Reversible Blockage of Neurite Development and Growth Cone Formation in Neuronal Cultures of a Temperature-Sensitive Mutant of *Drosophila*

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It has been suggested that the membrane recycling process in the *Drosophila* mutant *shibire^{ts1}* (*shi^{ts1}*) is reversibly blocked at restrictive temperature (>29°C) in various cell types, including neurons, as a result of enhanced vesicle fusion and disrupted membrane pinch-off (Poodry and Edgar, 1979; Koenig et al., 1983; Kosaka and Ikeda, 1983a, b). We analyzed the neuronal development of *shi^{ts1}* cells at the single-cell level by using dissociated larval CNS culture. Compared to normal cultures, there was a substantial reduction in neuronal adhesion to the substratum and a retardation of growth cone formation in *shi^{ts1}* cultures at 30°C. Time-lapse studies showed that neurite initiation and elongation in *shi^{ts1}* cultures were suppressed at the restrictive temperature. Differentiated *shi^{ts1}* neurons exhibited accelerated reabsorption and retarded arborization of neurites at 30°C. The above processes were reversible since normal outgrowth was restored when the temperature was lowered. These findings provide a new line of evidence that growth cone activity determines neurite initiation, elongation, and branching. Furthermore, if the primary defect of the *shi^{ts1}* mutation indeed resides in a membrane recycling mechanism common to different cell types, our results would also lend strong support to the notion that membrane recycling is crucial in the regulation of neuronal membrane adhesion and growth cone activity.

The development of neurons and the functional organization of nervous systems are dependent upon macromolecules encoded by genes. By altering relevant genes, mutants with specific biochemical, anatomical, or behavioral abnormalities can be produced. This approach has been effectively employed to probe the development and function of the nervous system of *Drosophila melanogaster* (Benzer, 1973; Hall, 1982).

Several temperature-sensitive paralytic mutants of *Drosophila* have been isolated (Grigliatti et al., 1973; Siddiqi and Benzer, 1976; Wu et al., 1978; Singh and Siddiqi, 1981; Kulkarni and Padhye, 1982; Ganetzky and Wu, 1985). Among them, the sex-linked recessive mutant *shibire^{ts1}* (*shi^{ts1}*) becomes paralyzed above the restrictive temperature (29°C) and gradually recovers when the temperature is lowered (Grigliatti et al., 1973).

Previous studies indicate that paralysis in *shi^{ts1}* flies is caused by a temperature-dependent disruption in synaptic transmission. At the neuromuscular junction, a progressive diminution of the excitatory junctional potentials (Ikeda et al., 1976; Siddiqi and Benzer, 1976; Koenig et al., 1983) and a reduction in the frequency of spontaneous miniature excitatory junctional potentials (Koenig et al., 1983) occurs as the temperature is raised above 29°C. These effects appear to be presynaptic, since *shi^{ts1}* muscle fibers respond normally at high temperature to direct electrical stimulation (Ikeda et al., 1976) and to the putative transmitter L-glutamate (Koenig and Ikeda, 1983). Moreover, studies of *shi^{ts1}* genetic mosaics showed that the transmission block occurs when the motor neuron is mutant regardless of the genotypes of the muscle (Koenig and Ikeda, 1983). Defects in *shi^{ts1}* flies are not limited just to the neuromuscular junction. Synaptic transmission between photoreceptors and laminar neurons may be blocked at high temperature, as suggested by electroretinogram analysis (Kelly and Suzuki, 1974). In addition, radiochemical studies have indicated that the storage and release of the neurotransmitter ACh at high temperature are altered in the CNS of *shi^{ts1}* larvae (Wu et al., 1983a).

Changes in membrane fusion and retrieval that could explain the disruption in synaptic transmission have been observed. When *shi^{ts1}* flies are held at or above 29°C for over 2.5 min, a decrease in the number of synaptic vesicles and a corresponding increase in the number of cisternae or large vesicles can be found in presynaptic terminals of neuromuscular junctions of tibial muscles (Poodry and Edgar, 1979) and dorsal longitudinal flight muscles (Koenig et al., 1983). Synaptic vesicles reappear upon returning to room temperature. Temperature-induced vesicle fusion in the sarcolemma of indirect flight muscles has also been observed in *shi^{ts1}* flies (Costello and Salkoff, 1986). Evidence for a defective membrane retrieval mechanism at high temperature was provided by Kosaka and Ikeda (1983a), who showed an accumulation of many pitlike structures in the plasma membrane at presynaptic terminals of various kinds of motor, sensory, and central neurons. In fact, a reversible blockage of endocytosis, presumably due to disrupted membrane pinch-off, has been directly demonstrated in the *shi^{ts1}* garland cells (Kosaka and Ikeda, 1983b).

This temperature-induced membrane defect appears to disrupt the development of different cell types, including myocytes in embryonic culture (Buzin et al., 1978), and flight muscles (Costello and Salkoff, 1983) and imaginal disk-derived tissues (Poodry et al., 1973; Suzuki, 1974) in pupae. It has been reported that high-temperature treatments during metamorphosis alter the development of the giant fiber pathway in *shi^{ts1}* flies (Hum-

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mon and Costello, 1984; 1987). This raises the possibility that the *shi^{ts1}* mutation may directly affect growth cone activity and neurite extension. In this study, we confirmed this possibility by utilizing the culture system of larval CNS neurons (Wu et al., 1983b). We have extensively investigated, in time-lapse experiments, the effects of high-temperature treatments on neurite initiation, elongation, and arborization in *shi^{ts1}* neurons at the single-cell level. The changes were correlated to growth cone formation and cell membrane adhesion properties. The role of membrane recycling in neuronal morphological development is discussed and the abnormal membrane fusion and retrieval process found in various *shi^{ts1}* cell types are evaluated as the mechanism underlying the altered neuronal morphology reported here. A preliminary account of some of the results reported here has appeared previously (Kim and Wu, 1986).

Materials and Methods

Animal stocks. The wild-type strain Canton-S and the mutant allele *shi^{ts1}* of *Drosophila melanogaster* raised at room temperature were used. Among the available alleles of the *shi* locus, *shi^{ts1}* has been studied most extensively (Grigliatti et al., 1973; Poodry et al., 1973; Suzuki, 1974; Kosaka and Ikeda, 1983a, b). A different allele *shi^{ST139}* (Siddiqi and Benzer, 1976) was also examined.

CNS cell culture. A revised Schneider's *Drosophila* culture medium (Schneider and Blumenthal, 1978) containing 10% heat-inactivated fetal bovine serum, penicillin (50 units/ml), and streptomycin sulfate (50 µg/ml) was used. Dissociated larval neurons can be grown in this medium for several weeks (Wu et al., 1983b). All the above solutions and chemicals were obtained from GIBCO (Grand Island Biological Co., Grand Island, NY).

Preparation of the primary cultures of dissociated CNS cells was essentially the same as that reported previously (Wu et al., 1983b). Brains and ganglia dissected from mature third instar larvae were cut into small fragments in culture medium and were incubated for 40 min to 1 hr at room temperature (22 ± 1°C, permissive temperature) in Ca²⁺- and Mg²⁺-free solution (137 mM NaCl, 2.7 mM KCl, 0.36 mM NaH₂PO₄·H₂O, 11.9 mM NaHCO₃, 5.6 mM glucose; see Schneider and Blumenthal, 1978) containing 0.33–0.50 mg/ml collagenase (Type I, Sigma Chemical Co., St. Louis, MO). The tissue fragments were then dissociated into single cells by repeated passage through the small opening (100–200 µm) of siliconized glass pipettes with fire-polished tips. Fifty to 100 µl of cell suspension were plated onto 35 mm tissue culture dishes (Corning) or onto round glass coverslips (12–15 mm in diameter) kept in plastic dishes. The plating surface of tissue culture dishes or coverslips was precoated with poly(L-lysine) hydrobromide (*M_w* > 30,000, Sigma Chemical Co.) in 0.2 M Tris buffer (1 mg/ml), pH 8.4 (Letourneau, 1975; Banker and Cowan, 1977). As an exception, plain glass coverslips were used in the phase-contrast microscopy of growth cones (Fig. 1, Table 2). Cells showed increased growth cone activities on this less adhesive substratum.

To prevent evaporation of the culture medium, the dishes were kept in a humidified chamber. Cultures were routinely incubated at 22 ± 1°C (permissive temperature). For temperature shifts, an incubator at 30 ± 1°C (restrictive temperature) was used. Culture ages are given below as hours or days after plating.

Dissociated neurons were plated at densities ranging from 100 to 400 cells/mm². After 2–3 hr incubation, more than 50% of the dissociated cells initiated axonlike processes. At this stage, dissociated cells could be classified into 3 categories by their sizes and morphology as previously described (Wu et al., 1983b). Type I cells were large (>8 µm) neuroblasts, usually accompanied by smaller daughter cells; type II cells were relatively small (2–3 µm) monopolar cells that frequently had long (>20 µm) neurites; type III cells were oval or spindle-shaped cells of intermediate size (4–10 µm) and usually had thick and short processes. Type I cells were rare (<5% of the total population) and were excluded from this study. In the following experiments, the observations on type II and III cells were combined because consistent results were obtained from them.

Scoring cultures for neurite growth. For the time-lapse study of neurite growth, cultured neurons were scored with phase-contrast optics (Nikon Diaphot inverted microscope with a 40×, 0.55 N.A. objective) at the

ages of 3 hr and of 1, 2, 3, and in some cases 4 d. Previous studies showed that dissociated larval neurons enter a morphological steady-state after 3 d in culture, coincident with the duration of pupation (Wu et al., 1983b).

To examine the effects of high temperature, *shi^{ts1}* cultures were observed immediately before and after 24 hr incubation at 30°C. Cultures of 2 hr and 1 d of age were treated in this manner. The same temperature-shift experiments were carried out on normal cultures. For each experiment, both control and experimental cultures were prepared and observed at the same time.

The lengths of primary neurites of single cells were directly measured with a reticle in the eyepiece of the microscope or determined on photographs. Neurites shorter than 4 µm in length were excluded from analysis because they cannot be reliably distinguished from irregularities in cell shape. To simplify the measurement, only the longest primary neurites were recorded for bipolar or multipolar cells.

To ensure that the same cells were recorded during the time-lapse studies, fixed areas were identified with markings (2 cross lines) on the bottom of the tissue culture dishes. For each single culture, an area of 0.65–1.45 mm² was examined.

In a later phase of this study, a video camera system with contrast enhance circuit (Hamamatsu Photonics C2400-07) was used in conjunction with phase-contrast optics (Zeiss IM-35 with a 100×, 1.3 N.A. objective). The finer details of growth cone morphology were resolved on the video monitor (Fig. 1).

Scanning electron microscopy. Specimens for scanning electron microscopy (SEM) were prepared based on the methods described previously (Wolosewick and Porter, 1979). Dissociated cells were plated onto the poly(L-lysine)-coated round glass coverslips (12 mm in diameter). The coverslips with cells were washed briefly in *Drosophila* saline (Wu et al., 1983b) to remove cell debris. Cells were fixed with 1% glutaraldehyde in *Drosophila* saline for 10 min.

To observe the effects of high temperature on neurite arborization and growth cone morphology, cultures were kept at 22°C for at least 16 hr after plating and then shifted to 30°C for 4–8 hr prior to fixation, as specified in different experiments. Solutions used in wash and fixation procedures were prewarmed to 30°C before application.

Following fixation and dehydration through an alcohol series, cultures were immersed in hexamethyldisilazane (HMDS, Sigma Chemical Co.) for 5 min and air-dried in a ventilation hood at room temperature, as previously described by Nation (1983). The specimens were sputter-coated with gold and palladium and observed with a scanning electron microscope (JEOL JSM 35C) at an accelerating voltage of 13 or 14 kV.

In the SEM experiments, nerve terminals with flattened veil-like lamellipodia and/or filopodia were defined as growth cones. Fine processes projecting from the lamellipodia were taken as filopodia. In some cases, lamellipodia degenerated to club shapes, and the fine projections remaining at these structures were considered as filopodia. Since the diameter of filopodia is rather constant at about 0.15 µm (Bray, 1973b; Landis, 1983), filopodia could be distinguished from nonfilopodial processes by their thinner diameters as well as their positions.

Results

Qualitative observations of individual *shi^{ts1}* neurons

At room temperature, the morphology of *shi^{ts1}* neurons was apparently normal. Neurite outgrowth and growth cone activity were readily observed (Fig. 1). Neuroblasts underwent asymmetrical division to give rise to more daughter cells (Fig. 1).

This culture system contains neurons of heterogeneous developmental stages because the larval CNS undergoes vigorous development before entering metamorphosis (White and Kankel, 1978). Temperature-sensitive changes in *shi^{ts1}* cultures may reflect the ensemble effect of neurite initiation, reabsorption, elongation, and retraction, as schematically illustrated in Figure 2. In order to analyze each of these processes, time-lapse observations on individual neurons were necessary.

Figures 3 and 4 show tracings of representative populations of single cells in normal and *shi^{ts1}* cultures subject to temperature shifts at the ages of 0 and 1 d. During the experiments, single neurons remained at fixed positions, and their neurites main-

Figure 1. Neurons in dissociated cell cultures from normal and *shi^{ts1}* larval CNS at 22°C. Asymmetric divisions of neuroblasts and initiation of neurite outgrowth of their daughter cells were much the same in normal (*a, b*) and *shi^{ts1}* (*c, d*) cultures. Panels *a* and *b*, and *c* and *d* are sequential records (phase-contrast micrographs) taken 1 d apart. Active movements of filopodia and lamellipodia were observed in both normal (*e, f*) and *shi^{ts1}* (*g, h*) growth cones. Panels *e* and *f*, and *g* and *h* are sequential records (video contrast-enhanced micrographs) taken 10 sec apart. During the observation periods no substantial migration of single neurons was observed. In this and the following figures, time-lapse observations on the same cells were ensured by the marking technique described in Materials and Methods. Scale bar, 10 μ m.

tained roughly constant directions of growth (Figs. 3 and 4). The overall pattern of neurite outgrowth in *shi^{ts1}* cultures was evidently altered by high temperature (compare Figs. 3; 4, *c, d*).

Following plating, incubation at 30°C for 24 hr suppressed neurite initiation and increased neurite reabsorption in *shi^{ts1}* neurons (Fig. 3). In contrast, the neurite outgrowth in normal cultures was not interrupted under the same condition (Fig. 3). This temperature-induced neurite retraction also occurred in *shi^{ts1}* cultures of older ages. As compared to normal neurons, temperature shifts to 30°C during the second day of incubation caused neurite retraction in most (>85%) *shi^{ts1}* neurons (Fig. 4, compare *a* and *c*). However, in *shi^{ts1}* cultures maintained at room

Figure 2. Schematic illustration of neurite initiation, reabsorption, elongation, and retraction.

temperature, the majority of the cells had grown continuously, as had those in normal cultures (Fig. 4, *b, d*).

Similar results were obtained from the strain *shi^{ST139}*, an independent isolate in a different genetic background (Siddiqi and Benzer, 1976). Therefore, the observed changes are not likely due to mutations at other sites.

Neurite retraction and reabsorption in *shi^{ts1}* neurons at 30°C can be separated from the cell death process. Degenerating and dying neurons generally become spherical and finally detach from the culture dish (Wu et al., 1983b). At 30°C, neurite retraction and reabsorption of *shi^{ts1}* neurons occurred within hours, whereas the cell bodies retained their initial morphology for days and could reinitiate neurite outgrowth when the temperature was lowered (about 80, 50, and 5% for 1, 2, and 3 d treatments, respectively). Therefore, cell death may not be a significant factor causing neurite retraction and reabsorption within the time periods reported here.

Quantitative measurements

By tracing individual cells at successive time intervals, the morphological changes of *shi^{ts1}* neurons induced by temperature shifts can be monitored at a more refined level. Cellular responses such as initiation, elongation, retraction, and reabsorption of neurites (Fig. 2), and elaboration of branching pattern were quantitatively analyzed.

Neurite initiation. Newly seeded cells that were devoid of neurites but subsequently initiated neurite outgrowth were studied first. In *shi^{ts1}* cultures, neurite initiation was suppressed during high-temperature treatments (Fig. 5, *d, e*) as compared with untreated cultures (Fig. 5*f*). The suppression by high temperature is at least partially reversible. In both first-day (Fig. 5*d*) and second-day (Fig. 5*e*) treated cultures, neurite initiation showed a rebound following the downshifts of temperature. In contrast, neurite initiation in normal cultures was not inhibited or was even slightly accelerated during the 30°C incubation (Fig. 5, compare *a* and *b* to *c*).

Neurite reabsorption. Cells with neurites in *shi^{ts1}* cultures exhibited an increased occurrence of complete neurite reabsorption at 30°C. They either reinitiated neurite outgrowth upon returning to low temperature or eventually detached from the substratum. Figure 6 compares the cells that reabsorbed their neurites in *shi^{ts1}* and normal cultures within the 3 d observation period. In *shi^{ts1}* cultures, the frequency of neuritic reabsorption was markedly increased by high-temperature treatment during either the first- or second-day incubation, which was not observed in normal cultures (Fig. 6).

To examine whether neurite reabsorption in *shi^{ts1}* neurons

Figure 3. Effects of high-temperature treatment during the first day of incubation on normal and *shi^{ts1}* neurons. In this figure and Figures 4–6 and 8, periods of 30°C treatment are indicated (black bar) and temperature for the remaining time is 22°C (open bar). All single neurons in randomly selected fixed areas (see Materials and Methods) in the cultures were traced from phase-contrast micrographs in 3 successive days. In this figure and Figure 4, the cells were selected from these records to reflect the rough proportions of different cell types and morphological responses observed in the population. In *shi^{ts1}* cultures following the 30°C treatment, neurite initiation was greatly suppressed and retraction of neurites was seen in both mono- and multipolar cells. In normal cultures, cells showed continuous neurite outgrowth throughout the 3 d period.

varies with different degrees of differentiation, we traced the responses of neurons with respect to the initial neurite length. Cells were divided into 3 categories according to the initial length of their neurites (<10 μ m, 10–20 μ m, and >20 μ m). The distributions of initial neurite lengths in normal and *shi^{ts1}* neurons were similar (Fig. 7, inset). In both normal and untreated *shi^{ts1}* cultures, less than a third of cells in each category reabsorbed their neurites during 24 hr of incubation (Fig. 7). In contrast, *shi^{ts1}* cells incubated at 30°C showed a significantly higher percentage (~70%) of neurite reabsorption, regardless of the initial neurite length (Fig. 7). The remaining *shi^{ts1}* cells which did not completely reabsorb their neurite invariably showed a decrease in neurite length (see below). Therefore, the temperature-induced neurite reabsorption and retraction in *shi^{ts1}* neurons does not appear to depend on the extent of previous neurite outgrowth.

Neurite extension and retraction. The complete reabsorption of *shi^{ts1}* neurites may result from gradual retraction without compensating extension. This notion is consistent with the temperature effects on the length and growth rate of *shi^{ts1}* neurites. Neurite growth rate as a function of culture age is calculated from the difference in length between successive days (Fig. 8). Neurons that completely reabsorbed their neurites in response to 30°C incubation were excluded from the analysis. Although

there were fluctuations in the growth rates of neurites in all cultures, positive rates were always maintained except for *shi^{ts1}* cultures, which showed negative growth rates during exposure to 30°C (Fig. 8, *d, e*). The rate of neurite growth in *shi^{ts1}* neurons showed a rebound and was even accelerated upon returning to a permissive temperature (Fig. 8, compare *d* to *a* and *e* to *b*). Most strikingly, the highest neuritic growth rate (30.82 μ m/d) among all cultures occurred during the recovery period in the *shi^{ts1}* cultures exposed to 30°C in the first day of incubation (Fig. 8*d*).

Arborization of *shi^{ts1}* neurons. Nerve cells in more than 30 different cultures were observed 1 d after seeding. SEM was employed to resolve fine branches of their arbors. In *shi^{ts1}* and normal cultures kept at 22°C and in normal cultures exposed to 30°C, the degree of neurite branching was similar. However, neurites of *shi^{ts1}* neurons exposed to 30°C for 8 hr prior to fixation had fewer branches (Fig. 9). The histograms in Figure 10 further quantify the differences in branching patterns. The most prominent features of *shi^{ts1}* neurons following this temperature treatment were a complete absence of tertiary neurite branches and a higher percentage (about 65%, as compared to about 15% in control cultures) of cells having only primary neurites. The tendency of neurites to branch was further quantified in terms of the number of secondary neurites in every 50 μ m of primary

Figure 4. Examples of neuronal growth in normal and *shi^{ts1}* cultures with different temperature treatments. A decrease in neurite extension was detected in *shi^{ts1}* cells during the second day of incubation at 30°C (c). No suppression in neurite extension was observed in normal cultures under the same condition (a). Without high-temperature treatment, continuous growth was maintained throughout the 3 d period in both normal (b) and *shi^{ts1}* (d) cultures.

neurites and the number of tertiary neurites in 50 μm of secondary neurites (Table 1). It can be seen that in *shi^{ts1}* cultures exposed to 30°C, primary neurites branched fewer times and secondary neurites did not branch at all.

Growth cone of *shi^{ts1}* neurons

Growth cones play an important role in neurite extension and branching. For instance, growth cones with more microspikes (Haydon et al., 1985) and expansive lamellipodia (Argiro et al., 1984) sustain a greater rate of neurite elongation. Thus, the suppression in arborization (Fig. 9, Table 1) and negative growth rates (Fig. 8, d, e) of *shi^{ts1}* neurites at 30°C may be derived from defects in the growth cone.

Growth cone activity of *shi^{ts1}* neurons in 1-d-old culture was observed with phase-contrast microscopy. At 22°C, *shi^{ts1}* and normal growth cones were similar (see Fig. 1). About 30% of neurons had growth cones with filopodia and lamellipodia which could be positively resolved with the Nikon phase-contrast optics. [It was later confirmed with the video contrast-enhancement system at high magnifications that, at any given time, only about 50% of neurons in culture were in their active phase, displaying growth cones (see Materials and Methods).] After exposure to 30°C for 4 hr, the frequency of neurons with distinct growth cones remained the same in normal cultures but was reduced to less than half in *shi^{ts1}* cultures (see Table 2).

Growth cone morphology was further examined with SEM (Fig. 11). After the fixation and dehydration procedures, about 30% of the growth cones detectable with phase-contrast microscopy in the live preparation retained their morphology. Growth cones in normal cultures kept at 22 or 30°C, as well as *shi^{ts1}* cultures grown at 22°C, showed many filopodia, often (~50%) associated with veil-like lamellipodia (Fig. 11). In *shi^{ts1}* cultures incubated at 30°C for 4 hr, very few growth cone-like structures were detected at the tips of neurites (<20% of those

of control cultures). No growth cones exhibited lamellipodia, and only filopodia were occasionally observed (Fig. 11d).

Adhesive properties of *shi^{ts1}* neurons

Attachment of the growth cone and portions of the cell body and neurites to the substratum critically influences the neuronal development. Inadequate adhesion results in retarded advancement of growth cones and the retraction of neurites (Letourneau, 1975, 1985; Johnston and Wessells, 1980; Bray, 1982).

We found that the adhesion of *shi^{ts1}* cells to the substratum depended on temperature. Dissociated neurons in suspension were incubated at 30°C for 30 min prior to plating. After plating, the culture was further maintained at 30°C for an additional 2 hr and the cell adhesion efficiency was determined by scoring the density of cells with firmly attached somata. Unattached or incompletely attached cell bodies assumed a more spherical shape and vibrated upon gentle tapping on the microscope. At

Table 1. Degree of neurite branching in normal and *shi^{ts1}* neurons

Group	Temperature (°C)	Cells examined (n)	Secondary neurites (n) 50 μm primary neurite	Tertiary neurites (n) 50 μm secondary neurite
Normal	22	20	3.14	2.38
	30	20	3.31	1.58
<i>shi^{ts1}</i>	22	18	4.58	2.28
	30	33	1.35	0

The average number of secondary neurites in every 50 μm of primary neurites and the average number of tertiary neurites in the same length of secondary neurites were measured from scanning electron micrographs.

Figure 5. Effects of temperature shifts on neurite initiation. Open histograms represent data from normal neurons and stippled histograms from *shi^{ts1}* neurons in this and the following figures. Neurons initially devoid of neurites that subsequently began neurite outgrowth were counted. Data include all neurons of this category in the fixed areas examined. Upshifts in temperature decreased the proportion of cells that initiated neurite outgrowth in *shi^{ts1}* (*d, e*) but not in normal cultures (*a, b*). Data for control cultures are shown in *c* and *f*. Note that after returning from high temperature there was a rebound in neurite initiation in *shi^{ts1}* cells (*d, e*). Data were pooled from 4 normal and 5 *shi^{ts1}* cultures. Each histogram was constructed from 126–252 cells.

30°C, the adhesion efficiency of *shi^{ts1}* cells was greatly reduced, to one-third of that at 22°C (Table 3). Correspondingly, there was an increase in the number of cells that were not firmly attached to the substratum (data not shown). In contrast, the adhesion efficiency of normal cells was even increased at 30°C (1.31 times of that at 22°C; see Table 3).

It should be noted that, in addition to the cell body, *shi^{ts1}*

Figure 6. Effects of high temperature on neurite reabsorption. The percentage of cells that completely reabsorbed their neurites were scored on 3 consecutive days. Exposure to high temperature increased the proportion of cells with neurite reabsorption in *shi^{ts1}* cultures (*d, e*) but not in normal cultures (*a, b*). Results of control cultures are shown in *c* and *f*. Data were pooled from 4 normal and 5 *shi^{ts1}* cultures. Each histogram was based on a sample of 203–386 cells.

neurites also showed poor adhesion to the substratum at 30°C. At 2 hr after plating, neurites of some cells were not attached to the substratum even though their cell bodies were well anchored. At 30°C, more than 70% of attached cells in *shi^{ts1}* cultures displayed unanchored neurites as compared with less than 20% of cells in normal cultures.

Therefore, these experiments demonstrated that the *shi^{ts1}* mutation affects neuronal membrane adhesion properties. Regulation of this basic membrane property may be associated with the defective membrane fusion and retrieval mechanisms previously reported.

Table 2. Frequencies of neurons exhibiting distinct growth cones in normal and *shi^{ts1}* cultures

Group	Temperature (°C)	Number of experiments	Total cells examined (<i>n</i>)	Total cells with growth cones (<i>n</i>)	Frequency (%)
Normal	22	3	245	74	29.49 ± 5.52
	30		222	70	30.86 ± 6.83
<i>shi^{ts1}</i>	22	3	311	101	32.16 ± 6.65
	30		270	36	13.53 ± 4.06

Errors in the frequencies are SDs derived from 3 independent experiments, each including 56–125 cells. Growth cones were detected with the phase-contrast optics (Nikon Diaphot microscope, 40×, 0.55 N.A. objective). For each experiment, the cell suspension was equally divided and plated on uncoated coverslips and was grown for 1 d at 22°C. Afterwards, experimental cultures were incubated at 30°C for 4 hr prior to observation. See text for explanation.

Figure 7. Frequency of neurite reabsorption as a function of initial neurite lengths. Only the longest primary neurites were scored and cells were classified by their lengths. The initial distributions of neurite lengths in normal and *shi^{tsl}* neurons were similar immediately after plating (inset). The percentages of cells that completely reabsorbed all neurites following 1 d incubation at 22 or 30°C are shown in the lower histograms. Note that a large proportion (~70%) of cells in *shi^{tsl}* cultures exposed to 30°C reabsorbed their neurites regardless of the initial neuritic lengths. In comparison, in normal cultures, as well as the untreated *shi^{tsl}* cultures, only 15–35% of cells reabsorbed their neurites in the same periods. For each histogram, data were pooled from 574–933 cells.

Discussion

Morphology of *shi^{tsl}* neurons in culture

As reviewed in the introduction, manifestations of a defective membrane recycling mechanism in various *shi^{tsl}* cell types include (1) the depletion of intracellular vesicles coupled with an increase in cisternae-like structures, possibly due to vesicle fusion, and (2) an increase in pitlike structures and fingerlike invaginations on the plasma membrane, presumably associated with a disrupted membrane pinch-off mechanism (Kosaka and Ikeda, 1983a, b). As a result, endocytosis and synaptic transmission are blocked and the differentiation of nerve, muscle and epithelial structures is disrupted.

Our study was intended to examine isolated *shi^{tsl}* neurons in culture and to analyze the possible relationship between the membrane recycling mechanism and neuronal development. We found striking changes in growth cone formation and a weakened cell adhesion in these neurons at 30°C. Our results provide a new line of evidence supporting the view that growth cone activities are closely associated with neurite extension and branching (Letourneau, 1975; Johnston and Wessells, 1980).

Profound alterations in the morphological development of *shi^{tsl}* neurons that were induced by high-temperature treatment include (1) reversible suppression of neurite initiation, (2) grad-

Figure 8. Changes in growth rate in response to temperature treatments. The longest primary neurites were identified and followed in 3 successive days. Growth rate is shown as the change in neurite length per day. Positive growth rates were always maintained in normal cultures regardless of temperature (a–c). The same was true for *shi^{tsl}* cultures maintained at room temperature (f). Negative growth rate, reflecting neurite retraction over initiation and elongation, were detected only in *shi^{tsl}* cultures during incubation at 30°C (d and e). Note the rebound in growth rate upon returning to 22°C (d and e). Neurons that degenerated during the course of observation were excluded from the analysis. Error bars indicate SEM based on 26–70 cells.

ual retraction of neurites reflected by a negative growth rate, (3) abrupt neurite reabsorption, and (4) inhibition of arborization. Analysis of each of these components of neurite outgrowth may provide important clues to the relationships between the highly dynamic process of membrane recycling and the generation and maintenance of neuronal forms.

Growth cone formation

The growth cone consists of a protoplasmic enlargement at the tip of growing neurites that contains mostly membranous organelles and cytoskeletal elements and of thin peripheral regions that are composed of motile filopodia and lamellipodia (Ya-

Table 3. Temperature effects on cell adhesion as determined by plating efficiency

Group	Temperature (°C)	Number of experiments	Total cells attached (n)	Plating efficiency at 30°C normalized to 22°C
Normal	22	5	1218	1.31 ± 0.23
	30		1521	
<i>shi^{tsl}</i>	22	5	1656	0.34 ± 0.03
	30		537	

For each experiment, the dissociated cell suspension was equally divided for incubation at 22 or 30°C for 30 min prior to plating onto poly(L-lysine)-coated coverslips. For each culture, the number of cells attached on the substratum in 1.452 mm² area was scored. Plating efficiency at 30°C is normalized to that of 22°C, i.e., No. of cells attached at 30°C/No. of cells attached at 22°C. Errors represent SD of the normalized values. Student's *t* test indicates that the 30°C treatment significantly decreased the plating efficiency of *shi^{tsl}* cells ($p < 0.001$) but significantly increased that of normal cells ($p < 0.05$).

Figure 9. Scanning electron micrographs showing neurite branching. *a*, Normal neuron at 22°C. *b*, Normal neuron at 30°C. *c*, *shi^{ts1}* neuron at 22°C. *d*, *shi^{ts1}* neuron at 30°C. Scale bar, 10 μ m.

Figure 10. Temperature effects on neurite branching. Arborization of individual normal and *shi^{ts1}* neurons in scanning electron micrographs were analyzed. Histograms represent the distribution of neurons with the primary (1°), secondary (2°), and tertiary or higher ($\geq 3^\circ$) neurites as the highest degree of branching. No difference in neurite arborization was detected between normal and *shi^{ts1}* neurons at 22°C. The same pattern of neurite branching was seen in normal cultures at 30°C. Note the complete absence of tertiary branches and a higher percentage of cells with exclusively primary neurites in *shi^{ts1}* culture at 30°C. Data were collected from 34–71 single neurons.

mada et al., 1971; Landis, 1983). Although there has been a demonstration of neurite elongation on sufficiently adhesive substratum without filopodia or lamellipodia (Marsh and Letourneau, 1984), it is generally accepted that under normal conditions projection and retraction of growth cones lead to elongation (Yamada et al., 1971; Johnston and Wessells, 1980; Letourneau, 1985) and branching (Bray, 1973a) of neurites.

As previously reported, the marker particles on the surface of growing axons (Bray, 1970) and branch points of neurons (Bray, 1973a) in culture remained at a fixed distance from the cell body as the growth cone progressed a substantial distance. It is thought that membrane incorporation and retrieval occur mainly at the growth cone rather than on the existing neurites (Bray, 1970, 1973b; Landis, 1983). The leading edge of growth cones have been shown to be much more active than axons in the process of endocytosis (Bray, 1973b; Bunge, 1977) and in the assembly of microtubules (Bamburg et al., 1986). Microtubules constitute a major longitudinal structure of axons and are thought to be responsible for membrane vesicle transport.

High-temperature treatments of *shi^{ts1}* neurons induced striking alterations in growth cone formation before changes in the branching pattern of existing neurites could be detected. Although there have been no direct ultrastructural observations on membrane recycling in *shi^{ts1}* growth cones, it is likely that the same membrane defect observed in a wide variety of cell types and in other regions of neurons (see introduction) also exists in the mutant growth cone. Therefore, our results are consistent with the hypothesis raised by previous studies in other species that the growth cone is very actively engaged in membrane incorporation and retrieval to regulate its plasma membrane area.

Membrane adhesion properties

From previous studies, it is clear that growth cone morphology critically depends on the adhesion of lamellipodia and filopodia to the substratum (Letourneau, 1975, 1985). In principle, besides the change in the membrane surface area, active growth cones require constant shifting of adhesion sites in contact with

Figure 11. Growth cones of cultured neurons in SEM. *a*, Normal at 22°C. *b*, Normal at 30°C. *c*, *shi^{ts1}* at 22°C. *d*, *shi^{ts1}* at 30°C.

the substratum. As the lamellipodia ruffle and filopodia extend and retract, new adhesive sites in some areas must be established, while old sites in other areas must be broken. This process presumably involves redistribution within the growth cone membrane of the molecular components responsible for adhesion. The redistribution can be achieved by insertion and pinch-off of membrane vesicles or by lateral movements of membrane adhesion components.

As described in Results, *shi^{ts1}* cell bodies (Table 3) and neurites exhibited poor adhesion to the substratum. Apparently, the weakened adhesion is widespread in different neuronal regions, such as adhesive sites along neurites and within growth cones. Our observations on *shi^{ts1}* growth cones raise the possibility that a membrane recycling mechanism is involved in the incorporation and retrieval of membrane molecular components responsible for growth cone adhesion. Within 4 hr of exposure to 30°C, the number of growth cones in *shi^{ts1}* cultures dramatically decreased (Table 2). With the SEM, the remaining growth cones showed poor morphology, lacking any lamellipodia; this is an indication of weakened adhesion to the substratum. In contrast, growth cones in control cultures withstood the fixation and dehydration procedures, displaying extended filopodia and lamellipodia (Fig. 11, *a-c*). At high temperature, *shi^{ts1}* growth cones did not remain static and retained a normal appearance but showed signs of disintegration (Fig. 11*d* and unpublished results from video contrast-enhancement observations). This

indicates that the membrane defect in *shi^{ts1}* neurons does not simply stop the regulation of surface area but also affects its adhesive properties.

Mechanisms of altered membrane recycling in shi^{ts1} cells

The molecular mechanism of the *shi^{ts1}* defect still remains unknown, and many possibilities exist to explain the membrane recycling block in *shi^{ts1}* cells. According to our results, however, a simple block in the synthesis of membrane components is not a likely explanation. The observations of a rebound in neurite initiation (Fig. 5) and a higher than normal rate of neurite growth in *shi^{ts1}* cultures upon returning to permissive temperature (Fig. 8*d*) suggest that neither the total membrane pool nor the primary process of membrane synthesis was altered. The rebound in growth rate might have resulted from the blockage in the incorporation of newly synthesized membrane components into the growing tip during 30°C incubation.

Several other mechanisms that can account for the *shi^{ts1}* defects still remain to be fully investigated. (1) Integration of intracellular membrane, possibly vesicles, into plasma membrane is blocked at 30°C. (2) Membrane integration and fusion with the plasma membrane are not affected, but the inserted vesicle membrane fails to expand. (3) A block occurs in the pinch-off mechanism of the plasma membrane and internal tubular membrane. (4) Alternatively, the *shi^{ts1}* gene product could be important to a common mechanism underlying all of the above

processes. (5) Associated with these possibilities, a defect in the regulation of cytoskeletal elements must be considered.

Certain cytoskeletal components are thought to be responsible for the initiation, extension, and branching of neurites as well as the movements of growth cones (Letourneau and Ressler, 1984; Marsh and Letourneau, 1984; Bunge, 1986; Letourneau et al., 1986). For example, treatment of neurons with cytochalasin B, which disrupts microfilament function, causes microspikes to retract and growth cones to round up (Yamada et al., 1971; Marsh and Letourneau, 1984; for a review, see Landis, 1983). In addition, it is known that microtubules propel membrane vesicles from their site of synthesis in the cell body to the growth cone (Bray, 1973b). Microtubules are also required for the maintenance of neurites (Letourneau, 1985; Bunge, 1986). Other cytoskeleton-associated proteins such as vinculin (Sefton et al., 1981), large complexes called SAMs (Edelman, 1984), and membrane surface components like adherons (Schubert and LaCorbiere, 1985) have also been implicated in cell adhesion mechanisms.

Biochemical studies of *shi^{ts1}* nerve and muscle proteins have been initiated. So far, our preliminary results of SDS gel electrophoresis have indicated no qualitative changes of the major cytoskeletal proteins. In 2-dimensional gel analysis, no changes in migration rate have been detected in α -actinin, actin, myosin, α - and β -tubulin, and tropomyosin (J.J.-C. Lin, Y.-T. Kim, and C.-F. Wu, unpublished observations). However, we cannot exclude the possibility that there is a subtle change in these proteins that is not readily detectable in SDS gel electrophoresis or that the *shi^{ts1}* defect is related to a minor regulatory protein.

Intracellular free Ca^{2+} is thought to be important in regulating membrane fusion and cytoskeletal elements. Ca^{2+} action potentials supported by a high density of Ca^{2+} channels in growth cones have been demonstrated in cultured neurons (Anglister et al., 1982). Recently, it was suggested that Ca^{2+} influx induces retraction of growth cones (Cohan et al., 1986). Therefore, a possibility exists that intracellular free Ca^{2+} concentration in *shi^{ts1}* may be increased at the restrictive temperature. Nevertheless, Ca^{2+} influx in both adult and larval *shi^{ts1}* muscles are not apparently altered by high temperature since their Ca^{2+} action potentials appear normal (Ikeda et al., 1976; C.-F. Wu, unpublished observations). Furthermore, the muscle membrane excitability in *shi^{ts1}* is not altered at the restrictive temperature. Therefore, an increase in intracellular free Ca^{2+} due to release from internal storage is also not likely, otherwise it would increase the Ca^{2+} -dependent K^{+} current which can suppress the generation of action potentials in muscle (Elkins et al., 1986; Singh et al., 1986).

These hypothetical mechanisms of the *shi^{ts1}* defect should be investigated by using additional techniques such as immunocytochemistry and ultrastructural examinations. Further studies of the *shi^{ts1}* gene product will not only provide insights into how membrane fusion, adhesion, and endocytosis regulate neuronal morphology but may also extend our knowledge of these important cellular processes.

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