The \textit{Drosophila} NSF Protein, dNSF1, Plays a Similar Role at Neuromuscular and Some Central Synapses

FUMIKO KAWASAKI AND RICHARD W. ORDWAY

Department of Biology, The Pennsylvania State University, University Park, Pennsylvania 16802

Kawasaki, Fumiko and Richard W. Ordway. The \textit{Drosophila} NSF protein, dNSF1, plays a similar role at neuromuscular and some central synapses. \textit{J. Neurophysiol.} 82: 123–130, 1999. The N-ethylmaleimide sensitive fusion protein (NSF) was originally identified as a cytosolic factor required for constitutive vesicular transport and later implicated in synaptic vesicle trafficking as well. Our previous work at neuromuscular synapses in the temperature-sensitive NSF mutant, \textit{comatose \textit{comt}}, has shown that the \textit{comt} gene product, dNSF1, functions after synaptic vesicle docking in the priming of vesicles for fast calcium-triggered fusion. Here we investigate whether dNSF1 performs a similar function at central synapses associated with the well-characterized giant fiber neural pathway. These include a synapse within the giant fiber pathway, made by the peripherally synapsing interneuron (PSI), as well as synapses providing input to the giant fiber pathway. The latency (delay) between stimulation and a resulting muscle action potential was used to assess the function of each class of synapses. Repetitive stimulation of the giant fiber pathway in \textit{comt} produced wild-type responses at both 20 and 36°C, exhibiting a characteristic and constant latency between stimulation and the muscle response. In contrast, stimulation of presynaptic inputs to the giant fiber (referred to as the “long latency pathway”) revealed a striking difference between wild type and \textit{comt} at 36°C. Repetitive stimulation of the long latency pathway led to a progressive, activity-dependent increase in the response latency in \textit{comt}, but not in wild type. Thus the giant fiber pathway, including the PSI synapse, appears to function normally in \textit{comt}, whereas the presynaptic inputs to the giant fiber pathway are disrupted. Several aspects of the progressive latency increase observed in the long latency pathway can be understood in the context of the activity-dependent reduction in neurotransmitter release we observed previously at neuromuscular synapses. These results suggest that repetitive stimulation causes a progressive reduction in neurotransmitter release by presynaptic inputs to the giant fiber neuron, resulting in an increased latency preceding a giant fiber action potential. Thus synapses presynaptic to the giant fiber appear to utilize dNSF1 in a manner similar to the neuromuscular synapse, whereas the PSI chemical synapse may differ with respect to the expression or activity of dNSF1.

INTRODUCTION

The N-ethylmaleimide sensitive fusion protein (NSF) acts within a protein complex that functions in the docking and fusion of intracellular vesicles with the appropriate target membrane. This complex is thought to form by assembly of membrane proteins of the vesicle and target membranes called SNAREs (soluble NSF attachment protein receptors), followed by recruitment of NSF and the soluble NSF attachment proteins (SNAPs). The ATPase activity of NSF is thought to play a critical role in the structure and function of this complex on the basis of both in vitro and in vivo evidence indicating that ATP hydrolysis by NSF leads to complex disassembly (reviewed in Hanson et al. 1997; Hay and Scheller 1997; Rothman 1994; Rothman and Wieland 1996; Südhof 1995).

Despite substantial in vitro biochemical evidence supporting a role for NSF in synaptic vesicle trafficking, only recently has NSF been shown to play an in vivo role in this process [see Kawasaki et al. (1998) for a recent review of literature relevant to this study]. In part, this progress developed from analysis of a \textit{Drosophila} NSF mutant, \textit{comatose \textit{comt}}. This mutant was originally identified in a screen for temperature-sensitive paralytic mutants and was shown to exhibit a conditional neuropathological defect (Siddiqi and Benzer 1976). Our previous work demonstrated that mutations in the dNSF1 gene are responsible for temperature-sensitive paralysis in \textit{comt} (Ordway et al. 1994; Pallanck et al. 1995a). More recently, our electrophysiological and ultrastructural analysis at neuromuscular synapses in \textit{comt} has revealed that dNSF1 functions in the priming of docked vesicles for fast calcium-triggered fusion (Kawasaki et al. 1998). Work at the squid giant synapse using injection of NSF peptides has led to some similar conclusions, with some important differences (Schweizer et al. 1998). Although recent experiments suggest that NSF proteins may also function postsynaptically (Lin and Sheng 1998; Nishimune et al. 1998; Osten et al. 1998; Song et al. 1998), our experiments in \textit{comt} indicate a strictly presynaptic role for dNSF1 (Kawasaki et al. 1998; Kawasaki and Ordway 1999).

Biochemical analysis in \textit{comt} has also been informative. Recent work by the Pallanck laboratory (Tolar and Pallanck 1998) has characterized neural SNARE complexes in \textit{Drosophila} and carefully analyzed the status of this complex on the plasma membrane. A different conclusion has been reported elsewhere on the basis of a less exhaustive biochemical analysis in \textit{comt} (Littleton et al. 1998).

Taken together, the electrophysiological, ultrastructural, and biochemical analyses in \textit{comt} lead to the conclusion that the priming of docked vesicles relies on dNSF1-mediated disassembly of plasma membrane SNARE complexes. Models of NSF function incorporating these observations are presented elsewhere (Kawasaki et al. 1998; Tolar and Pallanck 1998).

Despite this progress, a number of unresolved issues remain. For example, early models of NSF function have proposed that it is a general purpose protein, perhaps a single protein performing the same function in membrane trafficking events throughout the organism. However, previous work has dem-
onstrasted the existence of two closely related NSF genes in Drosophila, dNSF1 and dNSF2, as well as multiple transcripts derived from the dNSF2 gene (Pallanck et al. 1995b). This diversity raises the question of whether specific NSF gene products may perform specialized functions within the organism. Thus our recent progress defining the synaptic function of a specific NSF gene product, dNSF1, provides an opportunity to address whether this single NSF protein performs an analogous function at all synapses. Here we utilize cont mutations and the well-characterized giant fiber neural pathway in Drosophila to compare the function of dNSF1 at different chemical synapses. Our results show that dNSF1 appears to function in a similar manner at neuromuscular and some central synapses, and that one synapse may differ with respect to the function or expression of dNSF1.

Some of this work has been communicated in preliminary form (Kawasaki and Ordway 1997, 1998).

METHODS

Dissection

Male or female flies 1–4 days of age were anesthetized using CO2 and then mounted over a hole in an air tube and secured with wax as described (Koenig et al. 1989). Air was delivered to the tracheal system using an aquarium pump. The fly was mounted laterally and dissected in recording solution (see Microelectrode recordings) to expose the lateral surface of one set of the dorsal longitudinal flight muscles (DLMs) as well as the thoracic ganglion of the CNS. For simultaneous recordings from the DLM and tegrotergochanter muscle (TTM), TTMs were exposed and the first dorsoventral muscles were removed to expose the anterior part of the DLM. Temperature was maintained at 20°C during dissection.

Brain stimulation

The head of the fly was twisted and mounted ventral side up. The membrane between the prefrontal and the anterior tentorial pit was cut along the midline to make an approach for a stimulation electrode to the head capsule. The glass stimulation electrode filled with recording solution was inserted through the incision and advanced between the frontal tracheal sacs to the brain. The tip diameter of the stimulation electrode was ~80 μm. The duration of the stimulus was 0.2 ms. Stimulation voltages used to elicit the long latency and giant fiber pathway responses were typically 10–15 and 90–100 V, respectively.

DLM motor axon stimulation

The posterior dorsal mesothoracic nerve (PDMN), through which the DLM motor axons project from the thoracic ganglion to the muscle, was cut and pulled into a suction electrode for stimulation.

Temperature control

Temperature control was achieved using a Medical Systems TC-202 temperature controller and PDMI micro-incubator (Medical Systems, Greenvale, NY). Temperature shifts from 20 to 36°C typically required ~4.5 min.

Microelectrode recordings

Microelectrode recordings were carried out by conventional methods with the use of a Dagan LX2–700 amplifier (Dagan, Minneapolis, MN) and glass microelectrodes filled with 3 M KCl (~30 MΩ). The recording solution consisted of (in mM) 128 NaCl, 2 KCl, 4 MgCl2, 1.8 CaCl2, 5 HEPES, and 36 sucrose. The pH was adjusted to 7.0 using NaOH. During recording, the 3-ml preparation chamber was perfused at 0.5 ml/min.

Electrophysiological data acquisition and analysis

Data were acquired on-line using a Power Macintosh 7500 computer, Pulse software (HEKA elektronik, Lambrecht, Germany), and an Instrutech ITC-16 laboratory interface (Instrutech, Great Neck, NY). Data were low-pass filtered at 3 kHz and acquired at 10 or 20 kHz. Stimulation was achieved using the Pulse program to trigger a Dagan S-900/S-910 stimulator (Dagan, Minneapolis, MN). Latency measurements were carried out using cursor measurements in the Pulse program. Microsoft Excel was utilized for data tabulation and statistical analysis. With the use of an unpaired Student’s t-test, statistical significance was assigned to comparisons with P \(<0.05\).

Drosophila lines and transformation rescue

All Drosophila lines used were cultured at 20°C. The three temperature-sensitive comt alleles used in this study contain point mutations leading to single amino acid changes. Missense mutations in comtST17 and comtST53 have been reported previously (Pallanck et al. 1995a); comtST7 contains a missense mutation converting proline 398 to serine. Rescue experiments utilized a transgene construct containing a wild-type dNSF1 cDNA under the control of an hsp70 heat shock promoter (Pallanck et al. 1995a). For rescue experiments, comt flies bearing a transgene were subjected to three heat shocks separated by 24-h intervals (all heat shocks were at 38°C). A 15-min heat shock on the first day was followed by 30-min heat shocks on the second and third days. Recordings were obtained ~24 h after the last heat shock. In the case of the comtST53 rescue experiments, the third heat shock was omitted. As is the case for temperature-sensitive paralytic behavior (Pallanck et al. 1995a), controls in which comt flies lacking the transgene were heat shocked demonstrated that phenotypic rescue was entirely dependent on the presence of the transgene.

RESULTS

Giant fiber and long latency pathways

The morphology and physiology of the giant fiber pathway was originally defined in the pioneering work of the Wyman (King and Wyman 1980; Sun and Wyman 1997; Tanouye and Wyman 1980) laboratories and has been subjected to further detailed characterization in a number of other studies (e.g., see Bacon and Strausfeld 1986; Engel and Wu 1996; Gorczyca and Hall 1984; Phelan et al. 1996; Trimarchi and Schneiderman 1995). As shown schematically in Fig. 1C (see legend), the giant fiber axon projects from the brain to the thoracic ganglion, where it forms an electrical synapse with a peripherally synapsing interneuron (PSI). The PSI forms a chemical synapse on each motor neuron innervating the DLMs, and this represents the only chemical synapse within the giant fiber pathway to the DLM motor neuron. The giant fiber neuron also makes an electrical synapse on the TTM motor neuron. Elements of the giant fiber pathway to be discussed here include the giant fiber neuron, the PSI, and the DLM and TTM motor neurons.

Excitation of the giant fiber pathway can be elicited by electrical stimulation of the brain and monitored by intracellular recording of postsynaptic action potentials in the DLMs. By varying the stimulus intensity, the giant fiber neuron may be stimulated directly or, alternatively, indirectly through stimulation of its presynaptic inputs. Higher stimulus intensities
activate the giant fiber neuron directly and thus produce a DLM potential through subsequent activation of the PSI and the DLM motor neuron. This giant fiber response indicated in gray in Fig. 1A exhibits a characteristic latency between brain stimulation and the DLM potential (∼1.2 ms) as described previously (Tanouye and Wyman 1980). As discussed below, lower stimulus intensities activate the giant fiber neuron indirectly through its presynaptic inputs; this long latency response exhibits a latency of ∼2.0 ms and is indicated in black in Fig. 1A.

A previously described long latency response (Tanouye and Wyman 1980) exhibiting a latency of ∼2.0 ms was attributed to stimulation of presynaptic inputs to the giant fiber pathway on the following basis. After activation of the giant fiber neuron, action potentials appear in both the DLM and TTM. Because the pathway to the DLM includes an additional chemical synapse, the DLM potential occurs ∼0.4 ms after the TTM potential (Tanouye and Wyman 1980). Thus this difference in TTM and DLM response latencies serves as a signature of the giant fiber pathway. To confirm that we observe the same long latency response previously described, we examined whether this response results from activation of inputs to the giant fiber pathway. Simultaneous DLM and TTM recordings were performed using lower intensity stimulation to produce long latency responses. Both DLM and TTM potentials were elicited with the 0.4-ms difference in latencies characteristic of the giant fiber pathway (Fig. 1B). Thus we define long latency responses as those elicited by stimulation of presynaptic inputs to the giant fiber pathway.

Our previous work at neuromuscular synapses in comt has defined a role for dNSF1 in the priming of docked synaptic vesicles for fast calcium-triggered fusion (Kawasaki et al. 1998; Kawasaki and Ordway 1999). These experiments utilized direct stimulation of the motor axon, together with microelectrode and voltage-clamp recordings, to demonstrate a progressive, activity-dependent reduction in neurotransmitter release at restrictive temperature. In microelectrode recordings from the DLM, the reduction in neurotransmitter release is seen as a graded decrease and then loss of the DLM action potential, revealing the underlying postsynaptic potential, which continues to decrease in amplitude. This reduction was dependent on the stimulation frequency. comt<sup>ST77</sup> was most sensitive, exhibiting a marked reduction in response to 1-Hz stimulation. In comt<sup>ST53</sup> and comt<sup>TP7</sup>, 10-Hz, but not 1-Hz, stimulation markedly reduced neurotransmitter release. Thus, as shown in Fig. 2 (see caption), when giant fiber or long latency responses are elicited at 1 Hz in comt<sup>TP7</sup> or comt<sup>ST53</sup>, the DLM action potential is not markedly reduced.

Giant fiber and long latency responses were examined in wild-type and comt flies at both permissive temperature (20°C) and a restrictive temperature (36°C) at which comt is paralyzed (Fig. 2). In wild-type and comt flies at 20°C, the latencies of the giant fiber and long latency responses were constant for a 1-Hz train of stimuli (Fig. 2, A and D). Also in comt flies at 36°C, the giant fiber pathway exhibited wild-type latencies (Fig. 2, B and E). In contrast, stimulation of the long latency pathway under the same conditions produced responses with increased and variable latencies, ranging from wild-type to very long latency responses (Fig. 2, B and E). These responses were attributed to the long latency pathway on the basis of the following three observations. I) They were elicited with the same characteristic stimulation threshold observed for long latency responses in comt exhibits a conditional neurophysiological defect presynaptic to the giant fiber pathway.
wild type and in comt at 20°C (the giant fiber pathway also exhibits the same stimulation threshold in comt and wild type). 2) The shortest latencies observed were very similar to wild type. 3) Longer latency responses always appeared in place of, rather than in addition to, responses exhibiting wild-type latencies. The increase and variation in latency were reversible, recovering over ~20 min after the preparation was returned to 20°C (Fig. 2F).

The latency increase and variation seen in the long latency response of comt contrasts the constant latency responses observed in the giant fiber pathway under the same conditions. As described earlier, both response pathways include activation of the PSI chemical synapse with the DLM motor neuron, whereas only the long latency response includes activation of chemical synapses presynaptic to the giant fiber pathway. Thus under these conditions, the chemical synapse between the PSI and the DLM motor neuron appears to be functioning normally, indicating that the observed latency changes occur in the presynaptic inputs to the giant fiber pathway. As described below, the persistence of apparently wild-type neurotransmission at PSI chemical synapses in comt was also observed at higher stimulation frequencies.

**Loss of dNSF1 function is responsible for increased latencies in the long latency pathway**

The same conditional neurophysiological defect observed in the long latency pathway of comt was also observed in two additional comt alleles, comt and comt. A comparison of latency values in comt, comt, and wild type are shown in Table 1. The similarity of results obtained in multiple comt alleles indicates that this phenotype results from a general loss of dNSF1 function rather than from any specific amino acid substitution in the dNSF1 protein. To further confirm that the comt mutations in dNSF1 are responsible for the latency changes, long latency responses were recorded from flies in which a transgene expressing the wild-type dNSF1 protein was introduced into a comt mutant background. Expression of wild type dNSF1 in a comt genetic background dramatically rescued both the temperature-sensitive paralytic behavior and the latency increases (Fig. 3 and Table 1). Although not shown, the behavioral and neurophysiological phenotypes in comt and comt were also completely rescued. These results indicate that the synaptic defect presynaptic to the giant fiber pathway results entirely from mutations in the dNSF1 gene.

**Temperature-induced spontaneous neural activity in comt**

Virtually no spontaneous DLM action potentials were observed at 20°C in either comt or wild type. At 36°C, comt flies exhibited a markedly elevated frequency of spontaneous DLM action potentials, whereas spontaneous activity in wild type was only slightly elevated (Fig. 4). Because this spontaneous activity was eliminated by cutting the DLM motor axon, it clearly represents an increase in neural activity. The spontaneous activity in comt often occurred as periodic high-frequency bursts of action potentials separated by intervals of lower frequency activity (Fig. 4). During this spontaneous activity, the amplitude of the DLM action potential was reduced during a burst and gradually recovered between bursts. This observation is consistent with two previous results obtained using

**TABLE 1. Response latencies during 1-Hz stimulation of the long latency pathway**

<table>
<thead>
<tr>
<th>Latency, ms</th>
<th>20°C</th>
<th>36°C 5 min</th>
<th>20°C 0 min</th>
<th>20°C 6 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>1.98 ± 0.05 (5)</td>
<td>1.08 ± 0.05 (5)</td>
<td>2.22 ± 0.05 (5)</td>
<td>1.98 ± 0.08 (4)</td>
</tr>
<tr>
<td>comt</td>
<td>1.92 ± 0.04 (6)</td>
<td>4.37 ± 0.57* (4)</td>
<td>3.71 ± 0.79† (4)</td>
<td>2.37 ± 0.05* (3)</td>
</tr>
<tr>
<td>TP7 HSdNSF1</td>
<td>1.96 ± 0.03 (4)</td>
<td>1.13 ± 0.02 (4)</td>
<td>2.22 ± 0.02 (4)</td>
<td>1.95 ± 0.05 (4)</td>
</tr>
<tr>
<td>comt</td>
<td>1.99 ± 0.06 (6)</td>
<td>5.39 ± 0.11* (4)</td>
<td>4.02 ± 0.38* (5)</td>
<td>2.61 ± 0.19* (4)</td>
</tr>
</tbody>
</table>

Values are means ± SE with number of experiments in parentheses. Response latencies at 20°C, after 5 min at 36°C, and at 20°C during recovery from exposure to 36°C (for 10 min). TP7 HSdNSF1 refers to transgenic flies in which the comt phenotype is rescued by expression of wild-type dNSF1 protein in a comt mutant background. * Values significantly different from wild type under the same conditions (P < 0.05). † P = 0.07.
direct stimulation of the DLM motor axon (Kawasaki et al. 1998). First, an activity-dependent reduction in neurotransmitter release at DLM neuromuscular synapses was observed during repetitive stimulation at restrictive temperature. Second, this reduction in neurotransmitter release was shown to recover at restrictive temperature in the absence of repetitive stimulation.

In the experiment in Fig. 2E at restrictive temperature in comt, a typical level of spontaneous neural activity was present and may have contributed to the observed reduction in action potential amplitude (compare Fig. 2, B and E). The frequency of spontaneous activity observed in this experiment was ~2.6 Hz, at which we estimate that <3% of traces contain a spontaneous event. Thus >97% (perhaps all) of the DLM potentials shown in Fig. 2E are evoked. Consistent with this, the responses are not randomly distributed. Each trace contains a single event, no events occur before stimulation, and the responses are clustered near the latency typically observed in the long latency pathway of wild type.

Importantly, the temperature-induced spontaneous activity in comt (as well as the small increase observed in wild type) recovered immediately on returning the preparation to 20°C, and thus latency values taken during recovery (as shown in Table 1) were recorded in the absence of spontaneous activity. As discussed below, this rapid recovery was further utilized to examine latency changes in the absence of spontaneous activity. Finally, the temperature-induced spontaneous activity in comt was rescued by transgenic expression of the wild-type dNSF1 protein.

Reduction in postsynaptic potential amplitude in comt delays action potential firing at central and peripheral synapses

Although no obvious pattern was observed in the latency increases occurring in the long latency pathway of comt, any such pattern may have been obscured by the effects of temperature-induced increases in spontaneous activity. To address this issue, response latencies were recorded in comt under conditions in which spontaneous DLM potentials are greatly reduced in frequency or absent. As mentioned earlier, the increased spontaneous activity observed at restrictive temperature in comt recovers immediately on return to permissive temperature. In contrast, the latency changes observed in the long latency pathway require >20 min for recovery. Thus long latency responses were recorded in the absence of spontaneous activity at 20°C during recovery from exposure to restrictive temperature. These experiments revealed a striking pattern in the latency changes: a progressive, activity-dependent increase in the response latency during the stimulus train (Fig. 5A and Table 1).

To compare central and neuromuscular synapses under similar conditions, we also directly stimulated the DLM motor axon at 20°C during recovery from exposure to restrictive temperature. As expected from our previous results in comt ST53 (Kawasaki et al. 1998), stimulation of the motor axon at 10 Hz,
but not 1 Hz, caused a progressive reduction of the DLM action potential and the underlying postsynaptic potential. As seen in Fig. 5B, this progressive decrease in the postsynaptic potential delayed firing of the muscle action potential, resulting in a progressive increase in the latency between motor axon stimulation and the DLM action potential. Thus the progressive increase in latency observed in the long latency pathway of comt (Fig. 5A) is precisely what would be predicted for an activity-dependent decrease in neurotransmitter release at presynaptic inputs to the giant fiber pathway. These observations indicate that both the DLM neuromuscular synapse and the presynaptic inputs to the giant fiber neuron exhibit a progressive, activity-dependent decrease in the postsynaptic potential, resulting in a progressive increase in the delay preceding the postsynaptic action potential. A further similarity between the behavior of these synapses is that the latency increase observed in the long latency pathway, like the activity-dependent reduction in neurotransmitter release at the DLM neuromuscular synapse (Kawasaki et al. 1998), recovers at restrictive temperature in the absence of stimulation. Thus synapses presynaptic to the giant fiber pathway appear to utilize dNSF1 as defined at the DLM postsynaptic potential. Similar results were obtained in 4 experiments.

**DISCUSSION**

The work presented here extends our earlier investigation of dNSF1 function at DLM neuromuscular synapses by analyzing the role of dNSF1 at chemical synapses associated with the giant fiber neural pathway. These experiments indicate that chemical synapses presynaptic to the giant fiber neuron utilize dNSF1 as described for the DLM neuromuscular synapse, in the priming of docked synaptic vesicles for fast calcium-triggered exocytosis (Fig. 7).

One important observation is that the long latency pathway in comt exhibits a progressive, activity-dependent increase in response latency during repetitive stimulation at restrictive temperature. This increase in latency occurs in the postsynaptic inputs to the giant fiber neuron and can be understood in the context of our previous observations at the DLM neuromuscular synapse. We suggest that a progressive, activity-dependent reduction in neurotransmitter release at presynaptic inputs to the giant fiber neuron results in a reduction in the postsynaptic potential and a progressive delay in the latency preceding the giant fiber neuron action potential.

The schematic diagrams of the giant fiber pathway shown here are highly simplistic representations of a much more complex pathway. In fact, the electrophysiological characteristics of the long latency pathway and the latency increases observed in comt raise the question of whether the long latency pathway includes only a single chemical synapse presynaptic to the giant fiber neuron. Although this issue remains unresolved, the electrophysiological observations allow some speculation. The difference between the response latency observed for the giant fiber and long latency pathways defines the latency between brain stimulation and activation of the giant fiber neuron. These values typically fall within the range of 0.6–0.9 ms. By comparison with the 0.4-ms time delay introduced by the PSI chemical synapse, these observations suggest that the long latency pathway may include two chemical synapses.
afferent or whether both dNSF1 and dNSF2 may function at the same synapse. We have observed that the activity-dependent latency changes suggests this as one possibility. Alternatively, the activity of dNSF1 may differ at this synapse, such that it plays a less critical role in absence of stimulation. Thus it appears that a residual vesicle priming process occurs at restrictive temperature. Whether this priming represents residual dNSF1 activity, dNSF2 activity, or priming by another mechanism remains unresolved; however, at present we favor the idea that a dNSF1-independent priming process is responsible (Kawasaki et al. 1998). Localization of the dNSF1 and dNSF2 proteins will begin to clarify whether synapses may differ in their complement of NSF proteins, and whether these differences may lead to diversity in synaptic mechanisms.

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Address for reprint requests: R. W. Ordway, Dept. of Biology, The Pennsylvania State University, University Park, PA 16802.

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