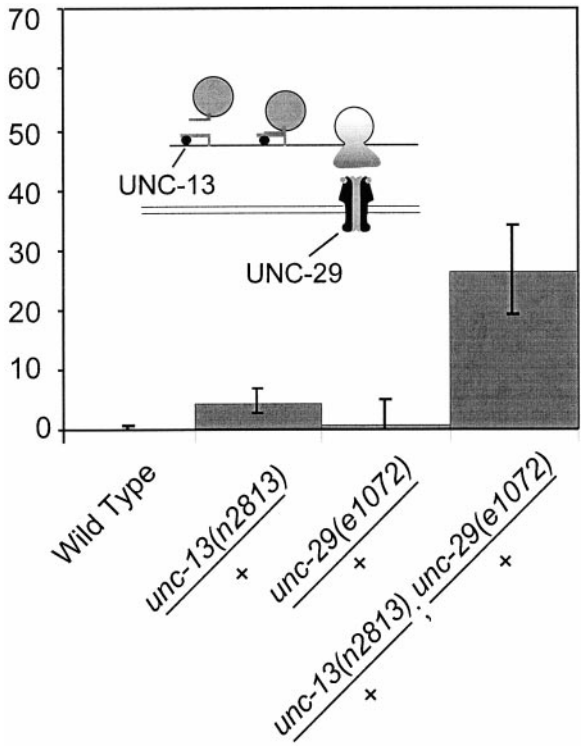


FIGURE 7.—Nonallelic noncomplementation occurs between genes that function across the synapse. *unc-13* does not complement a mutation in *unc-29*, which encodes an acetylcholine receptor subunit. Wild type, $n = 10(32)$; *unc-13 (n2813)/+*, $n = 6(39)$; *unc-29(e1072)/+*, $n = 2(48)$; and *unc-13(n2813)/+; unc-29(e1072)/+*, $n = 6(22)$. Resistance was measured after 12 hr of exposure to 0.7 mM Aldicarb.

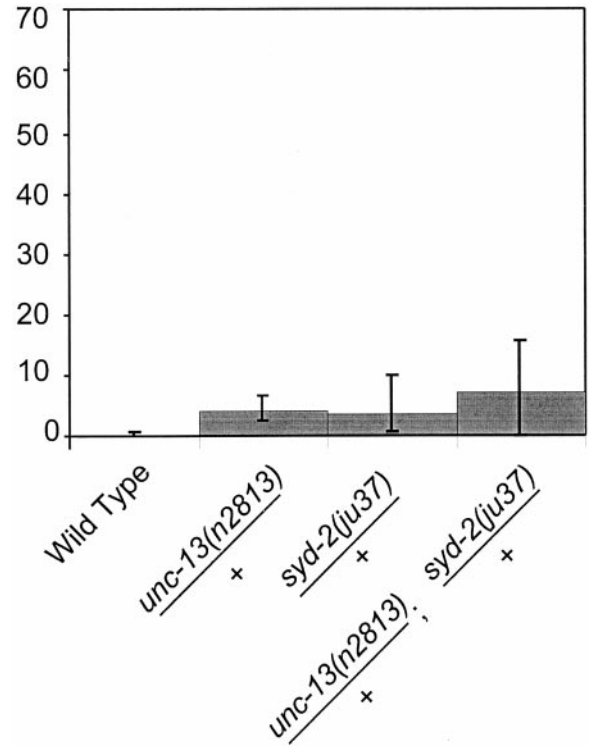


FIGURE 8.—Nonallelic noncomplementation does not occur between *unc-13* and genes required for development. *unc-13(n2813)* complements a mutation in *syd-2*, which is required for active zone formation. Wild type, $n = 10(32)$; *unc-13(n2813)/+*, $n = 6(39)$; *syd-2(ju37)/+*, $n = 3(26)$; and *unc-13(n2813)/+; syd-2(ju37)/+*, $n = 6(23)$. Resistance was measured after 12 hr of exposure to 0.7 mM Aldicarb.

data suggest that vesicle loading is not a rate-limiting step in synaptic transmission.

Finally, a mutation that affects synaptic development does not display nonallelic noncomplementation with *unc-13(n2813)*. *syd-2* encodes a liprin protein, which is a family of proteins that interact with receptor tyrosine phosphatases. *syd-2* is required for the development of synaptic termini (ZHEN and JIN 1999). Specifically, homozygous *syd-2* mutants exhibit a mislocalization of presynaptic proteins and have deformed active zones. *syd-2* mutant animals are moderately resistant to Aldicarb. However, *unc-13(n2813)* fully complements *syd-2(ju37)* [7% (0–16%) for *unc-13(n2813)/+; syd-2(ju37)/+*, 4% (1–10%) for *syd-2(ju37)/+*, and 4% (2–7%) for *unc-13(n2813)/+*; Figure 8]. These results indicate that the compromises in synaptic machinery caused by *unc-13(n2813)* do not synergistically interact with mutations in the developmental gene *syd-2*.

## DISCUSSION

Nonallelic noncomplementation occurs between synaptic function genes in *C. elegans*. Our analysis of this genetic interaction demonstrated that hypomorphic mutations, such as *unc-13(n2813)* and *unc-64(e246)*, which are recessive in behavioral assays, can act as weak poisons as heterozygotes in quantitative drug sensitivity assays. These poisons sensitize the process of neurotransmission to perturbations at other synaptic loci, resulting in nonallelic noncomplementation. In addition, it is the presence of these poisons rather than a simple decrease in the dosage of the gene product that is essential for nonallelic noncomplementation interactions at the synapse. Furthermore, in the cases where we observed nonallelic noncomplementation we have demonstrated that the increase in drug resistance of the double heterozygote is a synergistic effect and not simply an additive effect of the noncomplementing mutations.

The dosage model and the poison model have been proposed to explain how nonallelic mutations could exhibit noncomplementation. In the dosage model a decrease in expression at two loci cripples the process. In the poison model an altered gene product poisons the protein complex with which it is associated; thus there are not enough functional complexes. In the poison model the number of functional protein complexes is the limiting factor, not the amount of a single protein. Examples of both dosage-based (RANCOURT *et al.* 1995; JACKSON and BERG 1999; KIDD *et al.* 1999) and poison-based (STEARNS and BOTSTEIN 1988; HAYS *et al.* 1989;

Welch *et al.* 1993) nonallelic noncomplementation have been reported. In the instances of dosage-based nonallelic noncomplementation the gene products are required to coordinate developmental programs. For example, neuronal outgrowth across the CNS midline in Drosophila is compromised by a decrease in dosage of both *slit* and *robo*, the ligand and receptor, respectively, of this guidance pathway (Brose *et al.* 1999; Kidd *et al.* 1999). Thus a limiting factor in neuronal outgrowth is the concentration of its signals and receptors. This sensitivity to protein concentrations is a common feature of developmental pathways that must respond to protein gradients.

By contrast, we have demonstrated that synaptic function is not sensitive to gene dosage; however, synaptic function is sensitive to altered gene products. These observations suggest that neurotransmission is not as sensitive a process as developmental pathways to changes in protein concentrations. However, neurotransmission depends on the formation of a limited number of protein complexes. In processes requiring the correct assembly of protein complexes, a single faulty subunit can render a large number of gene products inactive by participating in and poisoning protein complexes.

How could poison alleles such as *unc-13(n2813)* or *unc-64(e246)* affect synaptic transmission? Synaptic transmission requires the action of a docking complex and then a fusion complex. We imagine that if a component of a complex is a poison protein, then the complex as a whole would not function or would function inefficiently. Therefore, poisons of the docking or fusion complexes could hinder vesicles from associating with the plasma membrane.

Not surprisingly, nonallelic noncomplementation was not observed between genes involved in processes removed from the exocytotic machinery. For example, *unc-13(n2813)* fully complemented mutations in genes responsible for acetylcholine synthesis or loading of neurotransmitter into vesicles. In addition *unc-13(n2813)* fully complemented a mutation in *syd-2*, which is required for development of neuromuscular junctions. Thus, nonallelic noncomplementation with *unc-13* is observed with other proteins in the UNC-13 pathway but no interactions were observed with genes involved in distant pathways such as neurotransmitter synthesis or synaptic development. Nonetheless, it is surprising that *unc-13(n2813*) can sensitize synaptic transmission to UNC-104 and DPY-23, proteins that are not involved in the formation of the SNARE complex. Heterozygotes of these genes must affect the complex indirectly by stressing the vesicular transport machinery. Thus, nonallelic noncomplementation with *unc-13* extends to processes involved in synaptic vesicle trafficking. Taken together, our results suggest that nonallelic noncomplementation would be a useful screening tool for uncovering new genes required for both SNARE complex formation as well as for genes acting at more distant steps in synaptic transmission.

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## APPENDIX: MAXIMUM-LIKELIHOOD MODELS OF GENETIC EFFECTS

The analysis of data in this article required statistical calculations to estimate the effects of particular mutations to test genetic hypotheses. We adopted a maximum-likelihood approach, which allows both parameter estimation and hypothesis testing using the same technique. Parameter estimations can determine the contributions to resistance by both the experimental genotypes and the balancer genotypes, which will appear in our experiments but are not informative. There are two hypotheses for the resistance of the double heterozygote: It is either due to the independent resistance from each locus—that is it is additive—or they interact synergistically. Note that nonallelic noncomplementation could also be caused by the independent additivity of resistance of the genotypes; that is, the double heterozygote will have greater resistance than either of the single heterozygotes. However, this is not true for the interactions observed.

**Estimating gene effects:** The *unc-64* null allele is homozygous lethal; thus it can be maintained only as a heterozygous strain balanced by another mutation [genotype *unc-64(js115)+/+ bli-5(e518)*]. Therefore, the effect of the *unc-64(js115)* null allele could not be measured directly; populations of *unc-64(js115)/+* heterozygotes are always accompanied by *bli-5(e518)/+* worms. This problem is compounded in the generation of double heterozygotes; populations of double heterozygotes containing the lethal *unc-64* allele are accompanied by double heterozygotes containing the balancing allele. Since the lethal and balancing mutations segregate in a Mendelian fashion, the total probability that a worm carries the lethal mutation is easily calculated. Since the worms are isogenic, each worm is treated as an independent trial for a genotype and thus we adopted

a binomial model of drug resistance. This model has no error terms, even though experimental error is introduced by variations in drug batches, humidity, agar, etc. These factors are unlikely to affect our statistical interpretations because wild-type worms were treated and measured in parallel with each of the drug trials. Data were included in the analysis only on days when wild-type worms, used as our control, behaved consistently with previously measured data.

Maximum-likelihood statistics involve the probability of observing the data given a model and model parameters. With the model described above, the probability of observing a given data set is

$$\Pr[\text{data}|p_1, p_2] = \prod_j \left( \binom{n_{1,j}}{i_{1,j}} p_1^{i_{1,j}} (1 - p_1)^{n_{1,j} - i_{1,j}} \right) \times \prod_j \left( \binom{n_{2,j}}{i_{2,j}} \left( \frac{p_1 + p_2}{2} \right)^{i_{2,j}} \left( 1 - \frac{p_1 + p_2}{2} \right)^{n_{2,j} - i_{2,j}} \right), \quad \text{(A1)}$$

where $p_1$ is the probability that an individual with the balancing allele is resistant; $p_2$ is the probability that an individual with the lethal allele is resistant; $n_{1,j}$ and $i_{1,j}$ are the starting number, that is, the initial number of worms on each plate, and ending number, that is, the number of resistant worms on each plate, from the $j$th trial for the balancing allele; and $n_{2,j}$ and $i_{2,j}$ are the starting and ending numbers of worms for the $j$th trial for the lethal allele. The likelihood function is the negative of the natural logarithm of the probability. The maximum-likelihood estimator (MLE) is the parameter value that maximizes the likelihood function. This can be found by solving for the derivative of the likelihood function, with respect to the parameters, equal to 0. For the two-parameter model described by Equation A1, that is the simultaneous solution of

$$\frac{\partial \text{ Log}(\Pr[\text{data}|p_1, p_2])}{\partial p_1} = 0$$

and

$$\frac{\partial \text{ Log}(\Pr[\text{data}|p_1, p_2])}{\partial p_2} = 0,$$

which yields

$$p_1^* = \frac{i_1}{n_1} \quad \text{(A2)}$$

$$p_2^* = \frac{2i_2}{n_2} - \frac{i_1}{n_1}, \quad \text{(A3)}$$

where $i_k = \sum_j i_{k,j}$, $n_k = \sum_j n_{k,j}$, and $p_i^*$ is the maximum-likelihood estimator for genotype $i$.

While the solutions in Equations A2 and A3 represent the only critical point for the likelihood function, they sometimes yield solutions outside of the range of possible susceptibilities [0, 1]. In that case there is no critical point and the MLE is on the boundary. If Equation A3 yields a $p_2^* < 0$, then the solution is

$$p_1^* = \frac{n_2 + i_1 + 2(n_1 + i_2) - \sqrt{(n_2 + i_1 + 2(n_1 + i_2))^2 - 8(i_1 + i_2)(n_1 + n_2)}}{2(n_1 + n_2)} \quad \text{(A4)}$$

$$p_2^* = 0. \quad \text{(A5)}$$

Likewise, if Equation A3 yields a $p_2^* > 1$, then the solution is

$$p_1^* = \frac{i_1 + 2i_2 - n_1 - n_2 + \sqrt{(n_1 + n_2 - i_1 - 2i_2)^2 + 4i_1(n_1 + n_2)}}{2(n_1 + n_2)} \quad \text{(A6)}$$

$$p_2^* = 1. \quad \text{(A7)}$$

The confidence intervals were calculated by finding the range of parameters for which the likelihood function was within 2 of the maximum value. The range of parameters was determined by numerically finding the value of $p_1$ that produced a likelihood score 2 units less than the maximum, while the likelihood function was maximized in $p_2$. The bounds for $p_2$ were calculated in a symmetric way.

**Testing genetic hypotheses:** Since single heterozygotes can exhibit a low level of drug resistance on their own, it was important to determine if the resistance of the double heterozygote could be explained simply by the two loci acting independently or if the resistance of the double heterozygote is caused by a synergistic interaction of the two loci. To this end we compared two hypotheses for the resistance of the double heterozygote. We call the first hypothesis the additive hypothesis, that is, that the resistance of the double heterozygote is given by $p_3 = 1 - (1 - p_1)*(1 - p_2)$. The other hypothesis, which we call the synergistic hypothesis, is that all three genotypes have independent resistances. The additive hypothesis could be rejected in favor of the synergistic hypothesis for two potential reasons: The resistance of the double heterozygote was either lower or higher than expected under additivity. If it is higher than expected under additivity then we can say that there is a synergistic effect. This is similar to a two-tailed test. There were no cases of synergism where the resistance was less than expected under the additive hypothesis.

The likelihood of a given model is analogous to Equation A1 for each genotype, and the total likelihood of a model is the sum of the likelihoods for each genotype (Edwards 1992). We used likelihood-ratio tests [analogous to the $G$-test (Edwards 1992)] to determine which hypothesis best represents the data. Since the different models have different numbers of parameters, we apply the Akaike information criterion (AIC; Sakamoto *et al.* 1986) and deduct one log-likelihood point per free parameter. The results from the hypotheses testing for the genotypes compared in this article are displayed in Table 1 (underlined values represent the accepted model).