Phenotypic Interaction between Temperature-Sensitive Paralytic Mutants *comatose* and *paralytic* Suggests a Role for *N*-Ethylmaleimide-Sensitive Fusion Factor in Synaptic Vesicle Cycling in *Drosophila*

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The temperature-induced paralysis of *comatose* (*comt*) mutants of *Drosophila* is suggestive of a function for *N*-ethylmaleimide-sensitive fusion factor (NSF) in the CNS. Mutations in the *para* gene encoding the α subunit of the voltage-gated sodium channel also result in a similar phenotype. We show that paralysis in *comt* flies is activity-dependent, and in the doubly mutant *comt para* flies *comt*-like paralysis does not set in until the effects of *para* are reversed by shifting to permissive temperatures. During recording from the thoracic flight muscles, we observed that *comt* flies showed a burst of spontaneous activity at restrictive temperature. This has been reported earlier as a unique characteristic of *comt* paralysis. The *comt para* double mutant showed this burst of activity not at restrictive but only on shifting back to permissive temperature. The unusual behavior and electrophysiology of the doubly mutant flies reported here indicates a role for NSF in synaptic vesicle cycling.

Key words: synaptic vesicle cycling; NSF; paralytic mutants; comatose*;* para*; flight muscle recording;* Drosophila

The fruit fly *Drosophila melanogaster*, in addition to being a favorable genetic model, has also been developed to take advantage of a variety of insightful techniques to address pertinent questions. The mutational approach, which seeks to cause lesions in genes, thereby leading to discernible and heritable phenotypes, has been the driving force behind an analysis of the nervous system in the fly. Several mutations are now known in a handful of genes that lead to a phenotype of reversible temperature-induced paralysis. *Paralytic* (*para*), a sodium channel mutant (Suzuki et al., 1971; Loughney et al., 1989), *shibire*, affected in endocytosis (Kosaka and Ikeda, 1983; van der Bliek and Meyerowitz, 1991), and *comatose* (*comt*), a *Drosophila N*-ethylmaleimide-sensitive fusion factor (NSF) mutant (Siddiqi and Benzer, 1976; Pallanck et al., 1995), are well known examples. Analysis of these mutants has been aided by the ability to do electrophysiological recordings from both the neuromuscular junction and the retina of the fly (Kelly and Suzuki, 1974; Siddiqi and Benzer, 1976; Kawasaki et al., 1998; Littleton et al., 1998).

The $para^{ts2}$ mutation causes the fly to reversibly paralyze at temperatures >33°C (Suzuki et al., 1971). Both the paralysis and recovery of *para* mutant flies are extremely fast (in the order of seconds). No action potentials can be elicited in the dorsal longitudinal muscles (DLMs) of the thorax by stimulation of the cervical ganglion in *para* mutant flies at 38°C (Siddiqi and Benzer, 1976). Molecular cloning showed that the *para* gene codes for the fly homolog of the α subunit of the voltage-gated sodium channel (Loughney et al., 1989). Because the *para*-encoded sodium channel accounts for the majority of voltage-gated Na^+ current in *Drosophila* (O'Dowd et al., 1989), we have been able to use it as a genetic tool for manipulating nerve activity.

The soluble NSF attachment protein (SNAP) receptor (SNARE) hypothesis postulated that docking and fusion of synaptic vesicles at the presynaptic membrane is mediated by integral membrane proteins on the surface of synaptic vesicles (v-SNAREs) and the target membrane (t-SNAREs). Complexes between cognate v- and t-SNAREs would sequester cytosolic proteins α-SNAP and NSF. The ATPase activity of NSF was believed to disassemble the SNARE complex and thereby drive vesicle fusion (Sollner et al., 1993). Subsequent experiments have shown that NSF may not be the bona fide fusogen. (Banerjee et al., 1996; Mayer et al., 1996). A currently held view is that of NSF functioning as a chaperone to prime vesicles to make them fusion-competent (Hanson et al., 1997). The *comt* gene codes for the fly homolog of NSF (Pallanck et al., 1995). The conditional neural phenotype of the *comt* mutation makes it a valuable system for studying the role of NSF *in vivo*. One of the earliest reports of the *comt* mutation describes the temperature-sensitive paralytic behavior and electrophysiological defects in nerve transmission (Siddiqi and Benzer, 1976). Flies mutated at the *comt* locus show a characteristic paralysis at tem-

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peratures >33°C and prolonged recovery at room temperature. The time taken for recovery is dependent on the period of exposure to restrictive temperature (Siddiqi and Benzer, 1976).

From the postulated role of NSF, it is likely that in the *comt* mutant fly, paralysis is a result of a block in synaptic vesicle cycling. The extended recovery time and its dependence on the period of exposure to restrictive temperatures suggest that multiple rounds of vesicle fusion and retrieval are mandatory for the *comt* phenotype to manifest itself (Littleton et al., 1998). In *para* mutant flies, at restrictive temperatures, action potentials are abolished, probably resulting in stoppage of synaptic vesicle exocytosis and recycling. If paralysis of *comt* flies depends on activity, then one would expect a phenotypic suppression of *comt* by *para*. We report here a set of experiments that document such suppression in behavior and electrophysiology. We find that the onset of paralysis in *comt* mutant flies is delayed for the period that action potentials (and therefore synaptic vesicle cycling) are blocked by *para* inactivation. This argues for the *comt* effect being activity-dependent. Furthermore the behavior of the *comt para* double mutant clearly argues for a role of NSF in synaptic vesicle cycling.

MATERIALS AND METHODS

Drosophila *strains and genetics*

Flies were cultured in standard sugar- and agar-containing yeast medium in glass vials or bottles. All stocks were reared at 22°C. The comt ^{tp7} and *para* ^{ts2} mutants are a part of the Tata Institute of Fundamental Research stock collection.

Male *para* ^{ts2} flies were crossed to *comt* ^{tp7} virgin females. The heterozygous female progeny were then allowed to mate with the *comt* ^{tp7} males. Male flies in the next generation were assayed for presence of recombinant chromosomes carrying both the *para* ^{ts2} and *comt* ^{tp7} mutant loci by using the temperature-induced rapid paralysis of the *para* ^{ts2} allele and the extended recovery time for *comt* ^{tp7}. Individual recombinant males were then used to set up lines with attached X females. Only male flies were used in all experiments. In each of the *comt para* double mutant lines the recombinant chromosome was complementation-tested in females versus both single mutants, *comt* and *para*. The male *comt para* flies were crossed to either homozygous *para* ^{ts2} or *comt* ^{tp7} virgin females. The female progeny from these crosses were assayed for both paralysis and recovery. Both *comt* and *para* are recessive mutations. The phenotype displayed in each case was that of the mutation, which was homozygous in these females.

Behavioral assays

Paralysis profiles. The apparatus used for measurement of paralysis is described by Ramaswami et al. (1993). A smooth glass chamber connected to a tube was enclosed in a sealed water jacket through which water circulated by a water bath (FE2; Haake, Karlsruhe, Germany) whose temperature was controlled with a precision of 0.5°C. Paralysis was empirically defined as the condition in which the animal lies on its back with little effective movement of the legs and wings. We define the restrictive temperature for a particular mutation as the temperature at which 100% of the mutant flies paralyze in 3 min. Under these conditions the restrictive temperature of *para* ^{ts2} was 33°C, whereas that of *comt* ^{tp7} was 35°C. Thus at 38°C both *para* ^{ts2} and *comt* ^{tp7} mutant flies would paralyze rapidly within 30 sec, ensuring synchronous paralysis. This assumes importance in light of the fact that recovery periods in *comt* mutants are proportional to the exposure time above restrictive temperatures. Hence, a fast, synchronized paralysis at 38°C translates into a synchronous recovery at 20°C. A paralysis profile for a mutant was plotted by counting the number of flies paralyzed at intervals of 15 sec at 38°C for a total exposure of 1 min. All flies tested were 1–2 d old. At least three batches of 10 flies each were tested for each experiment.

Recovery profiles. A fly was scored to have recovered when it could stand upright and walk around. Usually recovered flies were able to right themselves instantly after they had fallen as a result of mechanical agitation. Batches of 10 flies not >1–2 d old were subjected to a fixed protocol for paralysis (e.g., exposure to 38°C/1 min), after which they were tapped out into a vial and allowed to recover at room temperature (20°C). The number of flies standing was enumerated at specified times during recovery at 20°C. Each data point is an average of at least three trials.

Electrophysiology

All recordings were made from the DLMs in the fly thorax. Flies were anesthetized lightly by cooling in ice for a few minutes. Anesthetized flies were mounted upright in modeling clay such that the thorax was exposed for electrode penetration. Flies were allowed to recover for 10 min before recording. Both the ground and recording electrodes were heat-pulled glass microcapillaries (tip resistance, 3–5 MΩ) filled with 3 M KCl. The ground electrode was inserted into the head, and the recording electrode was inserted through the thoracic cuticle into the DLMs. The typical firing pattern of the thoracic muscles was used to confirm the position of the recording electrodes (Ikeda and Kaplan, 1970; Ikeda et al., 1976). A heater plate connected to a DC power supply was used to heat the preparation, and a digital temperature probe was used to monitor the temperature. The time taken to ramp from 20 to 36°C was ~5 min. After the desired temperature was attained, the power supply was switched off, and the preparation was allowed to cool while recordings were monitored continually. The time taken to cool from 36 to 32°C was ~2 min. Signals were amplified using an intracellular preamplifier (WPI, Waltham, MA), and data were acquired directly from the oscilloscope (Hitachi) display.

RESULTS

A novel behavior observed during recovery of *comt* ^{tp7}-*para* ^{ts2} double mutants

comt ^{tp7}, *para* ^{ts2}, and *comt* ^{tp7} *para* ^{ts2} flies were subjected to a heat treatment protocol of 38°C for 1 min to induce paralysis. The flies were then shifted to 20°C to observe recovery as described in the Materials and Methods. In this protocol, *comt* ^{tp7} flies paralyze within 1 min at 38°C after a burst of activity and recover at 20°C in 30 min (Fig. 1*a*). During a 1 min observation period after shifting to 20°C after paralysis, they essentially remain immobile (Fig. 1*b*). *para* ^{ts2} flies paralyze within 15 sec at 38°C and recover as rapidly after shifting to 20°C (Fig. 1*a*). Thus, it can be seen that the two mutations exhibit distinctly different profiles of paralysis and recovery.

The behavior of *comt* ^{tp7} *para* ^{ts2} flies when subjected to the assay was dramatic. These flies paralyze at 38°C in 15 sec, which is a time course similar to that of *para* ^{ts2} flies (Fig. 1*a*). It may be noted that the burst of activity preceding paralysis as seen in the *comt* flies does not occur in the double mutants. This is probably because a *para* inactivation instantaneously abolishes action potentials in the motor neurons. After shifting to 20°C, the flies appear to recover immediately, and after a period of 10 sec into recovery, almost all the flies are upright. During this period increased activity is observed, but by 30 sec, more than half of the upright flies paralyze again, and paralysis is complete by 1 min (Fig. 1*a,b*). This intervening spurt of activity is reminiscent of the one that precedes *comt* paralysis at restrictive temperature. Recovery after this second round of paralysis proceeds in a manner similar to that of *comt* flies (Fig. 1*a*), albeit somewhat faster.

para suppresses the spontaneous DLM firing in *comt*

It has been reported previously that *comt* flies, when heated to restrictive temperatures, show a spontaneous burst of firing from the motor neurons innervating the thoracic flight muscles (Kawasaki and Ordway, 1999). To investigate whether we could monitor the interaction between the *para* and *comt* mutations in terms of physiology, recordings from the thoracic muscles were made from *comt* ^{tp7}, *para* ^{ts2}, and *comt* ^{tp7} *para* ^{ts2} flies as described in Materials and Methods. Raising a *para* ^{ts2} mutant fly to restrictive temperature (33°C) is likely to result in a failure of action potentials (Siddiqi and Benzer, 1976). It should be noted that the restrictive temperature for *para* mutant flies is sharply defined.

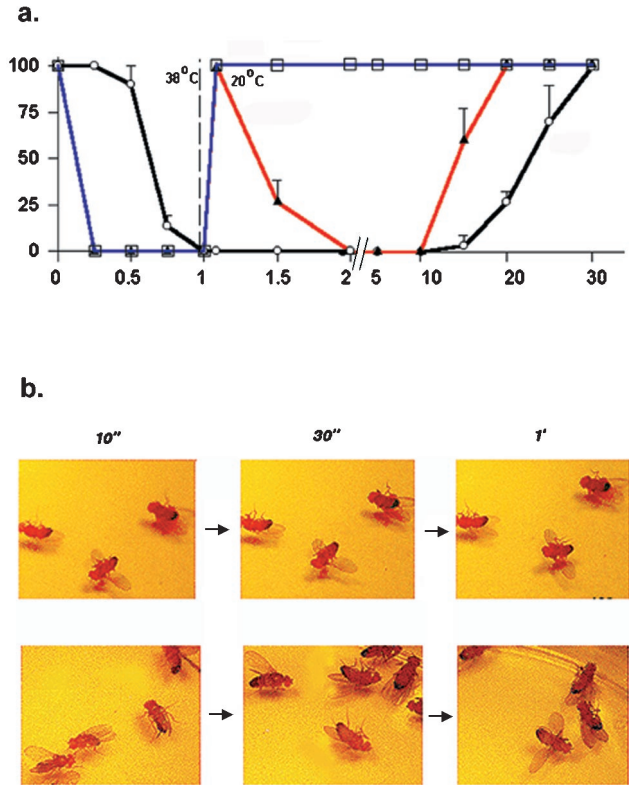

Figure 1. Novel behavior of *comt* ^{tp7} *para* ^{ts2} double mutant flies. *a,* Paralysis (at 38°C) and recovery (at 20°C) profiles of *comt* ^{tp7}, *para* ^{ts2}, and *comt* ^{tp7} *para* ^{ts2} mutants. *comt* ^{tp7}(○) flies paralyze completely within 1 min, and 100% recovery is seen by 30 min. *para* ^{ts2} (□) flies paralyze within 10–15 sec and recover as rapidly. *comt* ^{tp7} *para* ^{ts2} (▲) double mutant flies paralyze like *para* ^{ts2} and recover transiently at 20°C before reparalyzing at this temperature. Subsequent recovery is prolonged and occurs by 20 min. A break in scale is used on the *x*-axis to accommodate the different kinetics of paralysis and recovery in one graph. *b,* Photos of flies taken at indicated time points of recovery after paralysis. *Top panels*, *comt* ^{tp7} flies remain immobile during the observation period. *Bottom panels*, *comt* ^{tp7} *para* ^{ts2} flies recover briefly before reparalyzing.

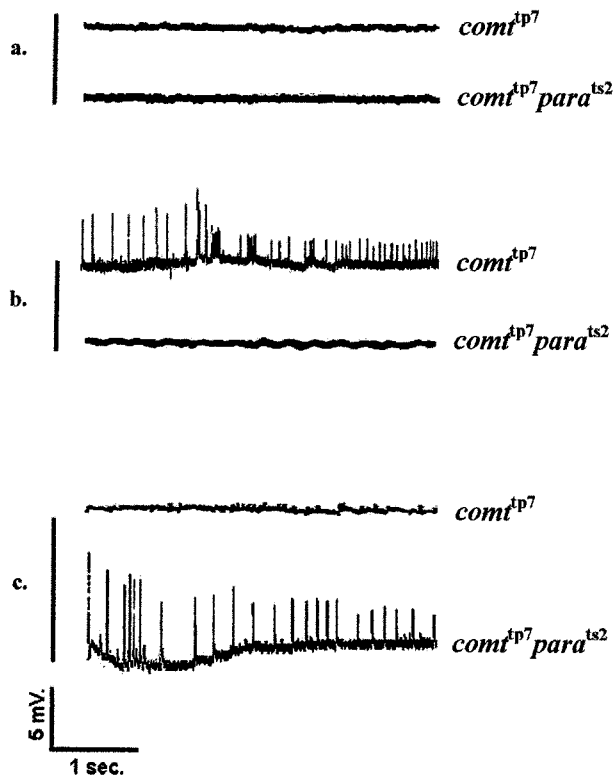

Figure 2. Recordings of spontaneous firing from the flight muscles, the DLMs, in *comt* ^{tp7} and *comt* ^{tp7} *para* ^{ts2} flies. *a,* At 20°C there is almost no spontaneous activity detected in the DLMs of either *comt* ^{tp7} or *comt* ^{tp7} *para* ^{ts2} flies. *b,* A characteristic spontaneous burst of activity is seen in *comt* ^{tp7} at 36°C, whereas this is completely suppressed in the double mutant. This activity in *comt* ^{tp7} continues for up to 2 min followed by quiescence. *c,* After cooling from 36to 32°C, *comt* ^{tp7} flies do not show any change in response, whereas the double mutant, now relieved of the *para* block, shows the typical *comt* burst of firing.

Hence in a *comt para* double mutant fly, exposure to restrictive temperatures would lead to a similar failure of action potentials. *comt* itself has a restrictive temperature higher than that of *para*. Heating the *comt para* flies to higher temperatures, which are restrictive for *comt* as well, would probably cause a suppression of the characteristic *comt* firing at this temperature. This allows the *para* mutation to be used as a temperature-dependent switch for controlling neural activity. Merely lowering the temperature to 32°C would restore activity. It would be interesting to see whether selective alleviation of the *para* block after inactivation at restrictive temperatures would now result in a delayed *comt*-like firing, offering a possible correlate for the behavioral interaction.

Recordings from the DLMs of *comt* ^{tp7} flies at 20°C showed hardly any activity but revealed the typical pattern of spontaneous firing at 36°C (Fig. 2*a,b*). This firing continued for up to 2 min at 36°C, followed by a progressive reduction in spike amplitude and eventual quiescence. Thereafter, on cooling the preparation to 32°C, no further activity could be detected. In *para* ^{ts2} flies the spontaneous activity was infrequent and usually undetectable at 20 or 36°C as well as on cooling to 32°C (data not shown). Recordings from the DLMs in *comt* ^{tp7} *para* ^{ts2} flies at 20°C did not reveal much activity (Fig. 2*a*). As expected, a shift to 36°C did not result in any discernible increase in activity (Fig. 2*b*). This is likely to be attributable to the abolition of action potentials by a fast onset of the *para* block. On cooling the preparation to 32°C, a robust *comt*-like burst of firing was observed from the DLMs (Fig. 2*c*).

It is likely that the temperature-induced burst of DLM firing marks the onset of paralysis in *comt* mutants. The *para*-mediated suppression followed by the delayed occurrence of this firing burst correlates well with the observations made in our behavioral experiments.

DISCUSSION

A role of NSF in synaptic vesicle cycling

Temperature-sensitive paralytic mutations in *Drosophila* have proved to be uniquely useful in the study of the synapse. Mutations in *para* and *comt* loci have been studied extensively in terms of their molecular nature and the physiological defects that they give rise to. The *para* locus encodes the fly homolog of the voltage-gated sodium channel, whereas the *comt* locus codes for NSF (Siddiqi and Benzer, 1976; Loughney et al., 1989; Pallanck et al., 1995; Kawasaki et al., 1998). The role of NSF in synaptic vesicle cycling has been a matter of debate. The SNARE hypothesis originally postulated that NSF is recruited to the site of vesicle docking by the formation of the 7S SNARE complex. Thereafter, the ATPase activity of NSF was postulated to drive membrane fusion leading to exocytosis at the synapse (Sollner et

al., 1993). Recent experiments have, however, shown that NSF may not be needed at the last step of fusion. These experiments point toward a prefusion priming role of the molecule (Banerjee et al., 1996; Mayer et al., 1996). Such a role would imply the existence of a pool of primed, fusion-competent synaptic vesicles. We have used the *para* mutation to genetically block all neural activity in a mutant NSF (*comt*) background in *Drosophila* to verify the presence of such a pool. In the *comt para* mutant fly, at restrictive temperature, action potential-driven synaptic vesicle cycling is abolished, causing the fly to paralyze instantly. On shifting to permissive temperature, these flies display an intervening spurt of activity during which the flies stand and walk around before undergoing a second round of paralysis. Recordings from the DLMs in these double mutants show that *para* completely suppresses the characteristic spontaneous burst of firing seen in *comt* at 36°C. Here again, alleviation of the *para* block achieved by cooling the preparation to 32°C results in a robust *comt*-like response. This delayed activity seen in both behavior and electrophysiology, when the *para* block is removed, is unlikely if *comt*/NSF impedes vesicle fusion at the last step of exocytosis. NSF may have a prefusion role at the synapse, possibly at a sorting step after vesicle retrieval by endocytosis. It should be noted that a postfusion role for NSF in resolving accumulated SNARE complexes on the plasma membrane after exocytosis has recently been proposed (Tolar and Pallanck, 1998). This role can be extended to the resolution of SNARE complexes on vesicle membranes after endocytosis and before the next round of fusion. Our results are in agreement with either alternative.

Paralysis in comatose mutants is activity-dependent

Experiments done with *comt* mutant flies have also investigated the probable causes of temperature-sensitive paralysis in these mutants. These experiments suggest that neural activity leading to multiple rounds of synaptic vesicle cycling is necessary to induce paralysis (Kawasaki et al., 1998; Littleton et al., 1998). In the present set of experiments we have addressed this possibility by manipulating neural activity using the *para* mutations in *comt para* double mutant flies. The behavior of flies carrying mutations at the *comt* locus is indicative of an activity dependence to their paralytic behavior. These flies paralyze in ~3 min if heated to 35°C. At subthreshold temperatures of 30–33°C, paralysis is not observed in 3 min. However, they paralyze if subsequently agitated mechanically whether during heating or after shifting back to permissive temperatures. Even while paralyzed, a behavior unique to *comt* (as different from *para* flies) is the spontaneous and periodic spurts of activity (our unpublished observations). The prolonged exposure of *comt* flies results in a concomitant increase in recovery times. This indicates an accretion of incompetence in the vesicle cycle. It has been suggested that mutations in the known temperature-sensitive alleles of *comt* lead to a general reduction in activity of the protein at restrictive temperature. Increased neural activity, either heat-induced or caused by mechanical or electrical stimulation, will result in rapid and progressive formation of newer SNARE complexes. The compromised activity of NSF would be unable to resolve these complexes fast enough for sustained synaptic transmission (Littleton et al., 1998). During recovery from paralysis, a slow buildup of docked fusion-competent vesicles could transiently restore neural function, leading to bursts of activity. This would result in deficient vesicle cycling, leading to failure of sustained and reliable synaptic activity, until such time as the docked pools build up again.

The behavior of the *comt para* doubly mutant flies reported here offers direct *in vivo* proof that the paralysis at restrictive temperatures in *comt* is a consequence of an activity-dependent block in synaptic transmission. The occurrence of a *comt*-like paralysis at the permissive temperature after relieving the *para* block suggests that neural activity is essential to cause paralysis. The spontaneous firing and eventual quiescence of the DLM motor axons below 33°C (i.e., below the *para*-restrictive temperature) in the *comt para* double mutant flies also correlates with the observed behavior. The requirement for a relatively constant quantal content per action potential would demand a very small fraction of docked and primed vesicle compared with a very large reserve pool of vesicles. A blockage in the vesicle cycle would lead to a failure of the synaptic release and consequent paralysis. If *comt*/NSF is involved not in vesicle fusion but in vesicle cycling, the observed behavior can be rationalized in the following manner. Temperature-dependent inactivation of *comt* will normally lead to paralysis by a threshold-dependent failure of the cycle. In the doubly mutant flies, abolishing action potentials and hence vesicle fusion and cycling by using the *para* mutation delays the crossing of this threshold by holding back a subset of vesicles. A direct consequence of this is that fewer rounds of vesicle cycling would have occurred in the double mutant compared with *comt*, which manifests as a reduction in the total recovery time after paralysis as seen in our assay.

To conclude, our results with the *comt para* double mutant flies are consistent with a role of NSF in synaptic vesicle cycling and confirm that the temperature-sensitive paralysis seen in *comt* flies is activity-dependent.

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