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A *Drosophila* mutant with a temperature-sensitive block in nerve conduction

(neurogenetics/paralysis/action potential)

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ABSTRACT **A mutant, *nap^{ts}* (no action potential, temperature-sensitive), is described in which axonal conduction fails at high temperature. Synaptic transmission at the larval neuromuscular junction is unimpaired. Larvae and adults are rapidly paralyzed at restrictive temperatures; they recover rapidly when the temperature is decreased. The mutant gene is recessive and is located on the second chromosome at map position 56.**

Single-gene mutations that affect specific components of the nervous system can provide insight into neurophysiological mechanisms underlying behavior. Temperature-sensitive paralytic mutants (1, 2) are particularly useful because expression of the mutant phenotype can be switched on or off. This permits the recovery of otherwise lethal mutations affecting processes such as nerve conduction, synaptic transmission, and muscle contraction. Analysis of such mutants can lead to identification of the molecular components of the system and the genes coding for them. Temperature-sensitive paralytic mutants of *Drosophila*, with varied physiological defects, have been reported (3, 4). Three such genes on the X chromosome are each represented by several alleles. We have extended the mutant search to the autosomes, which represents about 80% of the genome, and report the isolation and characterization of a new mutant with interesting properties.

MATERIALS AND METHODS

Isolation of *nap^{ts}*. Normal (wild-type Canton-S) *Drosophila* males were treated with the mutagen ethyl methanesulfonate (5) and mated according to the "free recombination scheme of Lindsley" (6) to encourage elimination of lethals and to produce progeny, some of which were homozygous for mutagenized second chromosomes. The progeny were screened for paralysis at 37.5°, at which temperature normal flies remain mobile for at least 30 min. Flies that became paralyzed within 5 min were recovered and bred to produce homozygous mutant stocks. Of 2000 chromosomes tested, five mutually complementing temperature-sensitive paralytics were recovered. One of them, *nap^{ts}* (no action potential, temperature-sensitive), is the subject of this paper.

Paralysis Tests. Flies were raised and maintained at 23°. Adult paralysis tests were done in glass vials (9.5 cm high × 2.5 cm wide). A polyurethane foam stopper was inserted to 6 cm from the bottom, and the lower 7 cm of the vial was immersed in a water bath. The vials were equilibrated for 5–10 min before the flies (4–7 days old, 10 flies per vial) were introduced by aspiration, and the time required for them to fall to the bottom of the vial and become immobilized was measured. For recovery, the vial was transferred to a 23° water bath, and the time required to regain full mobility was noted.

Mapping of the *nap^{ts}* Gene. Recombination tests placed *nap^{ts}* to the right of *purple* (map position 2-54.5) and 1.3 ± 0.5 map units to the left of *cinnabar* (position 2-57.5) on the second chromosome. The mutant gene is recessive. Flies heterozygous for *nap^{ts}* over a deficiency of salivary chromosome region 41B-42A were paralyzed at the same temperature and with the same kinetics as homozygous *nap^{ts}* flies. In addition, flies homozygous for *nap^{ts}*, but carrying a duplication for salivary region 41–43A, behaved like wild type. Thus, *nap^{ts}* is located cytologically between 41B and 42A. Adult paralysis, larval paralysis, and the neurophysiological phenotype all map together and therefore appear to result from a single gene defect.

Physiological Techniques. The larval preparation (7, 8) was bathed in 128 mM NaCl/2 mM KCl/5 mM $MgCl_2$/2 mM $CaCl_2$/35.5 mM sucrose, buffered at pH 7.1 with 5 mM *N*-2-hydroxyethyl-*N'*-1-ethanesulfonic acid (Hepes). Larval muscle membrane potentials were recorded intracellularly with glass microelectrodes filled with 3 M KCl (resistance, 30–60 MΩ). Larval recordings were made from the medioventral longitudinal muscles of the fourth or fifth abdominal segment.

A fine suction electrode (tip diameter, 5–8 μm) was used to draw in a loop of nerve for recording action potentials extracellularly. Signals were picked up by a differential amplifier (WP Instruments, DAM-5A) with bandwidth set at 0.1–3 kHz. Although the action potentials could be resolved in single traces on the oscilloscope, a signal averager (Tracor-Northern, NS-570A) was used to improve the signal-to-noise ratio.

For recordings from adult flight muscles, the fly was first lightly etherized and immobilized, dorsal side up, on a glass coverslip by fixing the head, legs, and tip of abdomen with myristic acid, a low-melting-point wax. These preparations remained in good condition for several hours. The technique for intracellular recording from dorsal longitudinal flight muscles has been described (4). The stimulus was applied to the cervical connective with a pair of tungsten electrodes and the response was recorded with a glass microelectrode. For direct-current injection, double-barreled glass microelectrodes were used; current was passed through one barrel and voltage was recorded through the other.

Temperature was regulated by a Peltier junction (Cambion Bipolar Controller 809-3011-01) and monitored by a thermistor (Bailey Instruments, BAT-4) in contact with the abdomen of the fly in the case of adults or within 1 or 2 mm of the larval preparation in solution.

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Abbreviation: ejps, excitatory junction potentials.

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RESULTS

Paralysis. At 23° nap^{ts} flies showed normal morphology, locomotor activity, flight, phototaxis, sexual courtship, and learning ability. At 32° or less, they did not pass out; but at 33° or more, paralysis occurred. Fig. 1 shows the kinetics of paralysis at 37.5° and recovery on return to 23°. The duration of exposure to 37.5° (up to 10 min or more) had little effect on recovery. Normal flies were not paralyzed by exposure to 37.5° for as long as 30 min.

The rapid paralysis and recovery, the sharp transition temperature, and the independence of recovery from duration of exposure, are features that nap^{ts} has in common with the X-chromosome mutant $para^{ts}$ (1). In contrast, the other X-chromosome temperature-sensitive paralytics, $shibire^{ts}$ and *comatose*, lack sharp transition temperatures and recover more slowly as the exposure to high temperature becomes longer (4).

When a *Drosophila* larva crawls on an agar surface, it typically extends its head forward and inserts its mouth hooks into the agar in two strokes, a superficial touch followed by a deep bite. Coincident with the bite, a wave of muscular contraction sweeps from tail to head, pushing the posterior part forward to begin the next cycle. This repetitive pattern produces a visible track that can be photographed. The nap^{ts} larvae produced normal tracks up to 32° but, as the temperature was raised, they became more erratic in their movements. Fig. 2 shows an erratic track produced by a particular nap^{ts} larva at 36°. At 37° this larva stopped tracking but continued to show slight twitching of the anterior segments. On transfer to lower temperatures, normal movement resumed. In contrast, normal larvae at 37° crawled rapidly, continuing to produce respectable tracks. Like nap^{ts} larvae, larvae of $para^{ts}$, $shibire^{ts}$, and *comatose* stopped tracking at high temperatures.

Neurophysiology of nap^{ts} Larvae. The larval neuromuscular preparation described by Jan and Jan (7, 8) was used. A mature third-instar larva was immersed in Ringer solution, cut along the dorsal midline, pinned out, and eviscerated (Fig. 3). The abdominal segments contain muscle fibers arranged in a regular pattern. The longitudinal fibers near the ventral midline are about 400 μm long, 80 μm wide, and 25 μm thick. Each is a single cell, easily penetrated with glass microelectrodes and essentially isopotential throughout its length (7). A nerve bundle from the ventral ganglion innervates the muscles in each lateral half segment (9). There is evidence for more than one axon per muscle fiber, as indicated by the observation of two stimulus thresholds, each corresponding to an endplate potential of different amplitude and time course (7). In the flies *Calliphora* and *Musca*, anatomical and physiological evidence also indicates multiple innervation (10, 11).

To perform intracellular recordings from a muscle fiber, the muscle was impaled with a microelectrode and the nerve innervating that half segment was cut at the ganglion end and stimulated with a suction electrode. The stimulus was adjusted to produce a maximal response. Excitatory junctional potentials

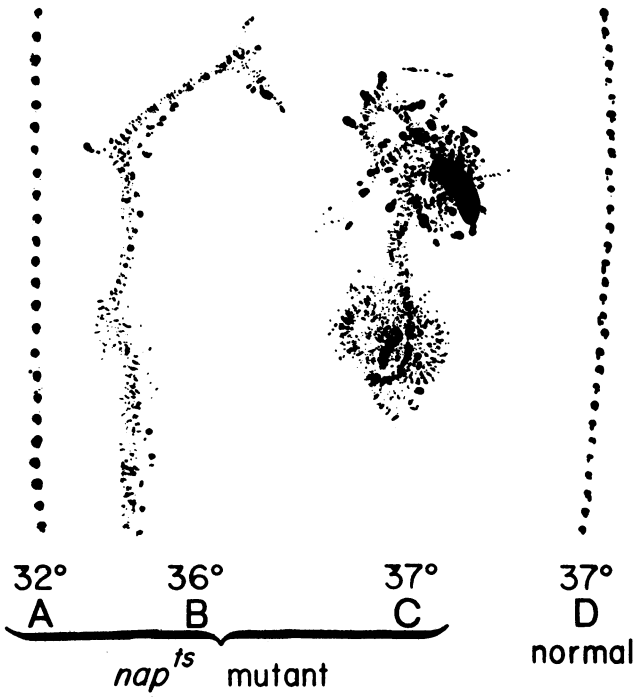

FIG. 2. Typical tracks made by normal and nap^{ts} larvae crawling on an agar surface at various temperatures. The mutant larva crawled normally at 32° (*A*) but became erratic at 36° (*B*). At 37°, it remained at the starting area (*C*, lower part of panel). When the dish was removed from the 37° incubator for photographing, the larva began to recover and resumed crawling, making the additional traces in the upper part of the panel, where the larva itself is visible. A normal larva kept on tracking at 37° (*D*).

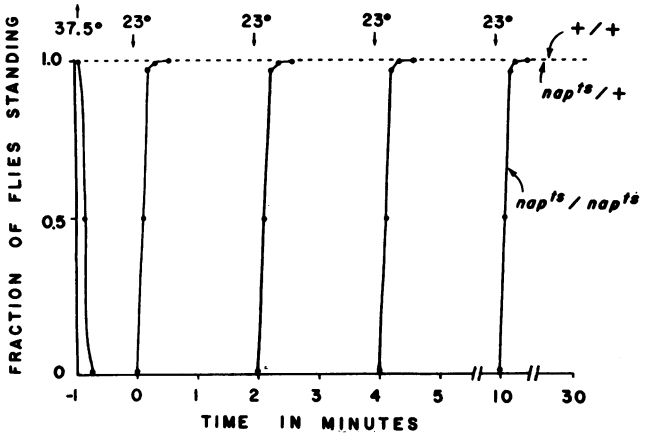

FIG. 1. Kinetics of paralysis and recovery in nap^{ts} adults. Four groups of flies (10 per group) were exposed to 37.5° at $t = -1$ min. One group was returned to 23° for recovery at each of the times indicated by arrows. Flies exposed to 37.5° for 10 min recovered as rapidly as those exposed for only 1 min. The broken line at the top represents the response of normal (+/+) and heterozygous nap^{ts} flies; both remained mobile at 37.5° for over 30 min.

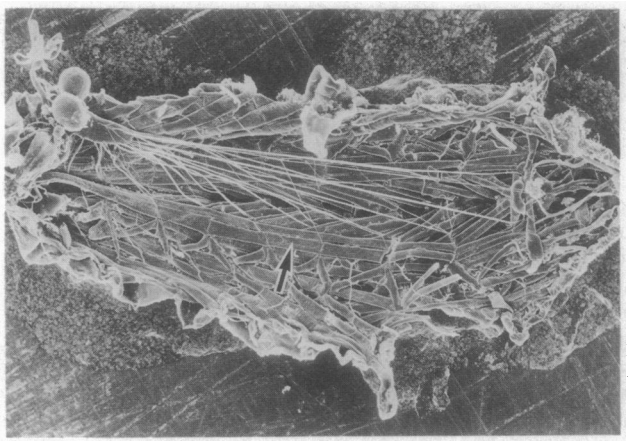

FIG. 3. Scanning electron micrograph of dissected larval preparation showing ganglion, nerves, and ventral muscles. Arrow points to ventrolateral longitudinal muscles from which intracellular recordings were obtained.

(ejps) up to 30 mV in amplitude were recorded. Under the physiological conditions used, the larval muscles did not fire action potentials.

At 23° the resting potential observed in *nap^{ts}* muscle fibers was about 40–50 mV, typical of that in normal larvae. Spontaneous miniature endplate potentials in *nap^{ts}* were also normal (about 1 mV in amplitude with a frequency of about 0.5/sec). Fig. 4 shows the effect of increasing temperature on the ejp. In the normal larva, the ejp persisted to well beyond 35°. The mutant larva, on the other hand, displayed normal ejps up to about 34°; at higher temperature the response disappeared. The ejp was fully restored by return to lower temperature. These effects could not be accounted for by changes in the resting potential, which remained intact in both mutant and normal larvae at high temperature. Although abrupt disappearance of the ejp was characteristic of the mutant, there was some variation in the critical temperature among mutant individuals (Fig. 5).

To determine whether the loss of the ejp above the critical temperature was a gradual reduction in amplitude or an all-or-none failure, we examined the ejp in mutant larvae with repeated stimuli of fixed amplitude at temperatures in the transition range. Fig. 6 shows that a small change of temperature could have a dramatic effect. In this particular preparation, which had a relatively low restrictive temperature, every stimulus yielded a response at 29° but at 31.2° none did. At intermediate temperatures, the response was always all-or-none, with the proportion of failures increasing as the temperature was raised. At a point where, for a fixed stimulus, the ejp first disappeared, increasing the stimulus voltage would usually restore the response temporarily (Fig. 7). However, the increased stimulus soon became insufficient, even when raised to many times above the original threshold.

Failure of the ejp at high temperature was not due to a defect in neuromuscular transmission. This was demonstrated by electrotonically evoking transmitter release. Propagation of nerve action potentials was blocked in normal larvae by adding

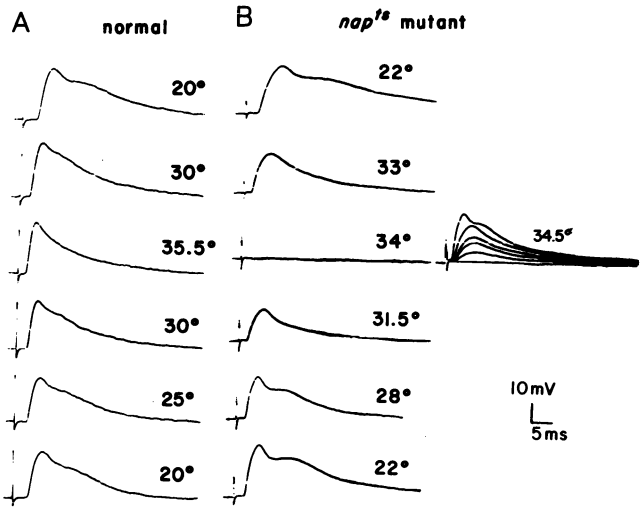

FIG. 4. Intracellular recordings from ventrolateral longitudinal muscles in normal and *nap^{ts}* larvae at various temperatures, showing ejps in response to nerve stimulation (0.2-msec duration, amplitude adjusted to produce maximal response). (*A*) Normal larva. The ejp persisted as temperature was raised to 35° and lowered again. (*B*) *nap^{ts}* larva. As temperature was raised to 34°, the ejp disappeared; it reappeared as the temperature was lowered. (*Inset*) At 34°, when the response had failed, a graded potential could still be induced electrotonically by using stronger stimuli (0.5-msec duration) of varied intensity.

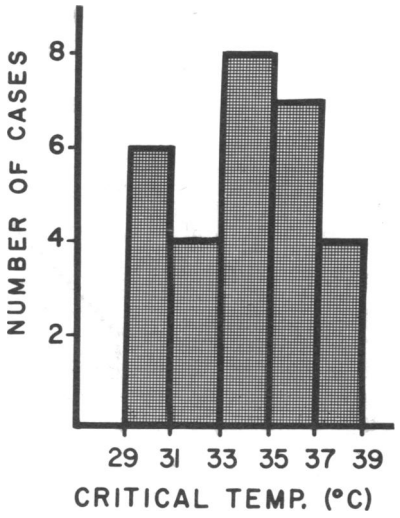

FIG. 5. Histogram of critical temperatures in various *nap^{ts}* larvae. The critical temperature was defined as the minimum temperature at which the ejp could not be evoked, even with a stimulus intensity severalfold greater than that required at low temperature. In each case, recovery occurred upon cooling. Mean critical temperature, 33.5°; $n = 29$.

tetrodotoxin (1 μg/ml) to the bath, and stimuli sufficient to depolarize the nerve terminals were applied via the suction electrode. The electrotonically evoked ejp was different from the normal ejp; it had short latency and graded amplitude with increasing stimulus intensity (Fig. 8). The effectiveness of this stimulation varied inversely with nerve length (12). In *nap^{ts}* larvae, at temperatures at which the active ejp disappeared, it was still possible to evoke an ejp electrotonically (Fig. 4*B*). This electrotonic ejp in *nap^{ts}* was comparable to that produced when the nerve was blocked by tetrodotoxin (at either low or high temperature) in normal or mutant larvae. Therefore, transmitter release and postsynaptic elements in *nap^{ts}* remained functional at the restrictive temperature. The all-or-none nature of the defect suggested a failure in nerve impulse propagation.

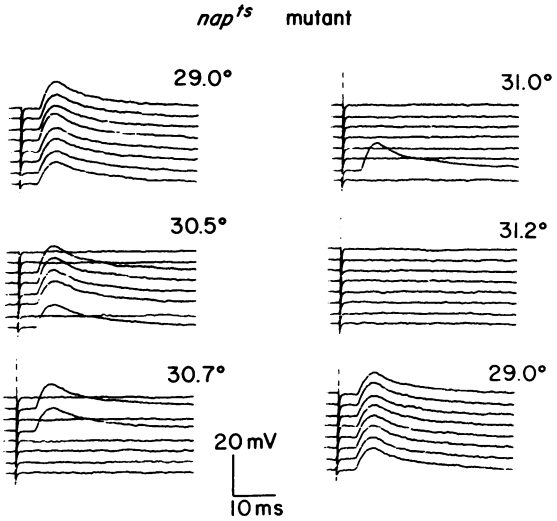

FIG. 6. Intracellular recordings from a *nap^{ts}* larval muscle fiber. There was an increase in the proportion of all-or-none failures, in response to a constant nerve stimulus, as the temperature increased within the critical range. Recovery was complete when temperature was lowered.

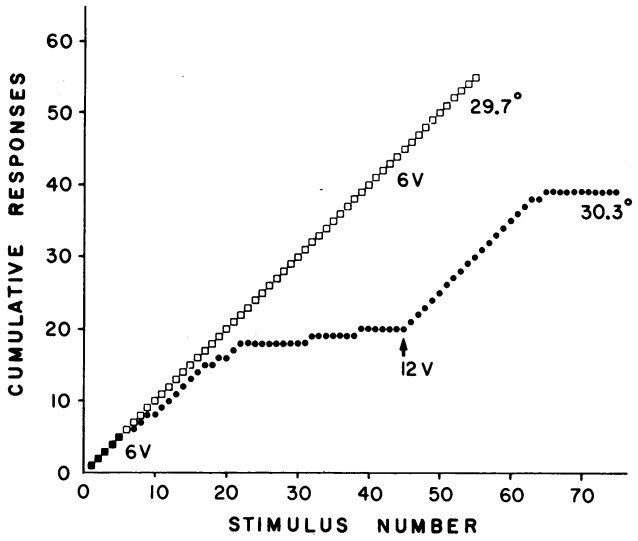

FIG. 7. Responses recorded from *nap^{ts}* larval muscle fiber near the critical temperature. Stimuli were applied to the nerve at 1-sec intervals. At 29.7° there was a one-to-one correspondence between stimulus and response. At 30.3° the response soon failed. When the stimulus intensity was increased to 12 V (arrow) the response was restored, but only temporarily. When the temperature was lowered, the response reappeared.

We therefore measured nerve action potentials directly. The nerve was not cut but was left in place, with the ganglion serving as an anchor. A fine-bore suction electrode was positioned near the ganglion end and a loop of nerve was drawn in for recording *en passant*. The stimulating electrode was placed near the neuromuscular junction at the nerve terminus and the nerve was stimulated antidromically. In this way, only a few nerve fibers in the immediate vicinity were activated. Typical recordings obtained from mutant and control larvae are shown in Fig. 9. The number of peaks observed (usually one to three) and their relative sizes depended on the precise location of the stimulating electrode and the stimulus strength. These responses could also be observed with the ganglion end of the nerve cut, so they were not ganglionic in origin. At high temperature, the responses in the mutant larva disappeared, different units dropping out at slightly different temperatures. They recovered at lower temperatures. In the control larva, at the same high temperature, there was a slight decrease in amplitudes but all peaks persisted. Because of their all-or-none character at threshold stimulus intensity and, in the case of *nap^{ts}*, their all-or-none disappearance at critical temperatures, the individual peaks recorded in this manner probably represented single or, at most, a few units.

The procedure was modified to record compound action potentials, to determine whether the *nap^{ts}* mutation had a similar effect on all the axons within a nerve bundle. The nerve was cut close to the ganglion and stimulated at that end with a suction electrode. The fine-bore suction electrode for recording was now positioned near the muscle, and a loop of nerve was drawn up. The compound action potential recorded in this way, comprised of many action potentials contributed by the various axons within the nerve bundle, is shown in Fig. 10. The exact waveform was variable and depended on the distance between stimulating and recording electrodes, the length of the nerve loop within the recording electrode, and the tightness of fit. The signal disappeared as the temperature was increased but reappeared on return to low temperature. The gradual disappearance of the compound action potential at high temperature can be explained by separate failure of various individual units as revealed in the previous experiment (Fig. 9).

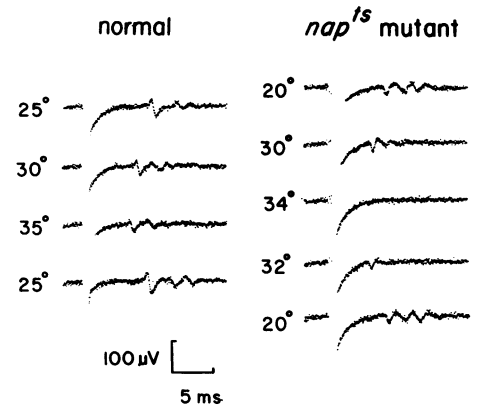

FIG. 9. Signals from a small number of single nerve units excited by stimulating antidromically near the nerve terminals and recording extracellularly from the segmental nerve bundle. In the *nap^{ts}* mutant, components dropped out individually as the temperature was raised and recovered when the temperature was lowered. Signals were averaged over 16 trials.

Calcium action potentials in larval muscle fibers can be produced under special conditions—e.g., in the presence of Ba^{2+}, Sr^{2+} or tetraethylammonium ions or in very high $[Ca^{2+}]$ (13). We compared such action potentials in *nap^{ts}* and normal larvae by adding 24 mM Sr^{2+} to the usual bath and injecting current directly into the muscle. Both mutant and normal larvae continued to give overshooting action potentials at temperatures up to 37° or more.

Neurophysiology of Adults. Intracellular recordings were performed on the dorsal longitudinal muscles in the adult thorax. These muscles are large single cells in which action potentials can be evoked by stimulating the cervical connective nerve bundle (4). At 20°, mutant and normal flies were similar but, as the temperature was increased, the evoked response in *nap^{ts}* flies abruptly disappeared. Reducing the temperature caused it to reappear.

Intracellular injection of depolarizing current elicited a repetitive discharge of spike-like action potentials which, in

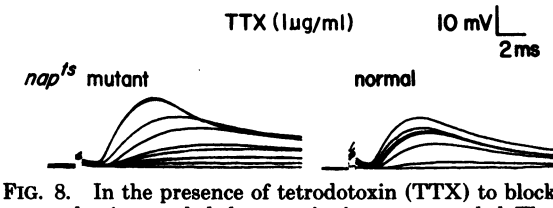

FIG. 8. In the presence of tetrodotoxin (TTX) to block active nerve conduction, graded electrotonic ejps were recorded. These were similar in *nap^{ts}* and normal larvae at 23°. (Compare to Fig. 4*B inset*, which is for *nap^{ts}* at high temperature without tetrodotoxin.)

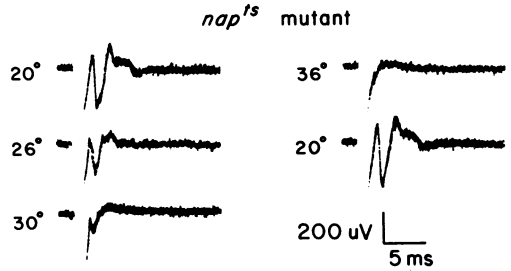

FIG. 10. Compound action potential recorded from nerve bundle in *nap^{ts}* larva, showing temperature dependence. Conduction was blocked at 36° but recovered when the temperature was lowered.

normal flies, occurred up to at least 37°. In *nap^{ts}*, the direct excitability of the muscle also remained intact at 37°, even though the cervically induced response was blocked, as observed with the mutant *para^{ts}* (4). These results are consistent with the conclusion from larval physiology that *nap^{ts}* produces a temperature-dependent block in nerve conduction but does not affect the muscle fibers noticeably.

DISCUSSION

The primary physiological basis for paralysis in the *nap^{ts}* mutant appears to be a reversible, temperature-dependent block in nerve conduction. Synaptic transmission and muscle response are essentially normal. The effect at the molecular level is unknown. An interesting possibility is that the defect resides in the nerve membrane, altering the ionic gating mechanisms involved in the propagation of action potentials. Such a defect could alter the voltage dependence, ion selectivity, or kinetics of opening or closing of the ionic channels responsible for impulse propagation. The mutation could affect the molecular structure of an ionic channel directly or could disturb the environment in which the channel operates by altering some other membrane component. The mutation does not affect all electrically excitable membranes because, as our experiments have indicated, muscle action potentials in *nap^{ts}* larvae and adults are inducible by direct-current injection, even at temperatures at which nerve action potential propagation fails. These muscle action potentials are due to electrically excitable calcium channels (13, 14), whereas nerve action potentials depend on excitable sodium channels.

There exist other *Drosophila* mutants in which membrane components are implicated. Jan *et al.* (12) showed that in the *Shaker* (*Sh*) mutant, transmitter release is abnormally prolonged at the larval neuromuscular junction. The effect is mimicked in normal larvae by potassium channel blocking agents, suggesting that the mutant might have altered potassium channels. In *Paramecium*, mutants have been described in which the Ca^{2+} action potential is lost or abnormally prolonged (15, 16), and voltage clamp experiments showed that ionic channels are defective in some of the mutants (17). Similar measurements may prove feasible in *Drosophila*, in spite of the relative smallness of its axons.

A functional membrane element such as an ionic channel could contain more than one molecular component, each coded for by a separate gene. Mutations in the different genes might lead to similar physiological effects. If two such mutations were combined in the same individual, the defect might be more extreme than that produced by either mutation alone. The mutants *para^{ts}* and *nap^{ts}* resemble each other behaviorally although their genes are located on different chromosomes. Both have relatively sharp critical temperatures and undergo rapidly reversible transitions between active and paralyzed states. In both mutants, blockage of action poentials occurs at high temperature. We made crosses designed to produce a double mutant of *nap^{ts}* and *para^{ts}*. The results indicated that this combination is not viable at a temperature (23°) at which the single mutants are fully viable. This suggests a possible interaction between the two gene products. In contrast, *shibire^{ts}* and *comatose* differ markedly from *nap^{ts}* in their kinetics of paralysis and recovery (4). Physiological experiments on *shibire^{ts}* adults indicate a defect in synaptic transmission (3, 4) whereas in *comatose* adults there is a decreased conduction velocity or a delay in transmitter release or both (4). The double mutant combinations of these mutants with *nap^{ts}* are viable and, in each case, show the phenotypes anticipated if those genes act independently of the *nap^{ts}* gene. The interaction between *nap^{ts}* and *para^{ts}* may therefore be significant.

A feature of *Drosophila* that may prove valuable is that the chromosome band containing the gene in question can be identified. If the gene codes for a protein such as a sodium channel molecule, a plasmid containing the gene (identifiable by hybridization to the specific chromosome region) might be used to synthesize large amounts of what is ordinarily a rather scarce membrane molecule.

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