Proc. Natl. Acad. Sci. USA
Vol. 73, No. 9, pp. 3253–3257, September 1976
Genetics

Neurophysiological defects in temperature-sensitive paralytic mutants of *Drosophila melanogaster*

(neurogenetics/neuromuscular junction/behavioral mutants)

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Contributed by Seymour Benzer, July 6, 1976

ABSTRACT **A new temperature-sensitive paralytic mutant of *Drosophila, comatose,* is compared behaviorally and physiologically with the previously known types, *para* and *shi.* All three have different properties with respect to kinetics of paralysis at high temperature and recovery from paralysis; *com* is hypersensitive to paralysis by cooling. Neurophysiological experiments indicate different mechanisms for paralysis in each of the mutants.**

The macromolecules composing neural structures are constructed according to genetic information and are thus subject to modification by mutation. In analyzing basic mechanisms in neurophysiology, mutations affecting specific neural elements are potentially incisive. Alteration of genes responsible for the synthesis of membrane components, transmitter molecules, or contractile structures could be employed to perturb or block axonal conduction, synaptic transmission, or muscle response. Of particular interest are temperature-sensitive mutants in which defects can be turned on or off at will.

A special class of *Drosophila* mutants is the reversible, temperature-sensitive paralytic, exemplified by the mutants *para* (for *paralyzed*) and *shi* (for *shibire,* meaning paralyzed in Japanese) isolated in Suzuki's laboratory (1, 2) and *com* (for *comatose*) isolated in our laboratory (3). In this paper, we characterize these three mutants behaviorally and neurophysiologically, and show that paralysis involves different physiological mechanisms in each case.

MATERIALS AND METHODS

Isolation of Mutants. The mutants $para^{ts-1}$ and shi^{ts-1} were obtained through the courtesy of Dr. David Suzuki and will be referred to as *para* and *shi.* New mutants were generated from the wild-type strain, Canton-Special (C-S), of *Drosophila melanogaster* by methods previously described (4). Descendants of mutagen-treated adults were exposed to 38° for several minutes to detect mutants that became paralyzed at the elevated temperature. By complementation tests, some of the new mutants were identified as alleles of *shi* and *para,* which are located on the X-chromosome at 52 and 54 recombination units, respectively (1, 2). Others belong to a new gene, *com,* which maps to approximately 40 units, between *fw* (at 38.3) and *wy* (at 40.7) on the X-chromosome (Fig. 1).

Kinetics of Paralysis. Flies were raised and maintained at 23°. For paralysis tests, adult flies 4–5 days old were placed in 18 × 150 mm thin-walled glass test tubes (10 flies per tube), a polyurethane foam stopper was inserted down to 3 cm from the bottom of the tube, and the lower 5 cm of the tube was immersed in a water bath at the desired temperature. The temperature rise reached to within 10% of its final value in 15 sec, as determined with a thermocouple in contact with the inside tube surface. The number of flies that remained standing (after the tube was gently tapped) was recorded as a function of time. Recovery was observed by transferring the tube to a water bath at 23°.

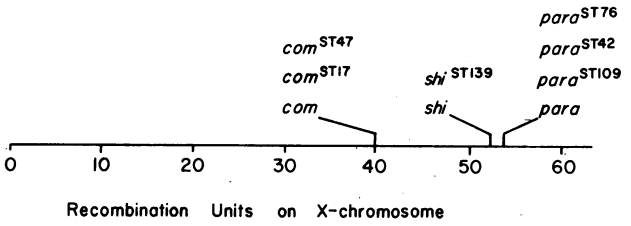

FIG. 1. Genetic map of temperature-sensitive paralytic mutations. Mutants *para* and *shi* were isolated in Suzuki's laboratory, along with a number of their alleles (not shown). The mutant *com* and its alleles were isolated at Caltech, along with the additional alleles of *shi* and *para* shown.

Neurophysiology. For observation of leg movements evoked by stimulation of the cervical connective, the fly was lightly anesthetized with ether and fixed on its back to a small cork, with soft dental wax, leaving the legs free to move. After recovery from the ether had been allowed, the stimulus was applied via a pair of metal electrodes placed in the neck. For intracellular recording from the flight muscles, the fly was mounted dorsal side up, with its legs stretched out and fixed with the wax. One side of the thorax was supported on a wax stilt, without obstructing the thoracic spiracles. The cork was placed inside a plastic cylinder (5 cm diameter, 5 cm high), wrapped inside with a tungsten heating wire. A plastic lid had openings for electrodes. Heating current was supplied by a proportional temperature device, controlled by a thermistor. The temperature was measured by a second thermistor close to the fly. To raise the heating chamber from room temperature to 38° required about 3 min. When it was desired to attain the paralyzing temperature more quickly, the chamber was prewarmed before the mounted fly was inserted.

For penetration of individual dorsal longitudinal flight muscle fibers, their cuticular terminations were located via morphological landmarks on the thorax (5). A sharpened metal needle was used to make a hole in the cuticle and a glass microelectrode, filled with 3 M KCl (resistance 20 MΩ) was inserted. In most preparations, one could record from each of the six dorsal longitudinal fibers on one side, in sequence, by advancing the electrode. The reference electrode was a relatively crude micropipette, filled with *Drosophila* Ringer's solution (6), inserted into the thorax. For direct intracellular stimulation of flight muscle, two separate micropipettes, filled with 3 M KCl, were inserted into the same fiber; one was used for current injection, the other for recording.

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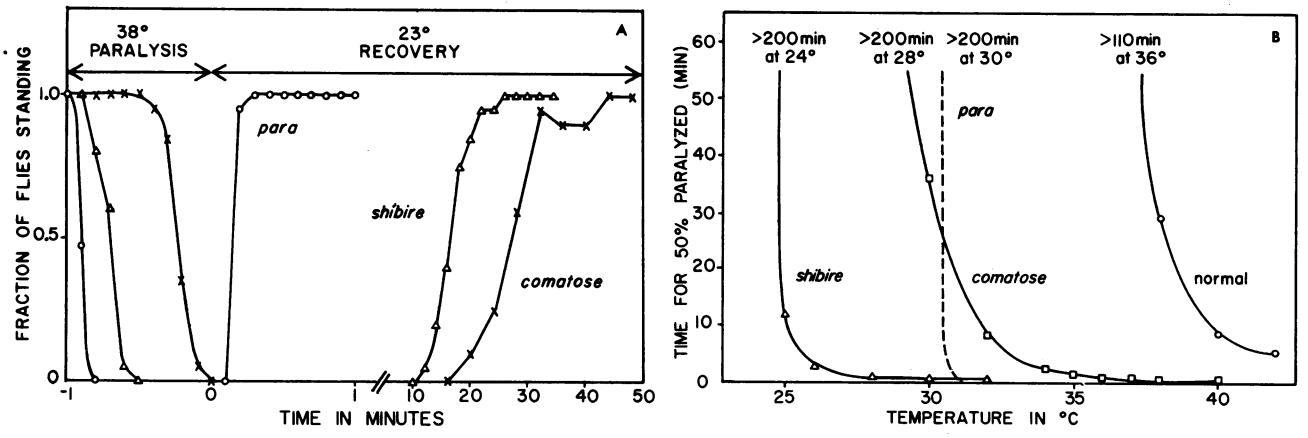

FIG. 2. Kinetics of paralysis. (A) Flies exposed to 38° at $t = -1$ min, then returned to 23° at $t = 0$. Twenty flies of each strain were used. (B) Time required for paralysis versus temperature. Twenty flies were used to obtain each point.

RESULTS

Paralysis by High Temperature. Fig. 2A illustrates the kinetics of paralysis for *para*, *shi*, and *com* mutants exposed to 38° for 1 min. While normal flies (C-S wild type) are not paralyzed by such exposure, *para* is paralyzed almost instantly; when returned to 23°, it also recovers quite rapidly (1). The mutant *com* is very different. It takes longer to pass out and, when returned to 23°, recovery is much slower. Under the conditions used, *shi* gives a result intermediate between the other two mutants (2).

For *com* and *shi*, there is not a sharp transition temperature; the higher the temperature, the more rapidly paralysis occurs. Fig. 2B shows the time required for half of the flies to become paralyzed. Thus, *shi* can become paralyzed even at 25° (only 2° above the 23° standard maintenance temperature used) if exposed long enough, and *com* may become paralyzed at 30°. Normal flies, if taken to high temperatures for long enough, will also collapse (Fig. 2B). Among these mutants, *para* is distinctive in the sharpness of its cutoff.

Recovery from Heat Paralysis. The rate of recovery depends upon the extent of previous exposure to high temperature. Fig. 3A shows data for *com*. Each mutant shows different properties in this respect, as shown by the curves of Fig. 3B. In the case of *com*, the time required for half the flies to recover is almost proportional to the duration of previous exposure at 38°. The mutant *shi* is different; it recovers rapidly after short exposure, but the recovery time escalates strongly as previous exposure increases. Recovery for *para* is rapid even after prolonged exposure. *para* also accommodates to a temperature slightly above that which induces paralysis; if the same temperature is maintained, the flies gradually recover their motility (1). This accommodation is not exhibited by *com* or by *shi*, which seem rather to become progressively more adversely affected. Age has an important effect: older *com* flies in particular are more readily paralyzed and take longer to recover.

Paralysis and Recovery in Larvae. In all these mutants, locomotor paralysis also occurs in larvae, although somewhat

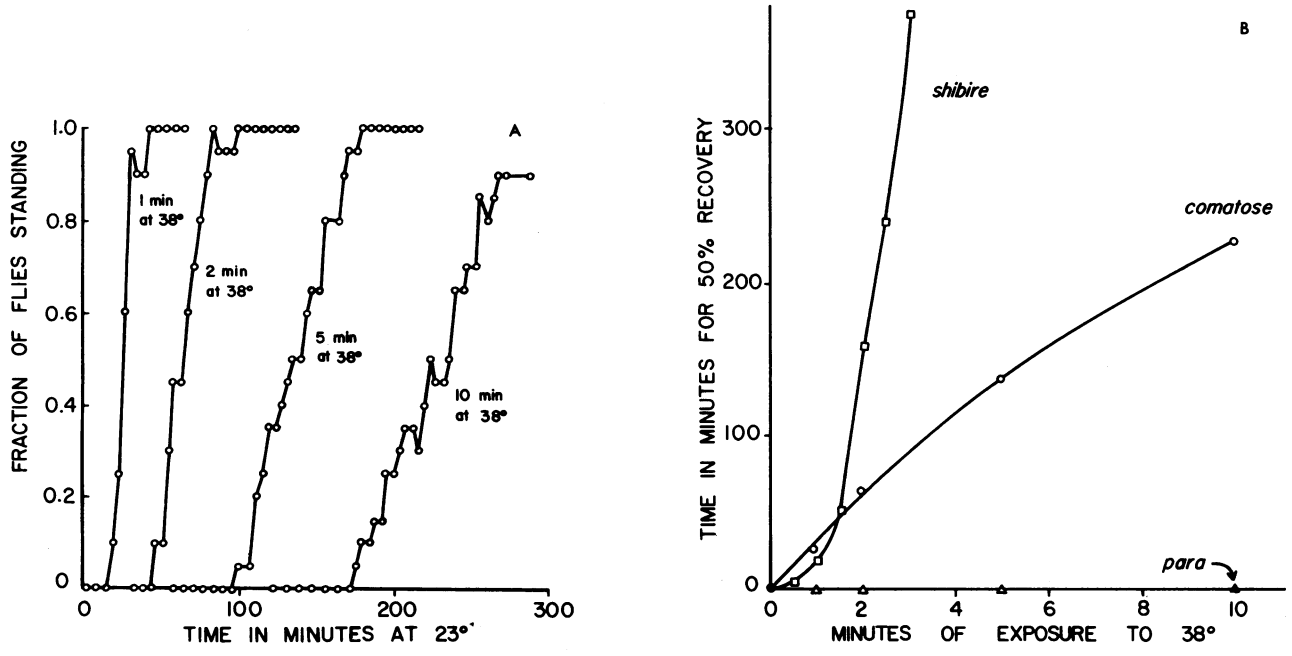

FIG. 3. Kinetics of recovery. (A) Recovery of *com* at 23° after exposure to 38° for various lengths of time. Twenty flies were used for each curve. (B) Dependence of time required for recovery at 23° on duration of previous exposure to 38° is different for the three mutant types. Twenty flies were used to obtain each point.

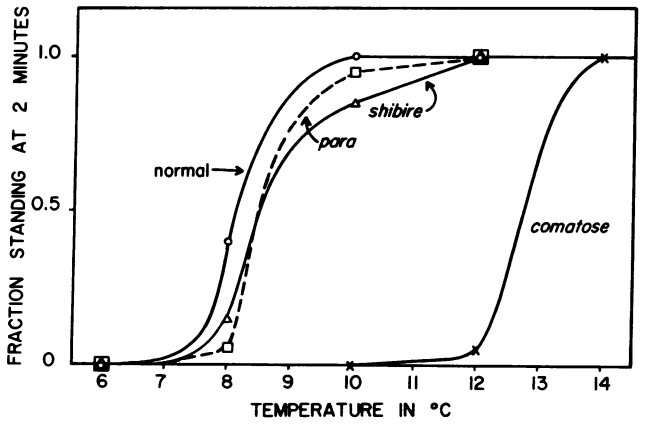

FIG. 4. Paralysis at low temperature. The mutant *com* is hypersensitive. Twenty flies were used for each point.

higher temperatures may be required than for adults. Thus, *para* larvae become immobilized at around 35° and recover rapidly when the temperature is lowered. *shi* larvae behave like *para* for short exposures, but recovery is slower after prolonged paralysis (2). The larvae of *com*, like the adults, are paralyzed in a minute or two at 38°, and remain so for a long time at room temperature before recovering.

Paralysis by Cooling. Chilling normal flies to temperatures below 10° causes rapid paralysis. As shown in Fig. 4, *para* and *shi* show similar responses, but *com* is exceptional in being markedly more sensitive to cold, responding at temperatures below 14°. A cold-sensitive mutant *Out-cold*^{ts} was reported by Søndergaard (7), who reported its location as 55.2 ± 0.2 on the X-chromosome, quite distant from *com*.

Accommodation to low temperature occurs in all these mutants and in normal flies, provided the temperature is not too low. This effect is illustrated in Fig. 5 for normal flies compared with *com*. The results for *para* and *shi* were the same as for normal flies.

Response to Cervical Stimulation. By placing two metal electrodes in the neck of a fly fixed on its back with wax, the cervical connective nerve bundle running between head and thorax can be stimulated. When a 0.2 ms stimulus of 3–4 V was applied to normal flies, a strong jerk of all the legs was evoked. As the temperature was raised, both spontaneous leg movements and the evoked response of these normal flies persisted up to 40° for several minutes.

The mutant *com*, after 1 or 2 min at 38°, ceased spontaneous leg movements and also stopped responding to cervical stimulation. On return to room temperature, this paralysis persisted; a several-fold increase in stimulus (to as high as 20 V) failed to evoke any perceptible leg movement. After the usual recovery period (Fig. 3), both spontaneous movement and the evoked response returned to normal.

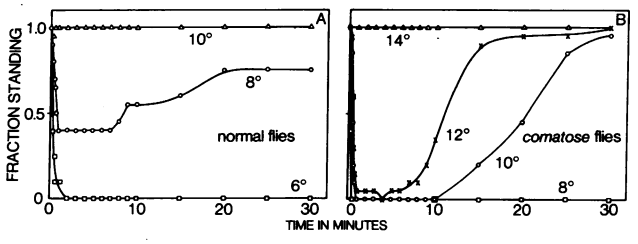

FIG. 5. Accommodation to low temperature, as shown by recovery from paralysis. (A) Normal flies; (B) *com* mutant. Twenty flies were used for each curve. The mutants *para* and *shi* behave similarly to normal flies.

The mutant *shi*^{ST139}, an allele of *shi* isolated by us, was used for the physiological studies. At 32°, spontaneous and evoked leg movements ceased within about 2 min; recovery at room temperature was rapid, occurring within a minute or two. Longer exposures required longer periods for recovery.

The physiological results with both *com* and *shi*^{ST139} parallel the paralysis behavior of these mutants, which is manifest primarily in leg movements. They indicate that one or more steps along the pathway from cervical nerve excitability to leg muscle contraction are blocked at high temperature.

In contrast, *para* (and the alleles isolated by us, *para*^{ST42} and *para* ^{ST109}), when raised to a temperature at which spontaneous leg movements ceased, continued to exhibit evoked leg jerks.

Some pathway from cervical connective to leg apparently remains intact and also the leg muscle itself is clearly responsive. Nevertheless, something must be blocked, arresting spontaneous leg movements. Cervical stimulation apparently overrides or bypasses that block. The observation (8) that a paralyzed *para* fly can be made to leap in response to a visual stimulus (provided the *Hyperkinetic* gene is also present) is consistent with the fact that at least some neural and neuromuscular pathways are intact in *para* at high temperature.

Intracellular Recordings from Flight Muscles. The longitudinal indirect flight muscles in the thorax lend themselves readily to intracellular recording. They are single cells, some as large as 120 by 60 μm, and are easy to impale with glass microelectrodes. Anatomical and physiological evidence shows that each dorsal longitudinal muscle fiber receives multiple terminals from a motor neuron in the thoracic ganglion, as well as a branch of the contralateral giant fiber (9–13).

Intracellular recordings were made from these muscles (see *Materials and Methods*). In normal flies, resting potentials ranged from −70 to −80 mV. Cervical stimulation evoked a single excitatory endplate potential with a latency at room temperature of 1.2 ms (Fig. 6A). As the temperature was raised to 39° the latency decreased (to 0.7 ms) and the rise became more rapid. This change in time course is to be expected due to increased conduction velocity and decreased synaptic delay with increased temperature (14, 15). The resting potential and the amplitude of the endplate potential remained undiminished for at least several minutes. Thus, a pathway from the cervical connective to the evoked potential in the muscle remained essentially intact in normal flies at 39°. When the temperature was lowered, the latency returned to its original value (Fig. 6A).

In *para*, as pointed out earlier, leg jerks could still be evoked by cervical stimulation at paralytic temperatures that block spontaneous leg movements. The evoked response in flight muscle also persisted at such temperatures. However, as the temperature was raised a few degrees higher, the cervically-evoked response observed in the flight muscle suddenly failed in an "all-or-none" fashion (Fig. 6B). When the temperature was lowered again below this critical value, the response abruptly reappeared. The response could be evoked again by raising the stimulus intensity, which abruptly produced an evoked potential of latency and amplitude normal for that temperature, but as temperature was raised further, the threshold rose very rapidly, so that even a ten-fold higher stimulus became inadequate. This "all-or-none" failure and the increased threshold of excitation suggest that temperature may affect the excitability of the nerve at the point of stimulation. Once the nerve is excited by a stronger stimulus, a normal muscle response occurs; the neuromuscular junction and the muscle seem unaffected. This phenomenon was observed in *para*, *para*^{ST42}, and *para*^{ST104}.

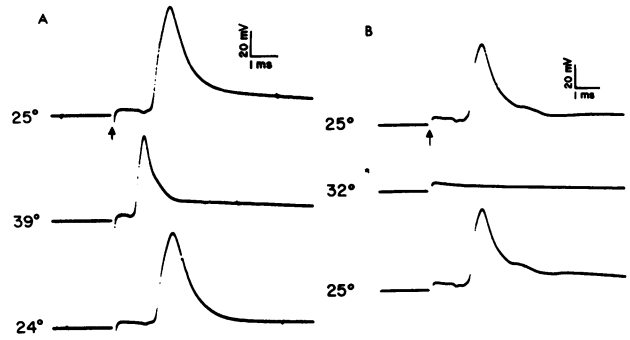

FIG. 6. Intracellular recordings of membrane potential in flight muscle following stimulation (arrow) of the cervical connective. (A) Normal fly. Evoked potential persists at 38°. Decreased latency is reversed by lowering temperature. (B) *para*^{ST42} fly.

A quite different result was obtained with *com*. As temperature was raised to 38° the response underwent a series of gradual changes (Fig. 7). After normal shortening due to rise in temperature, the latency became progressively longer; the amplitude gradually decreased. At first, an apparent regenerative component of the response became distinguishable from the junction potential. This may be due to the normal junction potential's being so large that the regenerative component is normally overshadowed. With time, the response became smaller and later; after about 1 min at 38°, no trace was visible. During the transition from the normal to the paralyzed state, when the evoked potential was reduced and delayed but still visible, increasing the amplitude of the stimulus (to the cervical connective) did not restore a normal response. Recovery at room temperature was slow and graded, following a similar pattern in reverse (Fig. 7).

The behavior of *shi*^{ST139} was quite different. As temperature was raised, the evoked muscle response declined in graded fashion (Fig. 8). The latency decreased at first, as in normal flies, then increased slightly, but the increase was small compared to that in *com*. Normal amplitude could not be reinstated by increasing stimulus strength. The effects occurred at lower temperatures than in *com*, consistent with the lower paralysis temperatures required for this mutant. When the fly was cooled again, after a brief exposure to high temperature, the action

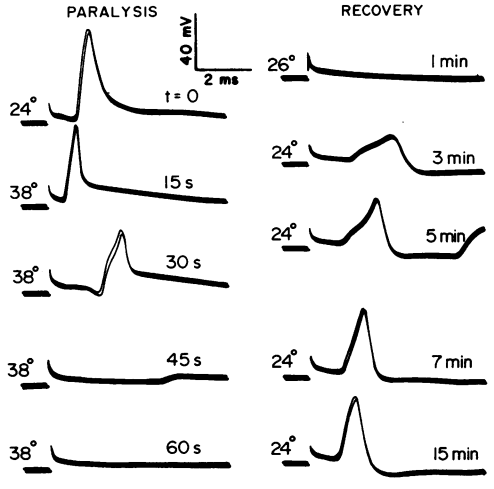

FIG. 7. Cellular recordings from *com* flight muscle, with stimulation of the cervical connective, as temperature is raised to 38°, then lowered for recovery. Two successive sweeps are shown for each time.

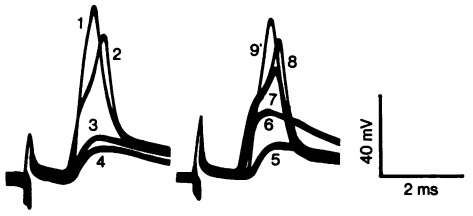

FIG. 8. Intracellular recordings from *shi*^{ST139} flight muscle, with stimulation of the cervical connective. At left, temperature was raised from 25° to 32° (traces 1–4). At right, temperature was gradually decreased again to 25° (traces 5–9).

potential rapidly recovered its normal size and shape (Fig. 8). Long exposures required longer times for recovery. Ikeda *et al.* (16) reported similar observations on *shi* stimulated via the mesothoracic nerve.

In *shi*^{ST139}, during the transition to paralysis, nerve conduction between neck and flight muscle was apparently unimpaired; a one-to-one relation was maintained between stimulus and response. An interesting phenomenon, characteristic of *shi* alleles, is an apparent derepression of the flight oscillator neural network (17) at elevated temperature; while records are made from flight muscle, spontaneous repetitive potentials typical of flight are seen (18). These decrease in amplitude as temperature is raised, *pari passu* with the decrease in cervically evoked potentials.

Direct Excitability of Muscle. The excitability of the flight muscles was tested by direct injection of depolarizing current. A longitudinal flight muscle fiber was impaled simultaneously with two KCl-filled electrodes (resistance 20 MΩ), one to record the intracellular potential, the other to inject current (200–600 nA, approximately 30 ms). The response of the muscle to cervical nerve stimulation was also monitored in the same preparation.

In normal flies, sustained depolarizing current induced a repetitive discharge of spike-like action potentials (19, 20). Fig. 9 illustrates a typical result. This response was somewhat variable, but most preparations of normal flies remained excitable up to 39° and down again to room temperature. At excessive temperatures (above 39 or 40°), the response was degraded to a roughly sinusoidal discharge and the amplitude was diminished. The response was sometimes lost irreversibly, presumably due to damage by the large doses of current.

In *para*, at temperatures where the cervically evoked response had completely disappeared, the direct excitability of the flight muscle remained intact (Fig. 9). The same was observed for *com* (Fig. 10). Ikeda *et al.* (16) showed that the flight muscle in *shi* also remains excitable by direct current injection at paralytic temperatures.

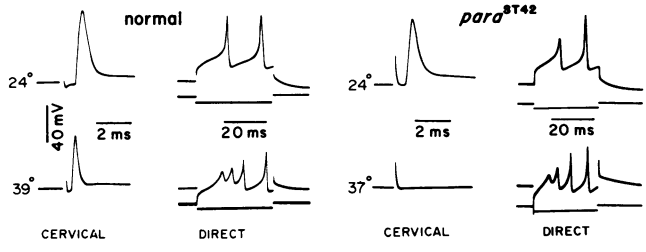

FIG. 9. Response of flight muscle to direct injection of depolarizing current, compared to stimulation of the cervical connective. At left, normal fly responds to both stimuli at both low and high temperature. In *para*, cervical stimulation fails at the high temperature; the muscle is nevertheless still excitable by direct injection of current.

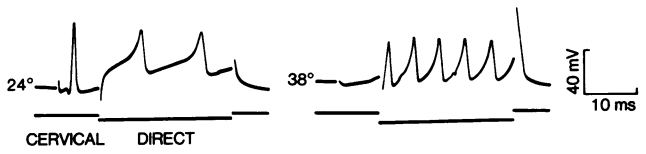

FIG. 10. Excitability of flight muscle in *comatose*. Cervical stimulation is followed by direct current injection in a single sweep. At 38°, cervical stimulus fails, but muscle is still excitable directly.

DISCUSSION

All three mutant types studied show different defects, indicating that mutants of this kind will indeed be a rich source of material for neurophysiology. In *com*, when the fly is heated, the cervically evoked endplate potential occurs progressively later and becomes weaker. Nevertheless, the muscle remains readily excitable by direct injection of depolarizing current. These experiments alone do not distinguish between a slowing of nerve conduction, delayed transmitter release at the nerve terminal, or some defect in response of the muscle to chemical excitation. In the *para* mutants, the threshold for excitability of a cervical pathway rises at temperatures a few degrees higher than the paralysis temperature, while nerve conduction velocity and muscle excitability are still normal. In *shi*, nerve conduction seems intact, but the evoked response gradually diminishes as temperature is raised. There appears to be a defect at the neuromuscular junction.

The clues obtained by studying flight muscle response may provide only partial answers, since a gene mutation affecting the nervous system need not necessarily act at only a single site. Genetic modification or loss of a specific membrane molecule, for instance, could alter the functions of various neurons, synapses, or muscles to different degrees. Paralysis observed in the whole fly would correspond to the part of the locomotor system that fails first as temperature is raised. In *para*, it is possible that the circuits that normally control spontaneous leg movement involve fine axons, which develop intractably high thresholds at a somewhat lower temperature than larger ones. If so, as temperature is raised, spontaneous leg movement will cease first; cervically evoked muscle responses, propagated via the giant axon, may fail later. In *shi*, at a temperature that paralyzes locomotor activity of the fly, the neural flight oscillator circuit turns on and continues happily, while evoking diminished muscle potentials that fail to produce wing flight movements. Also in *shi*, the corneal-positive on-transient of the electroretinogram, believed to be neural in origin, disappears at high temperature, without loss of the photoreceptor response (18). The same is true for *com* (our experiments). In *para*, on the other hand, the positive component remains intact (1). To identify precisely the defects in each mutant will require more detailed experiments.

Differences in the underlying basic mechanisms for the various mutants are apparent from their kinetics of paralysis and recovery. For *para* there is a rather fast, reversible transition between the active and paralyzed states, but the flies can gradually accommodate to raised temperature. For *com*, on the other hand, heating produces a cumulative effect; the time required for recovery is roughly proportional to the duration of exposure at 38°, as if the high temperature causes progressive inactivation of some substance necessary for neural function, and this substance is regenerated, at room temperature, at a constant rate. In the case of *shi*, there is a more complex, escalating effect of exposure time, possibly due to a combination of multiple effects. These kinetic data may be useful clues in identifying the specific macromolecules affected by the single gene changes.

We acknowledge the excellent technical assistance of Paul Christensen. This research was supported by Grant no. PCM 73-01972 from the National Science Foundation to S.B. and by a Gosney Research Fellowship to O.S.

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