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Temperature Modulation Reveals Three Distinct Stages of Wallerian Degeneration

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After peripheral nerve transection, axons distal to the cut site rapidly degenerate, a process termed Wallerian degeneration. In wild-type mice the compound action potential (CAP) disappears by 3 d. Previous studies have demonstrated that cold temperatures and lower extracellular calcium ion (Ca^{2+}) concentrations can slow the rate of Wallerian degeneration. We have incubated isolated sciatic nerve segments from wild-type and C57BL/Wld mice (which carry a gene slowing Wallerian degeneration) *in vitro* at 25 and 37°C. At 25°C we found that the degeneration rate of wild-type axons was slowed dramatically, with the CAP preserved up to 7 d post-transection. In contrast, at 37°C the CAPs were minimal at 2 d. When the temperature of wild-type nerves was raised to 37°C after 24–72 hr at 25°C, degeneration occurred within the subsequent 24 hr. Wld nerves, too, were preserved longer at 25°C but, on return to 37°C, degenerated promptly. Cooling the nerve within 12 hr after axotomy enhanced axonal preservation. Neither wild-type nor Wld nerves showed different degeneration rates when they were incubated with 250 μM or 5 or 10 mM extracellular Ca^{2+} for 1–2 d, suggesting that an abrupt increase in intracellular Ca^{2+} occurs at the time of axonal destruction. Wallerian degeneration, thus, appears to progress through three distinct stages. Initiation occurs at the time of injury with subsequent temperature-dependent and -independent phases. Nerves appear to remain intact and are able to exclude Ca^{2+} from entering until an as yet unknown process finally increases axolemmal permeability.

Key words: Wallerian degeneration; temperature; C57BL/Wld mice; axonal degeneration; calcium; in vitro

Transection of a peripheral nerve initiates the processes of Wallerian degeneration. In giant squid, crayfish, and fish the isolated axons may be preserved for months, with proteins continuing to be transported in the axoplasm and supplied by surrounding glia (Sheller and Bittner, 1992; Tanner et al., 1995a,b; Raabe et al., 1996). In contrast, injured axons in rodents degenerate within 3 d, and the nerve consequently becomes unable to conduct a compound action potential (CAP) on application of an external electrical stimulus (Luttges et al., 1976). For a CAP to be conducted, the plasma membrane and the transmembrane voltage-gated ion channels must remain intact, and the normal intracellular ion levels must be preserved. The existence of the C57BL/Wld (formerly known as the C57BL/Ola) mouse strain, carrying an autosomal dominant mutation (Wld^s) slowing the rate of Wallerian degeneration (CAPs can be conducted up to 3 weeks post-transection), demonstrated for the first time that rapid degeneration in mammals is not a necessary consequence of axotomy (Lunn et al., 1989; Perry et al., 1990a; Tsao et al., 1994). It is also clear that Wallerian degeneration is an active process, with an initiating mechanism that sets into motion a cascade of events leading to the final destruction of axons. The precise sequence of events that leads to axonal destruction is not known.

Experiments on Wallerian degeneration in mammalian, lower vertebrate, and invertebrate axons have all demonstrated that cold temperatures can slow the rate of progression (Gamble et al., 1957; Gamble and Jha, 1958; Usherwood et al., 1968; Wang, 1985; Bittner, 1988; Sea et al., 1995). This effect has been attributed variously to an alteration in the activity of degradative enzymes (changing the Q_{10} by threefold) or the rate of axoplasmic transport (Cancalon, 1982, 1985). Additionally, researchers have demonstrated a link between the intracellular and extracellular calcium ion concentration ($[Ca^{2+}]_{i/o}$) and the time course of axonal sealing and degeneration (Schlaepfer and Bunge, 1973; Schlaepfer, 1974, 1977; Krause et al., 1994; Fern et al., 1995; George et al., 1995; Eddleman et al., 1997, 1998). Axoplasmic Ca^{2+} concentrations of 100 μM induce vesicle-mediated sealing, although a higher $[Ca^{2+}]_i$ may be needed for the deleterious effects (Krause et al., 1994; Eddleman et al., 1997, 1998). The experiments of Schlaepfer and Bunge (1973), Glass et al. (1994), and George et al. (1995) suggest that degeneration proceeds only when $[Ca^{2+}]_o$ is >300 μM.

In wild-type axons axonal inability to continue conducting CAPs occurs simultaneously with axonal destruction (McDonald, 1972). In Wld mice the first electrophysiologically detectable event in Wallerian degeneration is axonal inability to continue conducting CAPs although the morphology remains relatively preserved (Tsao et al., 1994). Via monitoring the disappearance of CAPs and axonal morphology, this study also demonstrated that axonal degeneration had similar rates *in vivo* and *in vitro*. Using an *in vitro* system (described in Tsao et al., 1994), we have investigated the effect of temperature and media $[Ca^{2+}]$ on the time course of axonal degeneration (duration of prolonged CAP

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and, in some experiments, the rate of decline). We asked whether axonal loss in mouse nerves could be delayed by cooling the external media temperature to 25°C and, if so, when, after injury, cooling had to be initiated to prolong axonal survival. Finally, by varying $[Ca^{2+}]_o$, we addressed the question of whether intra-axonal Ca^{2+} increases gradually after axotomy or whether there is an abrupt transition from low $[Ca^{2+}]_i$ concentrations to high concentrations, accompanied by loss of the CAP and axonal cytoskeleton.

Parts of this paper have been published previously (Tsao and George, 1996; Tsao et al., 1997).

MATERIALS AND METHODS

Sciatic nerve preparation. C57BL/Wld mice were bred in the Johns Hopkins Hospital (Baltimore, MD) from mice originally supplied by Harlan-Olac (Bicester, UK). C57BL/6N mice (control, wild-type) were supplied by Harlan Sprague Dawley (Indianapolis, IN). Twelve-week-old mice were used in the experiments and were killed by cervical dislocation. The sciatic nerves were removed and, for the Ca^{2+} concentration experiments, desheathed with a microsurgical knife (Roboz Surgical Instrument, Rockville, MD) and then placed into 1 ml of serum-free Optimem I medium (Life Technologies, Gaithersburg, MD) or DME/F-12 medium (Sigma, St. Louis, MO) supplemented with $CaCl_2$ to give a Ca^{2+} concentration of 250 μM or 5 or 10 mM. Both media contained penicillin and streptomycin (Life Technologies), each at a concentration of 5 IU/ml. Media were gassed with 95% O_2/5% CO_2 to give a pH of 7.4 at 25 and 37°C in a humidified incubator and were changed every 3 d. All animals were cared for under the guidelines issued by Johns Hopkins University (Baltimore, MD).

To investigate the effect of temperature on the rate of Wallerian degeneration, we cultured one nerve from each mouse (Wld and 6N) at 25°C, with the contralateral nerve cultured at 37°C. To determine whether cooling nerves for a period of time at 25°C could slow or halt Wallerian degeneration subsequently at 37°C, we incubated wild-type nerves at 25°C for 24, 48, and 72 hr, followed by 24 hr at 37°C. Conversely, the time when degeneration could no longer be slowed by cooling was determined by incubating wild-type nerves for 3, 6, 9, 12, 14, 16, 18, and 24 hr at 37°C, followed by incubation at 25°C, for a total of 48 hr *in vitro*.

To examine the role of extracellular (media) Ca^{2+} concentrations on the degeneration rate, we incubated wild-type and Wld nerves for 48 hr in 250 μM or 5 or 10 mM Ca^{2+}. Other nerves were incubated first for 24 hr in 5 or 10 mM Ca^{2+}, followed by 24 hr in 250 μM Ca^{2+}, or for 24 hr in 250 μM Ca^{2+}, followed by 24 hr in 5 or 10 mM Ca^{2+}. A further permutation was introduced by using two temperatures, 25 and 37°C.

Electrophysiology and tissue examination. Between 1 and 11 d, both A (rapidly conducting, large myelinated fiber) and C (slowly conducting, small unmyelinated fiber) waves of the CAP were measured by placing the sciatic nerves in Ringer's solution [containing (in mM) 135 NaCl, 2.5 KCl, 10 D-glucose, 1 $MgCl_2$, 2.5 $CaCl_2$, and 6 HEPES, pH 7.4] at room temperature. The proximal and distal ends were placed onto bipolar silver electrodes (proximal end to the stimulating electrode and distal end to the recording electrode) while the middle of the nerve remained immersed in Ringer's solution. An inter-electrode distance of 0.5–1 cm was established so that the stimulating electrode did not interfere with the recorded signal. Adipose tissue adherent to the nerve at either the stimulating or recording end was found to decrease the CAP intensity; thus, care was taken to dissect the nerve free of such tissue.

Stimulating pulses of 200 μsec duration were delivered at 1 sec intervals via an optical isolation unit (Isoflex, A.M.P.I., Jerusalem, Israel), and the maximum fast monophasic A wave and the slower C wave were recorded. Using previously described methods (Perry et al., 1990b, 1992), we analyzed the maximal peak height and peak area of both A and C waves with the Sigavg Program (Cambridge Electronic Design, Cambridge, UK) after digitization (CED 1401) at 80 kHz and found them to be equivalent (Tsao et al., 1994). Selected nerves were fixed in 2% paraformaldehyde, osmicated for 2 hr, infiltrated with glycerol, and then teased apart and examined by light microscopy.

Statistical analysis. Data were analyzed with the Statview 4.0 program (Abacus Concepts, Berkeley, CA). Significance ($p < 0.05$) was assessed by using either ANOVA, followed by the Student's t test if ANOVA showed significance, or the paired t test.

RESULTS

Axonal degeneration *in vivo* and *in vitro*

We confirmed that axonal degeneration *in vivo* and *in vitro* in 6N nerves was comparable, with ~2.5% of the CAP (relative to control) remaining at 2 d, as previously reported (Tsao et al., 1994). The act of axotomy and removing the nerves for culturing was found to reduce the CAP A wave by ~30% within 3 hr, after which the nerve was stable for the next 21 hr (Fig. 1*A,B*). *In vivo* the CAP after axotomy was found to remain constant for the first 24 hr, actually rising in the first 6 hr (Fig. 2*A,B*). In all, 80% of the CAP was lost between 24 and 36 hr. A similar time course was found *in vitro* (Fig. 2*A,B*).

The effect of temperature changes on the rate of axonal degeneration

Nerves from 6N mice maintained at 25°C showed a prolonged presence of CAPs (A and C waves) (Fig. 3*A,B*). Light microscopic examination of teased fibers confirmed that myelinated axons remained intact (data not shown). The incubation of nerves at 25°C for 1–3 d, followed by 37°C for 1 d, demonstrated that previous cooling could not prevent the subsequent decrease in the CAP (Fig. 4*A,B*), because at the end of this additional 24 hr only small CAPs could be detected. The CAP at intermediate time points after the return to 37°C was not significantly different from that seen in nerves incubated continuously at 37°C.

If they were cooled to 25°C within 12 hr, 6N nerves that were incubated initially at 37°C had complete and prolonged preservation of the CAP A and C waves (mimicking the rate seen with continuous incubation at 25°C) (Fig. 5*A,B*). If the nerves were cooled after this time had elapsed, the CAP amplitude was found to be diminished at 48 hr.

Wld nerves also showed a delay in the degeneration rate on cooling to 25°C (Fig. 6*A,B*). On returning the nerves to 37°C after 3–4 d at 25°C, the CAP also returned to values not significantly different from those nerves incubated for the same length of time at 37°C (data not shown), similar to the effect seen in wild-type nerves. At 37°C *in vitro* the nerve survival was prolonged to 9 d, further extending previous data (Perry et al., 1990b; Tsao et al., 1994).

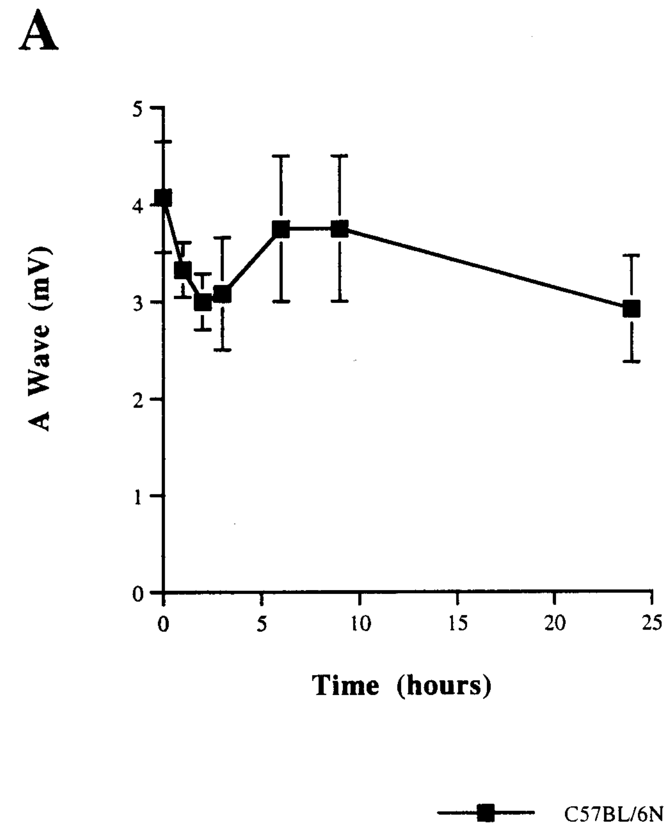
The effect of extracellular calcium ion concentration on the rate of Wallerian degeneration

Incubation of wild-type nerves in the presence of 250 μM or 5 or 10 mM external Ca^{2+} did not affect the degeneration rate (Fig. 7). Altering Ca^{2+} concentrations after 24 hr, with or without temperature adjustment, also failed to influence the rate of degeneration (data not shown). Similarly, the rate of axonal degeneration in Wld nerves was unaffected by the three Ca^{2+} concentrations (data not shown).

DISCUSSION

The effect of cooling on the rate of Wallerian degeneration

The effect of cool external temperatures slowing Wallerian degeneration *in vivo* is well known (Gamble et al., 1957; Gamble and Jha, 1958; Usherwood et al., 1968; Wang, 1985; Sea et al., 1995). In rats, Sea and colleagues (1995) showed that the time course for myelinated axons to degenerate after axotomy was 3 d at 32°C and 6 d at 23°C. The current results confirm that lowering the temperature *in vitro* can delay the progression of Wallerian degeneration (see Fig. 3*A,B*). The onset of degeneration appears to be halted in wild-type nerves in the first 1–4 d by maintaining



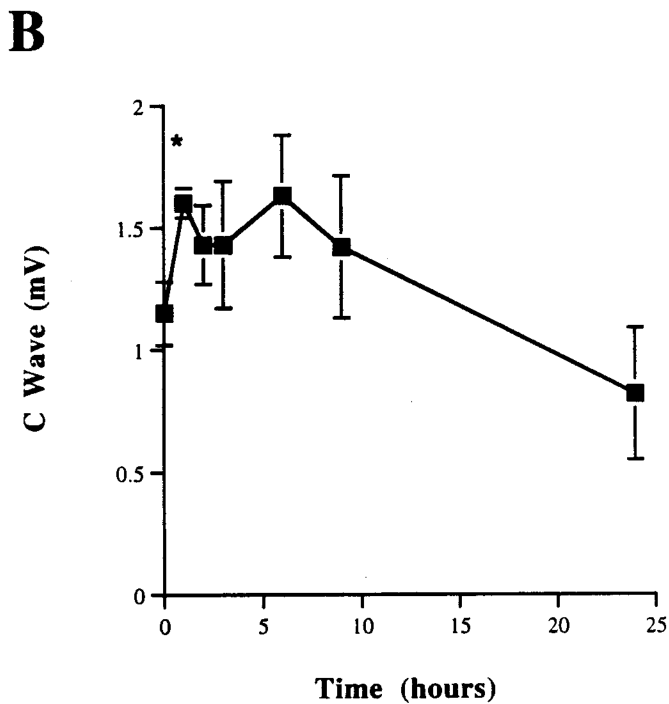
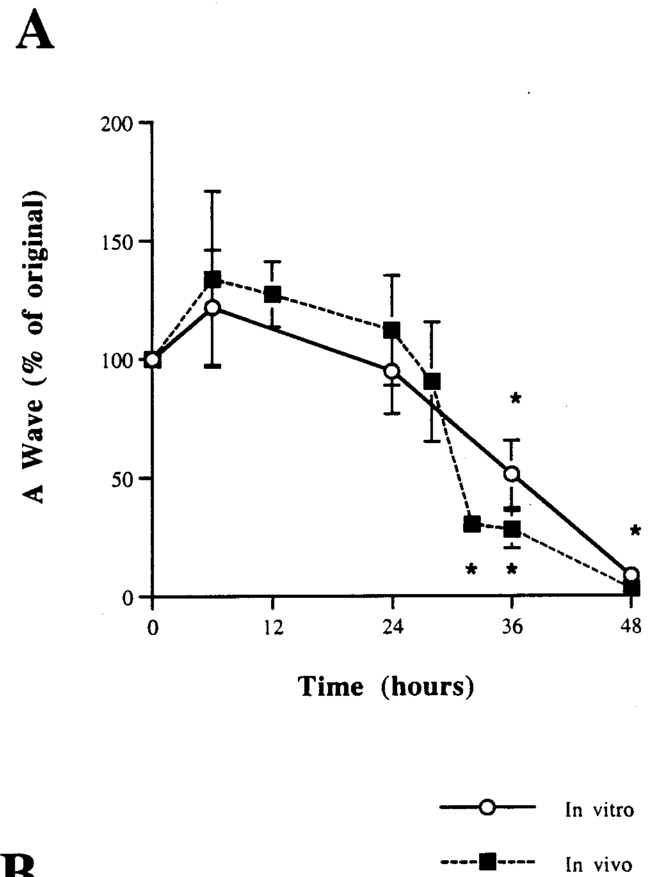


Figure 1. Compound action potential (CAP) from C57BL/6N sciatic nerves incubated for 0, 1, 2, 3, 6, 9, and 24 hr at 37°C in Optimem I medium. The CAP values demonstrate a decrease in CAP size in the first 3 hr after removal of the nerve to culture but stable values during the next 21 hr. *A*, A wave (from myelinated fibers). *B*, C wave (from unmyelinated fibers). $^*p < 0.05$, relative to time 0 hr.



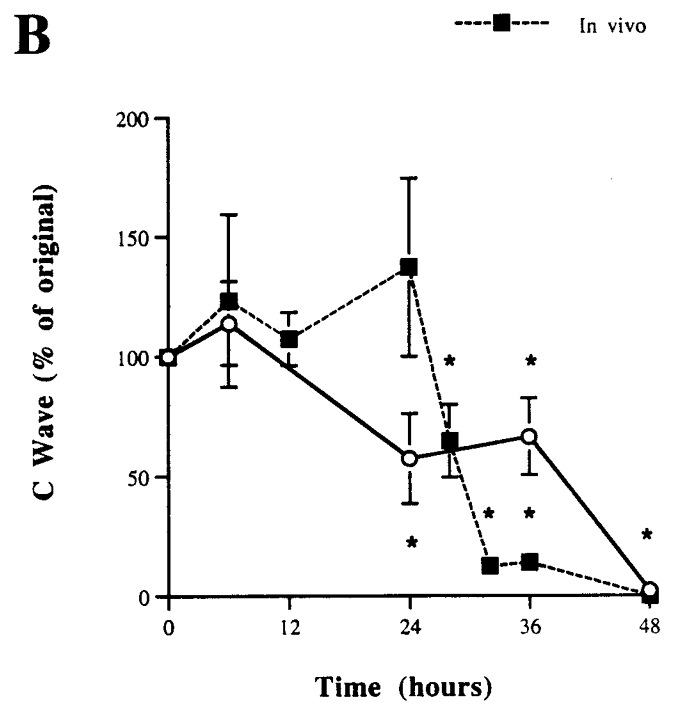


Figure 2. CAP from C57BL/6N sciatic nerves axotomized 0–48 hr *in vivo* and *in vitro*. *A*, A wave. *B*, C wave. In the first 6 hr after axotomy the CAP actually is increased. This is unlikely to be attributable to decreased temporal dispersion because the area of the waveform is increased. In both A and C waves, 80% of the CAP is lost between 24 and 36 hr. A similar time course for loss of the CAP is seen *in vivo* and *in vitro*. $^*p < 0.05$.

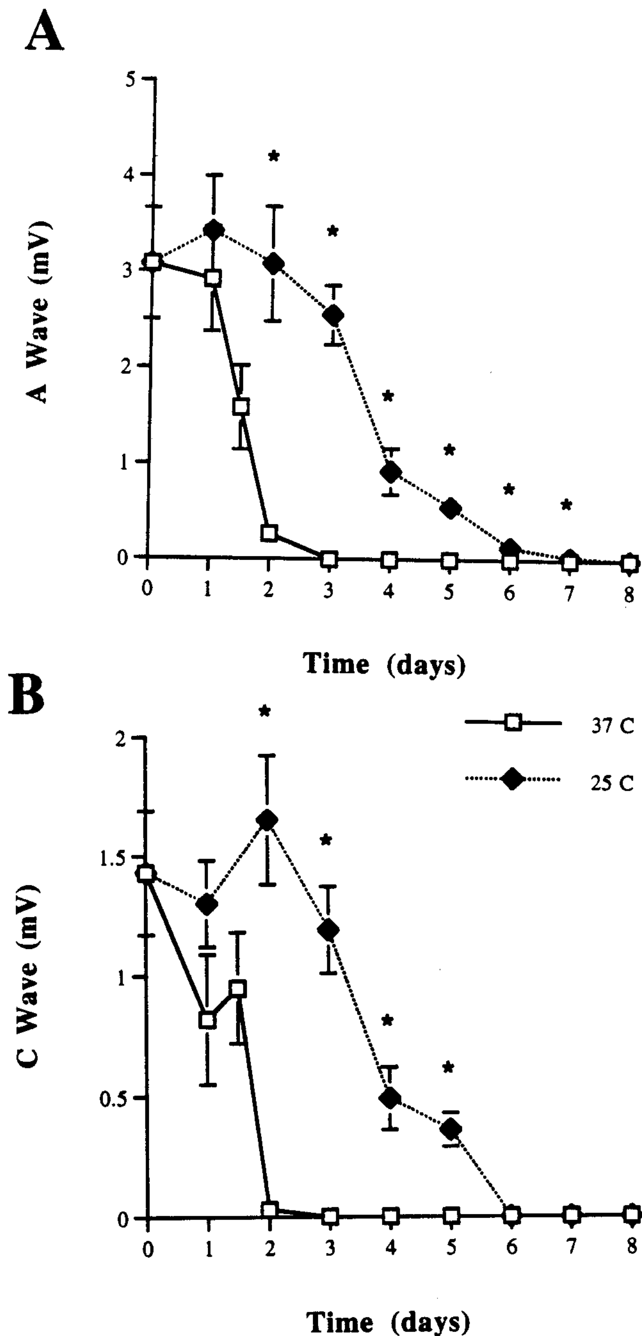


Figure 3. CAP from C57BL/6N sciatic nerves maintained at 25 and 37°C in Optimem I medium for 1–8 d. *A*, A wave. *B*, C wave. Note that the CAP disappears within 3 d at 37°C, whereas it is preserved to 7 d at 25°C. $^{*}p < 0.05$.

an external media temperature of 25°C. On raising the temperature to 37°C, however, the time to complete degeneration was not the customary 48 hr seen with a constant temperature of 37°C. Instead, loss of CAP occurred within 24 hr, with a rate similar to that seen in nerves continuously incubated at 37°C between 24 and 48 hr (see Fig. 4*A,B*). This suggests that cooling delays a "final" stage of Wallerian degeneration, but when that stage commences, it progresses rapidly to completion.

Knowing that a media temperature of 25°C would allow for the preservation of the axons and, thus, the CAP up to 7 d *in vitro*, we asked when after axotomy the cooling had to be initiated to delay Wallerian degeneration. We found that 100% of the CAP was preserved if the axotomized nerve was cooled from 37 to 25°C within the first 12 hr (all measurements were made at 48 hr for both A and C waves) (see Fig. 5*A,B*). These results suggest that there is a critical period, early after the initial injury and before the effects of Wallerian degeneration are manifest, during which activation of the sequence of events leading to axonal destruction may have begun but is not evident until later. One study supporting this hypothesis is that of Majno and Karnovsky (1958), which showed changes in nerve lipid composition within the first 6–12 hr after injury, a time when the CAP appears to be unaffected (see Fig. 2*A,B*). A change in temperature to 25°C would result in an alteration of both degradative enzymes and axonal transport along the Q_{10}'s (Cancalon, 1982, 1985), either of which might affect the subsequent degeneration rate. Alternatively, Wallerian degeneration might begin only after an initial period of time, after which the necessary degradative enzymes, such as calpains, become active.

Of note also are experiments on neural damage in head trauma that have revealed that axotomy from shear injury generally does not occur. Instead, subtle changes—an increase in axolemmal permeability, neurofilament compaction, and impairment of axoplasmic transport—appear within 1–6 hr, a time when axons still appear morphologically intact (Povlishock, 1992; Povlishock and Christman, 1995; Povlishock and Pettus, 1996; Povlishock et al., 1997). The injured axons finally begin separating from their cell bodies only after 6–12 hr. The current data are consistent with the hypothesis that changes in the first 12 hr are responsible for triggering the subsequent breakdown of the axolemma and cytoskeleton. Additionally, for the first time on the basis of these results, one could theorize that intervention during this early time might allow Wallerian degeneration to be reversed or halted completely. After 12 hr have elapsed, it appears that a second stage of Wallerian degeneration has begun, which either is temperature-independent or requires even lower temperatures to stop (see Fig. 5*A,B*).

Interestingly, Wld nerves also showed a temperature dependence in the degeneration rate (see Fig. 6*A,B*), suggesting that the Wld^{s} mutation actively acts to slow the progression of degeneration. A comparison of Figures 3 and 6 suggests that the early stages of Wallerian degeneration are prolonged similarly by cooling in both the 6N and Wld nerves. In contrast, the final stage in Wld nerves is slow and relatively unaffected by cooling, whereas the final stage of Wallerian degeneration in 6N nerves is rapid at 37°C but slowed by cooling. The data indicate that the signal initiating Wallerian degeneration is transduced in Wld axons but does not appear to propagate as rapidly as in wild-type axons.

The effect of extracellular calcium ion concentration on the rate of Wallerian degeneration

Intra- and extracellular Ca^{2+} concentrations are important in determining the rate of Wallerian degeneration and activation of degradative enzymes (Schlaepfer, 1974, 1977; George et al., 1995). Calcium ions are required both to help seal the damaged axon and to induce degeneration later. Axotomy is accompanied by the entry of calcium ions into the axoplasm, allowing the formation of membrane vesicles that help to seal the injured axon; this process requires a minimum axoplasmic Ca^{2+} concentration of 100 μM (Krause et al., 1994; Eddleman et al., 1997, 1998). An external level of <250 μM Ca^{2+} has been shown to retard degeneration of axons after axotomy (Schlaepfer and Bunge,

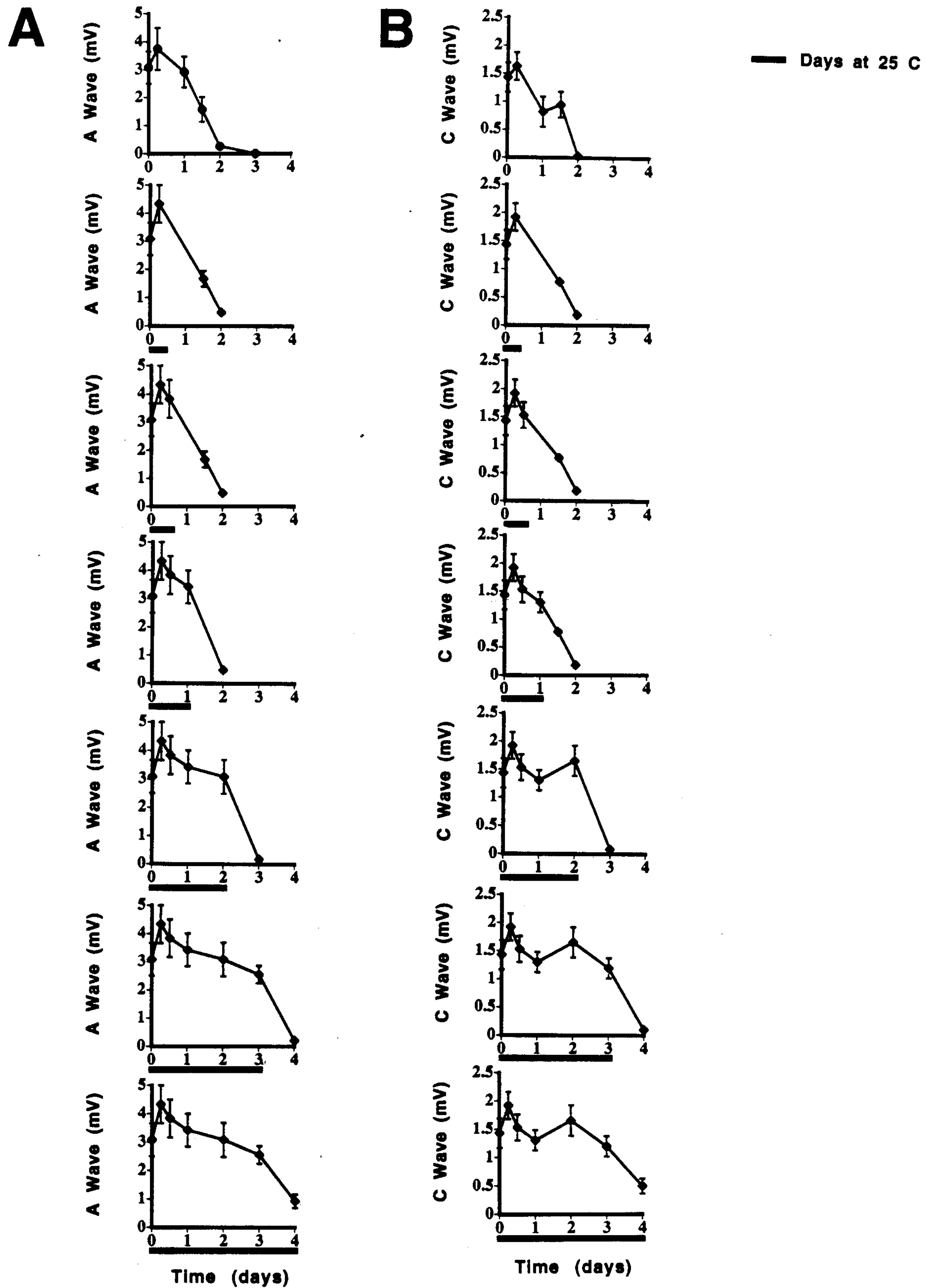


Figure 4. CAP from C57BL/6N sciatic nerves maintained at 25°C in Optimem I medium for a period from 6 hr to 3 d, followed by 1 d at 37°C. *A*, A wave. *B*, C wave. The CAP for nerves maintained for the duration of the experiment at both 25 and 37°C (from Fig. 1) is shown for comparison. Note that the CAP disappears within the subsequent 24 hr rather than the customary 48 hr seen with newly axotomized nerves.

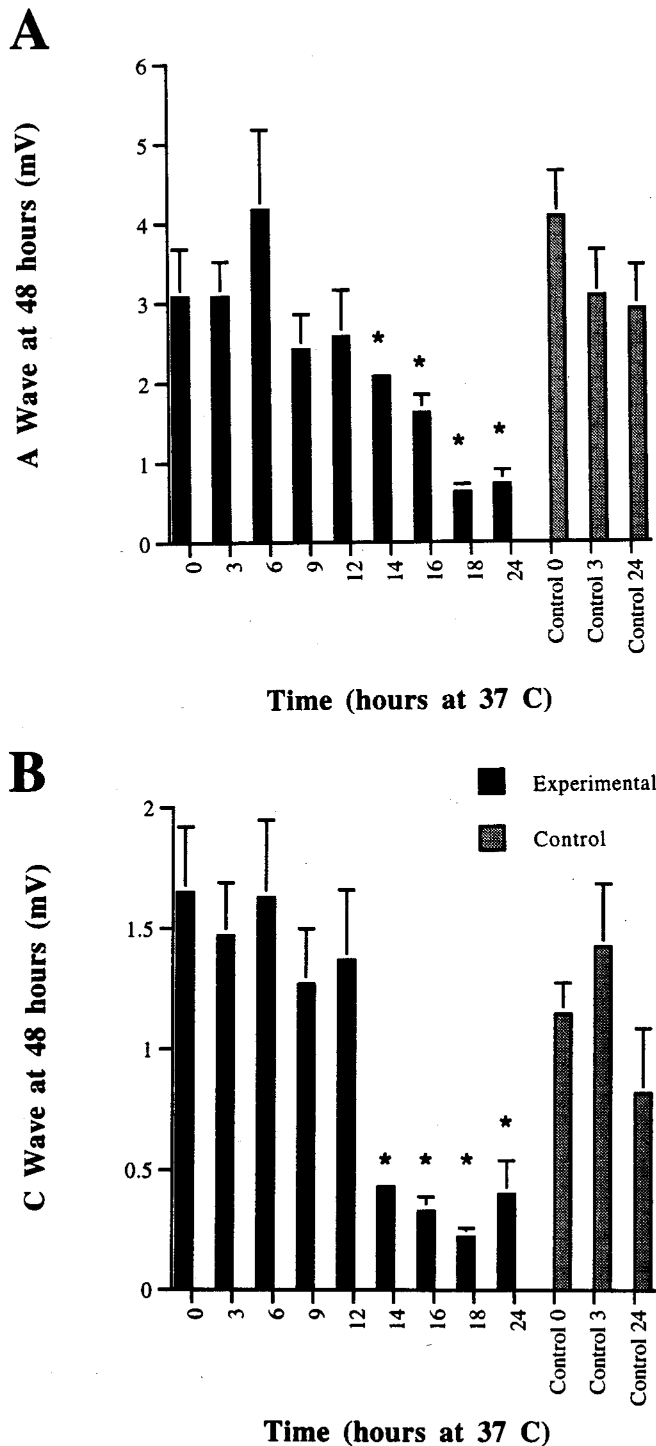


Figure 5. CAP from C57BL/6N sciatic nerves at 48 hr incubation in Optimem I medium. Nerves were incubated for 3–24 hr at 37°C before being cooled to 25°C. The CAP values at time 0, 3, and 24 hr at 37°C are shown for comparison to *demonstrate* the decrease in CAP size in the first 3 hr after removal of the nerve to culture. *A*, A wave. *B*, C wave. Complete preservation of both A and C waves is seen only if the cooling occurs by 12 hr after axotomy. $*p < 0.05$.

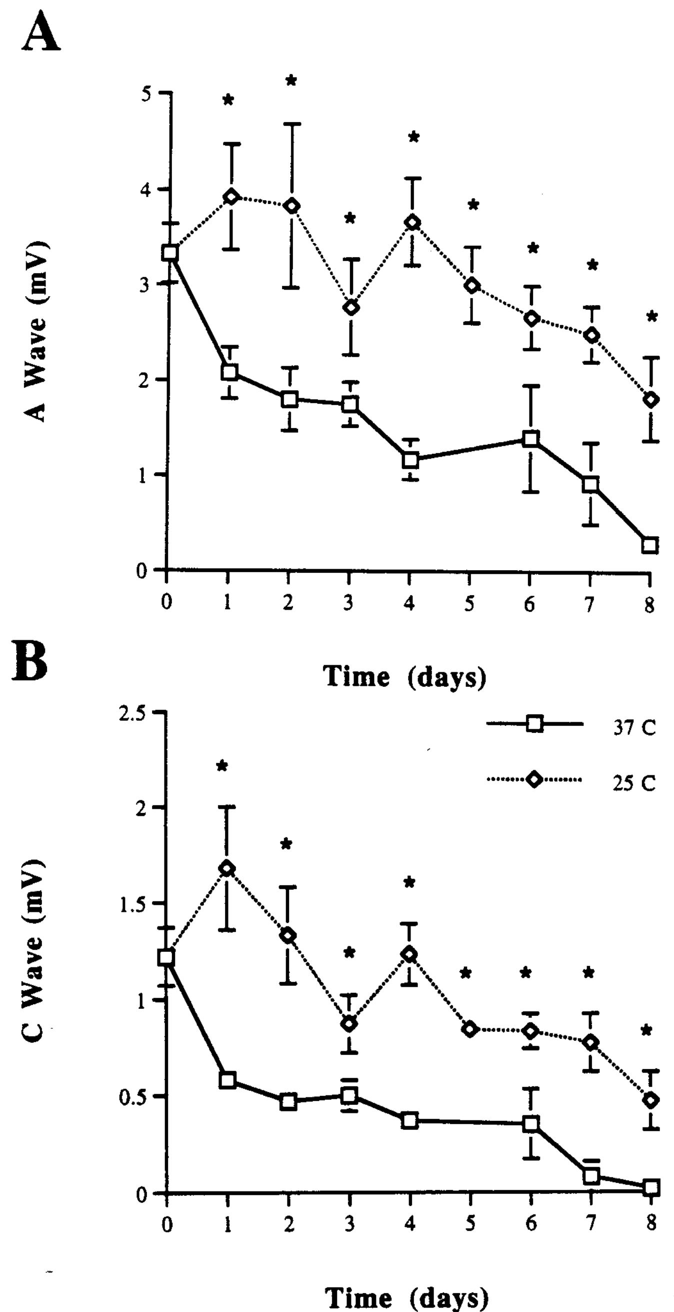


Figure 6. CAP from C57BL/Wld sciatic nerves maintained at 25 and 37°C in Optimem I for 1–11 d. *A*, A wave. *B*, C wave. Both waves are preserved with cooler temperatures. $*p < 0.05$.

1973; George et al., 1995). However, it is not known whether Ca^{2+} enters axons at a constant rate after injury or whether the ion exclusion is maintained until a time when large amounts of Ca^{2+} suddenly enter, reaching a concentration necessary for degradative enzyme activation. A $[Ca^{2+}]_o$ of 5 or 10 mM provides a larger ionic driving force for Ca^{2+} entry than a concentration of 250 μM does (physiological $[Ca^{2+}]_o$ is 1–2 mM). Our experiments examined whether or not maintenance of intact, desheathed sciatic nerves in low and high external Ca^{2+} concentrations could affect the rate of Wallerian degeneration and loss of the CAP. Incubation of nerves in both 5 and 10 mM external Ca^{2+} did not speed the time course for axonal degeneration in either wild-type or Wld nerves. Conversely, incubation of matched contralateral nerves in 250 μM Ca^{2+} also did not delay Wallerian degeneration (see Fig. 7). We were unable to lower media Ca^{2+} below 250 μM, because this caused the CAP to

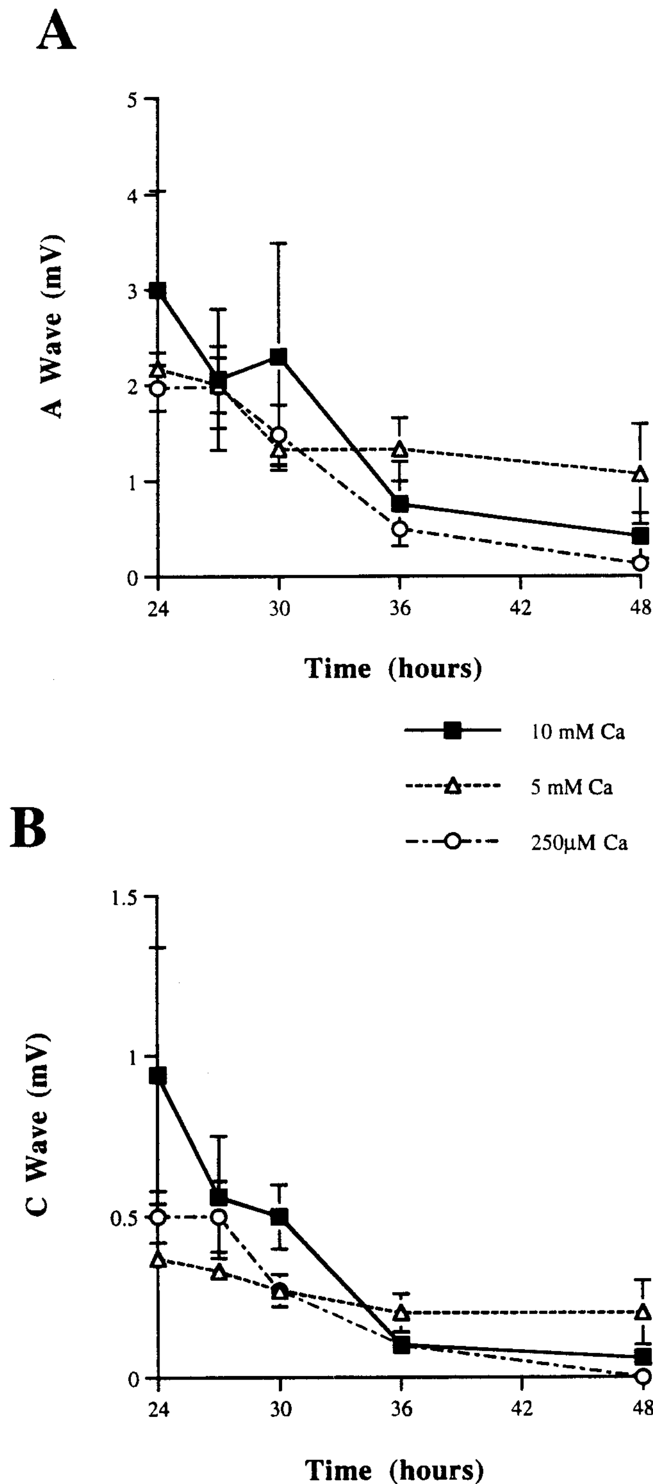


Figure 7. CAP from C57BL/6N sciatic nerves (desheathed) incubated with either 250 μM or 5 or 10 mM $CaCl_2$ for 48 hr at 37°C. *A*, A wave. *B*, C wave. $^*p < 0.05$.

disappear within 6 hr (our unpublished observations). In both high and low Ca^{2+} concentrations, the size of CAPs was similar at all of the time points that were studied. Because the rate of axonal degeneration is independent of the driving force for Ca^{2+} entry, either the transporter for Ca^{2+} influx is saturated easily even at 250 μM Ca^{2+}, or Ca^{2+} influx is a briefer event for which the duration does not impact on the overall rate of axonal degeneration. In the latter case plasmalemmal integrity may be maintained until a point, late in axonal degeneration, when there is rapid entry of extracellular Ca^{2+} and a culminating final catastrophic moment of axonal destruction, rather than a gradual creeping upward of $[Ca^{2+}]_i$ leading to a crescendoing degeneration rate.

Further supporting this hypothesis is an x-ray microprobe elemental analysis of rat sciatic nerves post-transection, which demonstrated a decrease in intracellular potassium (K^+) and chloride (Cl^-) ions and an increase in phosphorus (P) at 8 hr after injury in myelinated fibers (LoPachin et al., 1990). At 16 hr post-transection, elemental values remained the same as at 8 hr, with the exception of a newly elevated intracellular sodium ion (Na^+) concentration. The Ca^{2+} content of the axoplasm was unaltered at 8 and 16 hr, but its level in mitochondria was elevated significantly at 16 hr. Only after 48 hr, when all fibers had degenerated, was increased axoplasmic Ca^{2+} detected, with mitochondria at that time showing a >80-fold elevation in Ca^{2+}. This disruption of elemental homeostasis appears to be concurrent with a neuronal energy deficit seen 24–48 hr after peripheral nerve axotomy, which is accompanied by decreased activity of the membrane Na^+-K^+-ATPase (Stewart et al., 1965; Bachelard and Silva, 1966). The elemental changes noted by LoPachin et al. (1990) are consistent with Na^+-K^+-ATPase inhibition; with an intact plasma membrane Na^+ would be excluded, whereas K^+ still would continue to leak through open ion channels, with Cl^- following passively to maintain electroneutrality. With the K^+ gradient dissipated, Na^+ then could enter the axon, leading to the entry of Ca^{2+} via Na^+/Ca^{2+} exchange or the failure of pumping mechanisms for Ca^{2+} extrusion or sequestration.

Our data are not consistent with the hypothesis that the act of axotomy leads to a transient, massive increase in $[Ca^{2+}]_i$ along the entire length of the axon, which immediately activates the degradative proteases and lipases involved in Wallerian degeneration. A rise in intra-axonal Ca^{2+} concentration to >15 μM (a level certainly sufficient enough to activate most degradative enzymes) has been noted in *Aplysia* axons transected *in vitro* (Ziv and Spira, 1993). The authors of this study estimated that axonal Ca^{2+} concentrations quickly returned to basal levels of 0.05–0.1 μM after the 7–10 min that followed the sealing of the cut end within 0.5–2 min. They also determined that the elevation in Ca^{2+} was attributable to the influx of extracellular Ca^{2+} through the cut end of the axon and through the opening of voltage-gated Ca^{2+} channels along the entire length of the axon. If proteases and lipases are activated at the initial time of axonal injury, the delayed destruction of the axon that is seen (CAP remains nearly 100% at 24 hr after axotomy and then declines by 80% over the next 12 hr) occurs once sufficient plasmalemma and cytoskeleton have been degraded. In this case the effects of cooling would be similar at all phases of axonal degeneration, which our data do not support.

An alternative hypothesis is that the transient rise in Ca^{2+} might activate axonal kinases and phosphatases. The subsequent action of axonal transport on these modulators of protein phosphorylation might lead to the activation of Ca^{2+} channels or Ca^{2+} porters located at the nodes of Ranvier or along the length of the axolemma. The activation of mediators of Ca^{2+} influx later during the process of Wallerian degeneration then allows a catastrophic rise in axonal Ca^{2+} concentrations, leading to sudden, rapid destruction of the axon. This hypothesis gives rise to at least two phases of axonal degeneration and so is consistent with both

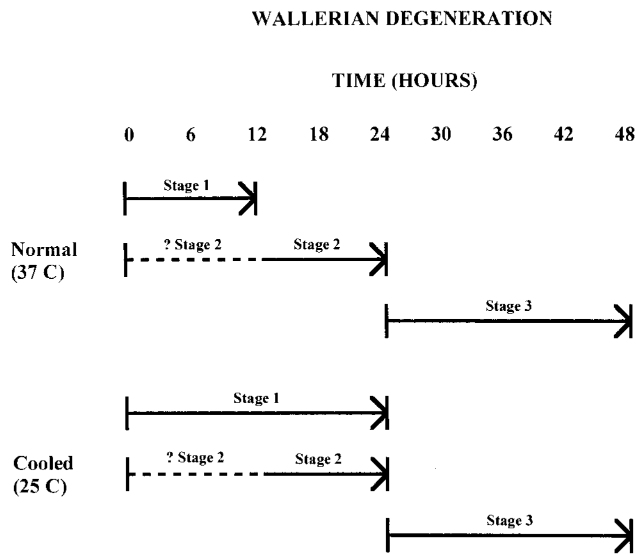


Figure 8. Schematic diagram of the proposed three-stage model of Wallerian degeneration.

the observed temperature effect and the insensitivity to external Ca^{2+} levels.

In the latter scenario, individual axons degenerate very rapidly at the end of the multiphasic Wallerian degeneration. The distribution of axonal calibers, concentration and rate of transport of the "factor" responsible for Ca^{2+} channel activation, and the ratio of the number of fibers of the sensory and motor lineages all affect the percentage of fibers that remain at any given time. The gradual decline in the measured CAP between 24 and 48 hr is, thus, a measure of a declining population of axons able to conduct CAPs.

Conclusions

The data lead us to propose a three-stage model of Wallerian degeneration (Fig. 8). The first stage occurs during the first 12 hr after axotomy at 37°C and is prolonged by low temperatures. Events during the first stage appear to initiate the process leading to Wallerian degeneration or "light the fuse." The second stage occurs during the period 12–24 hr after axotomy at 37°C. During this stage "the fuse is burning," because axonal degeneration is not yet evident (the morphology is normal, and the CAP remains close to 100% until 24 hr) but can no longer be delayed by cooling. It may be that this stage is independent of stage 1, because a delayed stage 2 is not seen when cooled nerves are warmed. Instead, nerves cooled during stage 1 appear to enter stage 3 immediately on warming, suggesting that the events of stage 2 may proceed despite the prolongation of stage 1. Alternatively, an external temperature of 25°C may not be low enough to alter the events of stage 2.

The third and final stage, which encompasses the rapid degeneration of axons during the period 24–48 hr (80% of the CAP is lost between 24 and 36 hr) at 37°C, occurs only after stages 1 and 2 are complete. This stage appears to correspond to the rapid enzymatic digestion of the axonal components. We speculate that catastrophic Ca^{2+} entry marks the transition between stages 1 and 2, which enable the Ca^{2+} influx, and stage 3, which results from protease and lipase activation by the Ca^{2+} influx.

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