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Temperature compensation of neuromuscular modulation in *Aplysia*

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Abstract

Physiological systems that must operate over a range of temperatures often incorporate temperature-compensatory mechanisms to maintain their output within a relatively narrow, functional range of values. We analyze here an example in the accessory radula closer (ARC) neuromuscular system, a representative part of the feeding neuromusculature of the sea slug *Aplysia*. The ARC muscle's two motor neurons, B15 and B16, release, in addition to ACh that contracts the muscle, modulatory peptide cotransmitters that, through a complex network of effects in the muscle, shape the ACh-induced contractions. It is believed that this modulation is critical in optimizing the performance of the muscle for successful, efficient feeding behavior. However, previous work has shown that the release of the modulatory peptides from the motor neurons decreases dramatically with increasing temperature. From 15° C to 25° C, for example, release decreases 20-fold. Yet *Aplysia* live and feed successfully not only at 15° C, but at 25° C and probably at higher temperatures. Here, working with reduced B15/B16-ARC preparations *in vitro* as well as a mathematical model of the system, we have found a resolution of this apparent paradox. Although modulator release decreases 20-fold when the temperature is raised from 15° C to 25° C, the observed modulation of contraction shape does not decrease at all. Two mechanisms are responsible. First, further downstream within the modulatory network, the modulatory effects themselves—experimentally dissected by exogenous modulator application—have temperature-dependencies opposite to that of modulator release, increasing with temperature. Second, the saturating curvature of the dose-response relations within the network diminishes the downstream impact of the decrease of modulator release. Thus, two quite distinct mechanisms, one depending on the characteristics of the individual components of the network and the other emerging from the network's structure, combine to compensate for temperature changes to maintain the output of this physiological system.

Introduction

The rates of individual biochemical reactions and elementary physiological processes vary intrinsically with temperature. Yet the larger physiological systems that are built from these processes must often maintain their output within a relatively narrow, functional range of values. Animals that are active over a range of environmental temperatures must therefore compensate, if they do not regulate their internal temperature, by building temperature-compensatory mechanisms into the structure of their physiological systems. We analyze here an example in the accessory radula closer (ARC) neuromuscular system of the sea slug *Aplysia*. This system is tuned by a complex network of neuromodulatory effects that, individually, vary greatly with temperature. Yet the overall output of the system does not.

The ARC muscle is one of the muscles of the buccal mass, a complex structure that produces rhythmic, cyclical movements of the animal's food-grasping organ, the radula, in several types of consummatory feeding behavior such as biting, swallowing, and rejection of unsuitable food

(Kupfermann 1974). The structure of the ARC system is summarized in Fig. 1. The muscle is innervated by two motor neurons, B15 and B16, which, when they fire, release their classical transmitter, ACh, to depolarize and contract the muscle (Cohen et al. 1978; *left-hand side* of Fig. 1). In addition, however, the two motor neurons release several families of modulatory peptide cotransmitters that shape the basal ACh-induced contractions (*right-hand side* of Fig. 1; for review and complete references see Weiss et al. 1993; Kupfermann et al. 1997; Hooper et al. 1999; Brezina et al. 2003a, 2005). Most important are the small cardioactive peptides (SCPs), released from motor neuron B15 (e.g., Lloyd et al. 1984; Cropper et al. 1987a; Vilim et al. 1996b), and the myomodulins (MMs), released from motor neuron B16 (Cropper et al. 1987b, 1991; Brezina et al. 1995; Vilim et al. 2000). The SCPs and MMs shape contractions through three main effects on the muscle: (1) they potentiate the contractions by enhancing the muscle's depolarization-activated Ca current that supplies Ca^{2+} essential for contraction (e.g., Brezina et al. 1994a, 1995; Brezina and Weiss 1995); (2) they depress the contractions by activating in the muscle a K current that opposes the ACh-induced depolarization, activation of the Ca current, and Ca^{2+} influx (Brezina et al. 1994b, 1995; Orekhova et al. 2003); and (3) they increase the relaxation rate of the contractions probably by modulating the muscle's contractile machinery (Probst et al. 1994; Heierhorst et al. 1994, 1995). The balance of the competing potentiating and depressing effects determines the net modulation of contraction size, which, together with the modulation of the relaxation rate, then produces the final shape of the contraction. Similar networks of modulatory effects shape the contractions of the other muscles of the buccal mass (e.g., Church et al. 1993; Evans et al. 1999; Keating and Lloyd 1999; Hurwitz et al. 2000).

In extensive work over many years, in the real system as well as with a series of increasingly realistic mathematical models, we have analyzed how the modulatory shaping of contractions dynamically tunes the buccal musculature to meet different behavioral demands, to perform efficiently each of the different types of feeding behavior. The modulation is critical for function: without the modulatory tuning, the constraint of the fixed neuromuscular properties would make many sequences of behaviors that the animal might be required to perform inefficient or even completely dysfunctional (Brezina et al. 1996, 2000b, 2003a, Brezina et al. 2005).

There is, however, a serious potential difficulty with this scenario. Vilim et al. (1996a) found that the release of the modulatory peptide cotransmitters from the motor neurons is extremely sensitive to temperature. Fig. 2 replots the data of Vilim et al. (1996a) for the release of the SCPs from the ARC motor neuron B15, measured by direct radioimmunoassay of the amount of released peptide (each point is the mean $\pm$ SE, smaller than the mean symbol size, of 4 preparations). Also plotted is the simultaneous release of the buccalins, peptides of another family that are co-stored in the same dense-core vesicles with the SCPs in B15 (as with the MMs in B16; "Buc" in Fig. 1) and co-released by the same release process (Vilim et al. 1996b, 2000). Release of both peptide families declines dramatically with increasing temperature: an increase of 10° C reduces release ~20-fold (see Fig. 2 legend).

Fig. 2 shows that release of the modulatory peptides is strong at 15° C, a physiological temperature for *Aplysia californica*, the species studied in all of this work. However, *Aplysia* do not live only at 15° C. At different times of the year and in different local environments—in deep water as compared with, for example, shallow, sun-heated tide pools—*A. californica* will encounter water temperatures ranging from well below 15° C to as high as 25° C (Kupfermann and Carew 1974). [Other *Aplysia* species, such as *A. fasciata* in the Mediterranean Sea or *A. dactylomela* in the Caribbean, live and feed at up to at least 29° C (Gev et al. 1984; Carefoot 1987), but the modulatory tuning of the feeding musculature has not been studied in these species.] *Aplysia* are, of course, poikilotherms that cannot maintain an internal temperature that is appreciably different from that of the surrounding water.

According to Fig. 2, there is little release of the peptide modulators at, say, 25° C.¹ Yet *Aplysia* live and feed at this temperature. Does this mean that the modulatory tuning is, after all, not important for functional, efficient feeding? In this paper we provide a resolution of this difficulty. Even though the release of the modulators declines dramatically with increasing temperature, the overall strength of the modulatory tuning does not decline. This is because the modulatory system incorporates mechanisms, individual effects with the opposite temperature-dependence as well as more general mechanisms that are inherent in the network structure of the system, that maintain its output throughout the entire temperature range.

Methods

Experiments

Preparation.—The preparation was the standard preparation used for recording motor neuron-elicited contractions of the ARC muscle (Cohen et al. 1978; Weiss et al. 1978; for recent use see, e.g., Orekhova et al. 2003; Horn et al. 2004). Briefly, the preparation consisted of the bilateral buccal ganglia, the two ARC muscles, and the connecting buccal nerves 3 through which the buccal motor neurons B15 and B16 innervate the ARC muscle. The cerebral ganglion, connected to the buccal ganglia by the cerebral-buccal connectives, was also retained. The buccal ganglia (but not the cerebral ganglion) were desheathed. One ARC muscle was pinned out in a separate subchamber and connected to an isotonic transducer (Model 60-3000, Harvard Apparatus, Holliston, MA) to measure the length of the muscle with a light counterbalancing load. The ipsilateral motor neurons B15 and B16 were impaled with standard intracellular microelectrodes and connected to an intracellular amplifier (Axoclamp 2A/B, Axon Instruments, Union City, CA). Under the control of external timers/stimulators (Pulsemaster A300, World Precision Instruments, Sarasota, FL, and Grass S48/S88, Astro-Med, West Warwick, RI), the motor neurons were stimulated with brief current injections to fire spikes in the desired pattern. All signals were sampled and recorded simultaneously by a computer using Digidata 1322A data-acquisition hardware and pCLAMP 8/9 software (Axon Instruments). When the experiment on the first ARC muscle was finished, the ARC muscle on the opposite side of the preparation was also used, whenever possible, for an independent experiment or a matched pair (see Results).

Experimental design.—Three basic experimental paradigms were used.

1. *Single motor neuron self-modulation.* Only one of the two ARC motor neurons, either B15 or B16, was fired in a relatively intense bursting pattern, generally at 10–15 Hz for 2 s every 5 s for 3 min, calculated to release the peptide modulators (see Fig. 3).
2. *Two motor neuron cross-modulation.* Both motor neurons were fired, one with brief bursts at regular infrequent intervals to elicit “monitoring” contractions of moderate size, the other with a short block of a more intense bursting pattern, interposed between the monitoring contractions once only during the experiment, to release the modulators (see Fig. 4). The monitoring neuron, either B15 or B16, was generally fired for 1 s every 100 s, B15 at 20 or 25 Hz and B16 at 25 Hz. Such a pattern was likely to have elicited little or no modulator release from the monitoring neuron itself (Cropper et al. 1990c; Vilim et al. 1996a; Brezina et al. 2003b). The modulator-releasing neuron was fired for 4 s every 10 s for 60 s, B15 at 20 Hz and B16 at 20 or 25 Hz.

¹As Fig. 2 implies, release of the peptide modulators has not been directly measured at temperatures higher than 19° C. However, measurements of downstream effects of the released modulators such as the SCP-induced elevation of cAMP in the ARC muscle (Fig. 1) strongly suggest that the release continues to decrease up to at least 22° C (e.g., Whim and Lloyd 1990), although one lesson of our work here that such indirect evidence must be treated with caution because the downstream effects are by no means a straightforward, linear readout of the release (see Discussion).

3. *Exogenous modulator application.* Motor neuron B15 was fired in the monitoring pattern, generally for 1 s every 100 s at 20 Hz, while exogenous SCP_B was applied to the muscle subchamber. Increasing amounts of SCP_B, calculated to produce a series of increasing known concentrations in the subchamber, were added cumulatively without wash (Fig. 7). Each concentration was left to work for at least 5 contractions (>8 min) before the parameters of the contraction were measured. Alternatively, a single concentration of SCP_B, 10⁻⁷ M, was applied and the time course of the development of its effects was followed for at least 10 contractions (>16 min) (Fig. 9A,B). In some of these experiments the SCP_B was then washed out, by perfusing the muscle subchamber (volume ~7 ml) steadily at 1.25 ml/min, while the reversal of the effects was followed with continuing monitoring contractions (Fig. 9C). When the effects had reversed completely (after at least 30 min but sometimes up to 1 hr of wash), the application of SCP_B was repeated at a different temperature. For additional details see Results and figure legends.

Temperature.—Temperature was controlled with a Peltier plate under the preparation, and continuously monitored with an IT-23 thermocouple microprobe (Physitemp Instruments, Clifton, NJ) inserted in the muscle subchamber and connected to a digital thermometer (BAT-12, Physitemp Instruments). Due to the relatively large volume of the muscle subchamber, the correspondingly large volume of any perfusing flow, and the long duration of many experiments (~1 hr at each temperature), it was not always possible to maintain the temperature completely steady throughout the entire experiment. However, the temperature generally varied by no more than 1° C. Except in a few experiments done at ambient room temperature (~22° C; Fig. 3), the temperature was controlled so that the 1° C range included, throughout the experiment, either 15° C or 25° C: these are the experiments referred to as having been done at “15° C” or “25° C”. When comparing the two temperatures sequentially in the same muscle or in the two muscles of the same preparation, the order in which the temperatures were tested was varied in different preparations; similar results were obtained regardless of the order.

Measurement of contraction parameters.—The peak amplitude of each contraction was measured relative to the baseline muscle length at the beginning of the motor neuron burst that elicited the contraction, or, in the case of closely spaced bursts that did not allow the muscle to relax fully to the baseline (Fig. 3), the baseline length at the beginning of the first burst. The relaxation rate of each contraction was then evaluated, with a program custom-written in *Mathematica* (Wolfram Research, Champaign, IL), by fitting a single exponential to the relaxation phase of the contraction from 90% to 33% of the peak amplitude (see Fig. 1, *bottom right*), or, with the closely spaced contractions, to the beginning of the next motor neuron burst if that came earlier. Over this upper middle portion of the relaxation phase, most contractions were in fact well fitted by single exponentials. The relaxation rate was then the reciprocal of the time constant of the exponential.

Modeling

The dynamical model of the B15/B16-ARC neuromuscular system and its modulation was taken from Brezina et al. (2003a,b) and analytically solved or numerically integrated in *Mathematica*. Specific changes in parameters, dictated by the experimental results, were made as described in the figure legends. The relaxation rate of the simulated contractions was measured in the same way as that of the real contractions.

Results

Single motor neuron self-modulation is not weaker at higher temperature

In the B15/B16-ARC neuromuscular system *in vitro*, the simplest way to observe the effects of the released modulators is with the single motor neuron self-modulation paradigm, in which just one motor neuron is stimulated both to release the modulators and to elicit the contraction that they modulate. Fig. 3 shows a representative experiment of this kind. Motor neuron B16 was stimulated to fire in a repetitive bursting pattern—similar to that which it in fact fires in the intact feeding animal (Cropper et al. 1990a; Horn et al. 2004; see Fig. 12)—that elicited a sequence of contractions. These contractions progressively changed in shape, in amplitude (*continuous waveform*; axis at left) as well as relaxation rate (*large black points*; axis at right). Previous studies have strongly suggested that much of the change in amplitude and especially relaxation rate is due to the progressive development of the effects of the modulators that such a pattern of firing also releases (e.g., Whim and Lloyd 1990; Brezina et al. 2003b).

Since modulator release declines with increasing temperature (Fig. 2), we initially expected that the observed modulation, too, would be weaker at higher temperature. We were therefore surprised to find that this was not the case. Fig. 3 shows the same pattern of firing repeated, in the two ARC muscles of the same preparation, at 15° C (A) and at ~22° C (B). At the higher temperature, the progressive changes in shape were, if anything, more pronounced. The same result was obtained in two other experiments with motor neuron B16 as well as two experiments with motor neuron B15.

We did not analyze these results in any detail because, although the single motor neuron paradigm is simple, its results are difficult to interpret. As the motor neuron firing continues, all of the system components shown in Fig. 1—modulator release, modulator concentrations, the various modulator effects, as well as processes that shape the basal contraction such as presynaptic facilitation and potentiation of ACh release (Cohen et al. 1978) and postsynaptic summation of the contraction waveform itself—continue to evolve at different rates (Brezina et al. 2003a), in continuing interaction with each other. Each feature of the contraction shape thus has multiple interpretations.

Two motor neuron cross-modulation is not weaker at higher temperature

More readily interpretable are the results of the two motor neuron cross-modulation paradigm, first introduced in this system by Whim and Lloyd (1990). One motor neuron is stimulated to fire brief bursts at regular infrequent intervals, which release little or no modulator themselves (Cropper et al. 1990c; Vilim et al. 1996a; Brezina et al. 2003b) but elicit regular “monitoring” contractions. The other motor neuron is then fired in a relatively short, intense bursting pattern to release the modulators. This pattern inevitably elicits a contraction, too, but afterward the contraction rapidly relaxes while the effects of the released modulators, some of which are very long-lasting (Brezina et al. 2003a; see below), persist to modulate the continuing monitoring contractions.² Fig. 4A1 shows a representative experiment. Following the modulator-releasing firing of motor neuron B16, the monitoring contractions elicited by motor neuron B15 were increased in amplitude (*continuous waveform*; axis at left) as well as relaxation rate (*large black points*; axis at right), and remained so for many minutes. From what is known about the system (see Introduction), both effects were presumably due to the MMs released from motor neuron B16. When, in other experiments, the roles of the monitoring

²The persisting modulation is not simply a long-lasting consequence of the (often quite large) contraction elicited by the modulator-releasing firing. This was shown by Whim and Lloyd (1990), who reversibly applied hexamethonium, a blocker of the ACh receptors in the muscle that mediate the contraction, during the period of the modulator-releasing firing. This completely blocked the contraction elicited by the modulator-releasing firing, but when the hexamethonium was then washed out to bring back the monitoring contractions elicited by the other motor neuron, these were found to be modulated as usual.

and modulator-releasing motor neurons were reversed, similar increases in contraction amplitude and relaxation rate were observed, in that case presumably due to the SCPs released from motor neuron B15. Previous work has shown that the SCPs and MMs increase the relaxation rate in an identical fashion and have identical potentiating effects on contraction amplitude (Fig. 1; Brezina et al. 1996, 2003a).

Parts *A* and *B* of Fig. 4 show the same experiment repeated, in the two ARC muscles of the same preparation, at 15° C and at 25° C. Panels *A2* and *B2* compare in detail three contractions expanded from *A1* and *B1*, in particular a control contraction before the modulator-releasing firing (contraction 1) with the modulated contraction ~2 min after the end of the modulator-releasing firing (contraction 2), at each temperature. Clearly, the observed modulation was not weaker at the higher temperature. The modulation of the relaxation rate, especially, was significantly stronger.

Quantification of the modulation of relaxation rate

In our analysis of the results of the two motor neuron cross-modulation experiments, we focused primarily on the modulation of the relaxation rate, and only secondarily on that of contraction amplitude. Even in the cross-modulation paradigm, the modulation of contraction amplitude is still a composite process with multiple interpretations. On the potentiating effect of the SCPs and MMs, mediated by enhancement of Ca current in the muscle, is superimposed a simultaneous depressing effect, mediated by activation of a K current (Introduction, Fig. 1). Furthermore, together with the SCPs and MMs both motor neurons B15 and B16 co-release the buccalins, which depress contractions (Cropper et al. 1988, 1990b; Vilim et al. 1994; Orekhova et al. 2003). Thus no net change in contraction amplitude, for example, could mean no modulator release, or conversely robust modulator release with the potentiating effect of the released SCPs, or MMs, balanced by an equally strong depressing effect of the SCPs or MMs, or of the co-released buccalins.³ To fully understand how the modulation of contraction amplitude is affected by temperature, these various effects would have to be studied in isolation, each with its own paradigm in a different type of reduced preparation (see, e.g., Brezina et al. 1994a,b; 1995, 2003a; Orekhova et al. 2003).

In contrast, the modulation of the relaxation rate is, as far as is known, a unitary process, which furthermore is activated, in an identical fashion, just by the SCPs and MMs: the buccalins do not modulate the relaxation rate (Cropper et al. 1988; Vilim et al. 1994). Analysis of the relaxation rate has, however, its own difficulty. In different preparations, the initial, “unmodulated” relaxation rate of the ARC muscle—the control relaxation rate against which the modulation is then evaluated—can be very different (Brezina et al. 1995; see below and Figs. 5, 8). We attempted to manipulate the control relaxation rate by altering the load on the muscle, with limited success. Where possible we also made matched pairs of measurements, at 15° C and 25° C, in the two muscles of the same preparation or successively in the same muscle. This reduced the variability somewhat, but considerable variability still remained.

³Indeed, although usually the modulator-releasing firing increased the amplitude of the monitoring contractions as in Fig. 4, occasionally, when the modulator-releasing motor neuron was B16, the amplitude was at first decreased, for the first one or two monitoring contractions after the modulator-releasing firing, before increasing in the usual way. Based on what is known quantitatively about the modulatory effects and their interaction (Brezina et al. 1995, 1996, 2003a), this was almost certainly a sign, not of weak modulation, but of strong modulation. The potentiating (Ca-current) and depressing (K-current) effects combine asymmetrically so as to give net potentiation at low modulator concentrations and net depression at high concentrations (see, e.g., Fig. 7, *A* and *B*). The depressing effect has fast intrinsic dynamics, and so will reverse rapidly after the modulator-releasing firing as the released modulator concentration falls, while the potentiating effect, with slow dynamics, will persist. Finally, the depressing effect is activated particularly strongly by the MMs, released from motor neuron B16. Thus, very likely, the transient net depression occasionally observed after the modulator-releasing firing of motor neuron B16 reflected exceptionally strong release of the MMs and activation of the depressing (K-current) effect. By extension, the depressing effect probably contributed to the net modulation of contraction amplitude much more generally, even when it was hidden in the usual net potentiation.

For this reason, we have first of all plotted in Fig. 5A, from each of the 40 cross-modulation experiments that we performed, simply the modulated relaxation rate measured ~2 min after the end of the modulator-releasing firing (i.e., that of contraction 2 in Fig. 4) against the control relaxation rate (that of contraction 1 in Fig. 4). There is considerable scatter of the control relaxation rate values, along the horizontal dimension of Fig. 5A, as of the modulated values, along the vertical dimension. No modulation is represented by a diagonal line through the points of equal control and modulated value (*grey line* marked “0% increase in relaxation rate”). The *blue symbols* indicate the experiments performed at 15° C and the *red symbols* at 25° C, with modulator release from motor neuron B15 (*circles*) or B16 (*squares*). (The two motor neurons gave similar results and are lumped together in Fig. 5B and in the following discussion.) Fig. 5B then shows the same measurements of control and modulated relaxation rate as in Fig. 5A plotted separately along one dimension for statistical comparison. The *box* in each column indicates the mean $\pm$ SE. The *thin black lines* join the corresponding control and modulated values that are plotted against each other in Fig. 5A; the *thick black lines* join the means. Together, the plots in Fig. 5, A and B, show a number of noteworthy features.

First, it can be seen that the modulator-releasing firing always brought about an increase—never any significant decrease—in the relaxation rate. Essentially all points in Fig. 5A lie above the 0% diagonal line; the lines joining the corresponding control and modulated values in Fig. 5B all slant upward. In individual experiments the increase could be small (e.g., experiment *a* in Fig. 5A, lying close to the 0% diagonal line) or large (experiment *b*, with >3000% increase in the relaxation rate). The mean increase, however, was statistically highly significant both at 15° C and at 25° C (see Fig. 5 legend).

Previous work has suggested that a major factor that determines the size of the increase in relaxation rate in this kind of experiment is the control relaxation rate that the modulation acts upon (Brezina et al. 1995). If the control relaxation rate is small, the absolute increase (the change in units of s^{-1}), and still more the relative increase (the change in %), is large. If the control relaxation rate is already large, the absolute and the relative increase is small. In either case, the modulation tends to bring the relaxation rate to about the same absolute level. A natural explanation is that there is some upper limit, some maximal rate, beyond which the muscle cannot be made to relax any faster by the modulation under those conditions. To demonstrate this again here, we performed 6 experiments with the two motor neuron cross-modulation paradigm, firing motor neuron B15 to release the SCPs, at 15° C, but in the presence of a saturating concentration of one of the SCPs, 10^{-5} M SCP_B (see Lloyd et al. 1984; Brezina et al. 1996). These experiments are shown by the *open blue circles* in Fig. 5, A and B. The exogenous SCP increased—essentially tripled—the relaxation rate to $>1.5 s^{-1}$, whereupon the modulator-releasing firing had little further effect. All of these experiments lie close to the 0% diagonal line in Fig. 5A; there was no statistically significant difference between their control and modulated means in Fig. 5B (see legend). We can reasonably take the mean modulated relaxation rate in these experiments, $\sim 1.8 s^{-1}$, to be the presumed maximal relaxation rate at 15° C. This upper limit is indicated by the *horizontal dashed line* across Fig. 5, A and B. Note that all of the other experiments at 15° C fall below the line.

Consider now the experiments at 25° C (*red symbols*) in Fig. 5, A and B. Clearly, these often had modulated relaxation rates considerably larger than any experiment at 15° C, exceeding by a considerable margin the limit just discussed. The *green lines* in Fig. 5A indicate matched pairs of experiments, at 15° C and 25° C, in the two muscles of the same preparation. In some cases at least, the two muscles had similar control relaxation rates—for example, the pair *c-d*—and in each of these cases the modulated relaxation rate was larger at 25° C than at 15° C. Finally, although in Fig. 5B the mean control relaxation rate was somewhat larger at 25° C than at 15° C (see also Figs. 7–9), statistical analysis (see legend) found that the difference was

not statistically significant. The mean modulated relaxation rate, however, was substantially larger at 25° C than at 15° C, and this difference was highly significant.

It appears, therefore, that in the two motor neuron cross-modulation experiments the modulation of the relaxation rate was not only not weaker at the higher temperature, but on the contrary was significantly stronger.

The modulation of relaxation rate reverses more rapidly at higher temperature

In Fig. 4 it appears that although the modulation of the relaxation rate was stronger at 25° C than at 15° C, it reversed more rapidly after the end of the modulator-releasing firing (as, apparently, did the modulation of contraction amplitude). The group data in Fig. 6, averaging together the time courses of the modulation of the relaxation rate in all of the experiments (without exogenous SCP) in Fig. 5, bear this out. The faster reversal of the modulation at 25° C than at 15° C was statistically highly significant (see Fig. 6 legend).

Modulation of the relaxation rate by exogenous modulator is intrinsically stronger at higher temperature

So far, with both of our motor neuron stimulation paradigms, we have studied the modulation, that of the relaxation rate in particular, as one overall input-output process, Motor neuron firing → Relaxation rate modulation, and determined what we will call its *cumulative* temperature-dependence. The overall process is composed of a sequence of more elementary steps, namely Motor neuron firing → Modulator release → Modulator concentration → Relaxation rate modulation (Fig. 1). Each of these steps will have its own *intrinsic* temperature-dependence, which will then combine with that of the other steps to give the cumulative temperature-dependence of the overall process. To understand the origin of the cumulative temperature-dependence, we must therefore know the intrinsic temperature-dependence of the individual steps. We already know that of the first step, Motor neuron firing → Modulator release (Fig. 2). What about the intrinsic temperature-dependence of the subsequent steps, in particular the last step, Modulator concentration → Relaxation rate modulation, the actual modulatory effect itself?⁴

To examine this, we performed experiments in which we fired motor neuron B15 to elicit regular monitoring contractions as in the two motor neuron cross-modulation paradigm, but then, instead of stimulating motor neuron B16 to release the modulators, we exogenously applied different steady concentrations of a modulator, SCP_B, directly onto the ARC muscle. Fig. 7 shows the results of one series of these experiments in which we applied cumulatively increasing concentrations of SCP_B to determine its dose-response relations at 15° C and at 25° C. Fig. 7A shows representative contractions at the two temperatures in the presence of 10⁻⁹, 10⁻⁸, 10⁻⁷, 10⁻⁶, and 10⁻⁵ (“-9” ... “-5”) M SCP_B, superimposed in each case on a control contraction for comparison. Fig. 7B then plots the dose-response relations for the net modulation of peak contraction amplitude, and Fig. 7C the dose-response relations for the modulation of the relaxation rate, averaged from 5 experiments each at 15° C (*blue*) and 25° C (*red*).

On contraction amplitude, previous work has shown that the potentiating (Ca-current) and depressing (K-current) effects of the modulators combine asymmetrically so as to give net

⁴Clearly, each of the “elementary” steps mentioned here can be broken down further, and, in particular, the last step Modulator concentration → Relaxation rate modulation can be broken down to Modulator concentration → Muscle cAMP level → Relaxation rate modulation (Fig. 1). Nevertheless, we study here the Modulator concentration → Relaxation rate modulation step as a unit, primarily because we can easily manipulate the input to this step, modulator concentration, but not so easily the muscle cAMP level. For the same reason, also, the muscle cAMP level is not an explicit part of our model of the system (see Brezina et al. 2003a) with which we seek to reproduce our experimental results in Figs. 10 and 11.

potentiation at low modulator concentrations and net depression at high concentrations (see Brezina et al. 1995, 1996, 2003a). It appears from Fig. 7, *A* and *B*, that both the net potentiation at low SCP_B concentrations and the net depression at high SCP_B concentrations—and therefore, presumably, both of the underlying potentiating and depressing effects—were larger at 25° C than at 15° C. Indeed, high SCP_B concentrations often produced at 25° C a very substantial net depression (Fig. 7*A*), such as is only rarely seen at lower temperatures with the SCPs—although it is seen with the MMs, which activate a much larger amplitude of the K current in the muscle (Brezina et al. 1995, 1996).⁵

The modulation of the relaxation rate, too, was larger at 25° C than at 15° C (Fig. 7*C*). Indeed, it was much larger: as Fig. 7*C* shows, it was necessary to scale the 15° C dose-response relation up ~5-fold before it reproduced, quite well at all concentrations, the magnitude of the dose-response relation obtained at 25° C.⁶

In another series of these experiments, we applied just a single steady concentration of the modulator, 10⁻⁷ M SCP_B, and followed systematically the time course of the development of the modulation, and in some experiments also that of its reversal upon washout of the SCP_B, at 15° C and at 25° C. From these experiments, Fig. 8 first presents, in the same way as did Fig. 5, the magnitude of the fully developed modulation of the relaxation rate. Fig. 8*A* plots the modulated relaxation rate against the control relaxation rate in each experiment at 15° C (*blue circles*) and 25° C (*red circles*). The *green lines* join matched pairs of measurements. (In this series of experiments the matched measurements were made not just in the same preparation, but in the very same muscle with two applications of SCP_B separated by wash.) Fig. 8*B* then plots the same measurements, and their means, for statistical comparison. Again, the modulated relaxation rate was always much larger at 25° C than at 15° C. At 15° C the modulated relaxation rate was smaller, but at 25° C considerably larger, than the presumed maximal relaxation rate attainable at 15° C (*horizontal dashed line*, taken from Fig. 5). In Fig. 8*B*, the difference between the mean modulated relaxation rates at the two temperatures was statistically highly significant (see Fig. 8 legend). (In this series of experiments, unlike in Fig. 5, the difference between the mean control relaxation rates—larger at 25° C than at 15° C—was also significant.)

Fig. 9 summarizes the time course of the modulation of the relaxation rate in these experiments. Fig. 9*A* shows simply the averaged absolute relaxation rates at each timepoint during the development of the modulation in all of the experiments at 15° C (*blue*) and at 25° C (*red*). Fig. 9*B* shows the same data first normalized within each experiment, as in Fig. 6, to discard

⁵Potentially, the changes in contraction amplitude were a problem for the interpretation of the modulation of the relaxation rate, because the relaxation rate depends to some degree on the amplitude: small contractions tend to relax faster than large contractions (Brezina et al. 2003b). However, in these and indeed in most experiments in this paper, the changes in amplitude were relatively small (the magnitudes in Figs. 4 and 7, *A* and *B*, are typical), considerably smaller than the several-fold potentiation, or complete suppression of contractions, that can occur (see Brezina et al. 1996). In some of the experiments in Fig. 9, we explicitly tested to what extent the changes in contraction amplitude could account for the changes in relaxation rate, by adjusting, once the modulation had developed, the firing frequency of the contraction-eliciting motor neuron so as to return the amplitude of the modulated contraction back to the control amplitude. The change in relaxation rate nevertheless remained very substantial. Although there was indeed an inverse relationship between contraction amplitude and relaxation rate, its slope was relatively shallow, so that the changes in contraction amplitude could account for only a small part of the observed changes in relaxation rate. We therefore made no special attempt to allow for the changing contraction amplitude in our analysis of the modulation of the relaxation rate in this paper.

⁶Our treatment of the relaxation-rate data in Figs. 7–9 is based on the simplest conceptual model—then formalized in our actual model in Figs. 10–12—of the effect of temperature: that it is only the modulation that is temperature-dependent. Effects of temperature on the basal relaxation rate are neglected. With the data in Fig. 5 this treatment appears to be adequate, but the data in Figs. 7–9 suggest that the basal relaxation rate, too, has a significant temperature-dependence. A completely realistic model would need to postulate a separate temperature-dependence for each process; our model here lumps the temperature-dependence of the basal relaxation rate into that of the modulation. Functionally (e.g., in Fig. 12), the result will be similar in either case: since the modulation is coupled, through the firing of the motor neurons, to the basal contraction, the final modulated relaxation rate will have a similar cumulative temperature-dependence no matter how that cumulative temperature-dependence has been decomposed into the intrinsic temperature-dependencies of the processes within the system.

magnitude differences and allow comparison just of the time course shapes at the two temperatures. Fig. 9C shows, similarly normalized, the time courses of the reversal of the modulation upon washout of the SCP_B. To characterize the time courses quantitatively, we fitted the experimental data with best single-exponential fits (*solid black curves*) as follows. First, statistical analysis (see Fig. 9 legend) showed that the normalized time courses of the development of the modulation in Fig. 9B were not significantly different at 15° C and 25° C. We therefore fitted all of the data in Fig. 9B simultaneously with the same exponential, with time constant $\tau = 179$ s. With this time constant, fitting the unnormalized data in Fig. 9A then yielded the magnitudes of the fully developed modulation at 15° C and 25° C. In this series of experiments, the modulation was ~3.2-fold larger at 25° C than at 15° C. (This result was already implicit in Fig. 8, which used the same data.) Finally, statistical analysis—and just simple inspection—showed that the time courses of the reversal of the modulation in Fig. 9C were very significantly different at 15° C and 25° C. We therefore fitted the data in Fig. 9C, over the range of times shown, with two different exponentials at the two temperatures, yielding $\tau = 3,995$ s at 15° C and $\tau = 462$ s at 25° C. Thus, the modulation of the relaxation rate by the exogenous modulator was not only intrinsically larger at the higher temperature, but, just like the modulation by the endogenously released modulator in Figs. 4 and 6, it reversed more rapidly when the modulator was removed.

Reproduction of the observed temperature-dependence in a mathematical model of the system

A dynamical model of the B15/B16-ARC neuromuscular system, including the basal ACh-induced contraction and the major components of the modulation—all of the variables $X(t)$ in Fig. 1—was previously constructed from a variety of experimental data by Brezina et al. (2003a,b). We now modified the model to incorporate into it temperature-dependence for the two steps that we have focused on in this paper. From the experimental data in Fig. 2, we modeled the intrinsic temperature-dependence of the release of the modulators (for details see Fig. 2 legend). The *continuous curves* in Fig. 2 show the modeled release, decreasing, like the experimentally measured release, ~20-fold for a 10° C increase in temperature. From the experimental data in Figs. 7–9, we then modeled the intrinsic temperature-dependence of the modulation of the relaxation rate (for details see Fig. 10 legend). Fig. 7C suggests that the magnitude of the modulation might increase ~5-fold, while Fig. 9A suggests ~3.2-fold, for a 10° C increase in temperature. Conservatively, we modeled it as increasing 3-fold. We also modeled a 5-fold increase in the rate constants of the modulation, so that the reversal of the modulation, in particular, would proceed faster at higher temperature as observed in Fig. 9C. Again, the 5-fold increase was conservative: Fig. 9C suggests a >8-fold increase.

With the incorporation of the intrinsic temperature-dependencies of these two steps, the model was then well able to reproduce the cumulative temperature-dependence that we observed, in particular, in our two motor neuron cross-modulation experiments. Fig. 10 shows a simulation of an experiment like that in Fig. 4. After the firing of the modulator-releasing motor neuron, the modeled relaxation rate in Fig. 10, just like the experimentally measured rate in Fig. 4, increases considerably more at 25° C than at 15° C, but then falls back to the basal rate much faster.

The modulation of contraction amplitude in the simulation in Fig. 10—not only the net modulation but each of its component effects, which can be inspected in the model—is smaller at 25° C than at 15° C, whereas in the real experiment in Fig. 4 it is not smaller, and may even be somewhat larger. Because we did not characterize separately the intrinsic temperature-dependencies of each of the component effects, we could not incorporate them into the model as we did that of the modulation of the relaxation rate, but presumably the modeling of such increases of the effects with temperature as were observed in Fig. 7, A and B, would largely

correct the deficiency seen in Fig. 10. Even without this correction, nevertheless, it is remarkable in Fig. 10 that the modulation of contraction amplitude is not *grossly* incorrect. Even though the modulator release decreases 20-fold as the temperature increases from 15° C to 25° C, the modulation of contraction amplitude decreases only moderately. This is because the system incorporates a second type of temperature-compensating mechanism, quite distinct from the intrinsic temperature-dependencies of the various steps, as the following analysis of the model reveals.

Second, structural mechanism of temperature compensation: saturating curvature of dose-response relations

For consistency we will discuss the second mechanism as it operates, once again, in the particular case of the modulation of the relaxation rate, but from its principles it will be seen that the mechanism will operate similarly for all of the modulatory effects, all those that contribute to the modulation of contraction amplitude as well. In the case of the relaxation rate, that there must be an additional mechanism becomes clear when we consider the magnitudes of the intrinsic temperature-dependencies that we modeled above. Modulator release decreases 20-fold for a 10° C increase in temperature, whereas the modulation of the relaxation rate increases, intrinsically, only 3-fold. Cumulatively, when the modulation is due to the released modulator, we should therefore expect the modulation still to decrease, to a first approximation, ~20/3-fold—more than 6-fold—when the temperature is raised from 15° C to 25° C. Instead, we see in Fig. 10 that the modulation does not decrease, and even increases. An additional temperature-compensating mechanism must therefore be operating in the model and, presumably, in the real system. Furthermore, it is not a mechanism that we ourselves have explicitly incorporated into the model. It must be implicit in the structure of the model network—present, for example, even in the previous model of Brezina et al. (2003a,b) which had no explicit temperature-dependence at all.

To demonstrate the second mechanism more formally, in Fig. 11A we have analytically solved the model equations to plot the cumulative steady-state temperature-dependence from the level of motor neuron firing to each of the subsequent levels in the sequence leading to the modulation of the relaxation rate. In other words, for the same representative pattern of motor neuron firing throughout (see Fig. 11 legend), Fig. 11A plots the temperature-dependence of modulator release (i.e., of the step Motor neuron firing → Modulator release; *light grey curve*), modulator concentration (Motor neuron firing → Modulator concentration; *light grey curve* again, identical to that for modulator release), and finally the modulation of the relaxation rate (Motor neuron firing → Relaxation rate modulation), either without (*dark grey curve*) or with (*black curve*) the incorporation of the intrinsic temperature-dependence of the step Modulator concentration → Relaxation rate modulation that we modeled above. The *light grey curve* of the temperature-dependence of modulator release is simply the curve already seen in Fig. 2, along which modulator release decreases 20-fold for a 10° C increase in temperature. Because modulator concentration is, in this model, simply a linear reflection of modulator release (see Eq. 8 in Fig. 11 legend), its cumulative temperature-dependence is identical. But the next level of the model, the modulation of the relaxation rate, does not at all follow this dramatic decrease with temperature. Even without any intrinsic temperature-dependence of its own (*dark grey curve*), the modulation of the relaxation rate decreases only very moderately. The incorporation of the intrinsic temperature-dependence of the modulatory effect then converts the moderate decrease into a positive increase (*black curve*). But fold-wise, it is the second mechanism, revealed in the great difference between the cumulative temperature-dependence of the modulator concentration and that of the modulation of the relaxation rate, even without its own intrinsic temperature-dependence, that is most significant under these circumstances.

What precisely is the nature of this second mechanism? Consider Fig. 11B, which shows simply the intrinsic steady-state dose-response relation of the modulatory effect, the step Modulator concentration $\rightarrow$ Relaxation rate modulation. This quasi-hyperbolic relation is everywhere curved concave-down.⁷ Consequently any change in the input modulator concentration will result in a smaller relative change in the output relaxation rate modulation. For example, the point labeled “Actual at 15° C” is the steady-state point reached with the motor neuron firing used in Fig. 11A at 15° C. When the temperature is now increased to 25° C, there is a 20-fold decrease in modulator concentration, and, if the modulation of the relaxation rate simply reflected the modulator concentration linearly, we would expect also a 20-fold decrease in the modulation of the relaxation rate, to the point labeled “Expected at 25° C with linear dose-response relation.” Instead, along the curved dose-response relation, the modulation of the relaxation rate decreases much less, to the point labeled “Actual at 25° C.”

The *inset* of Fig. 11A extends the main plot to higher temperatures. Even at much higher temperatures, together the two temperature-compensating mechanisms operating in the system—the “first” mechanism of compensating intrinsic temperature-dependence and the second, structural mechanism—do a remarkably good job of maintaining the overall output, the modulation of the relaxation rate. From 15° C to ~27° C, as already mentioned, the modulation (*black curve*) actually increases. Above 27° C it begins to decrease, but even at 40° C—where modulator release and concentration have decreased 1,745-fold relative to 15° C—it still has not decreased below that at 15° C. Only above 40° C does this happen.

Temperature compensation during realistic feeding behavior

The power of the second, structural mechanism will decrease as the motor neuron firing frequency and hence modulator release and modulator concentration decrease, because the system will be operating less along the saturated, horizontal limb and more along the vertical—although still concave-down—limb of the dose-response relation in Fig. 11B. The firing frequencies of the motor neurons B15 and B16 recorded with chronically implanted electrodes in intact, feeding animals (Cropper et al. 1990a,c; Horn et al. 2004) are lower than the frequency used in Fig. 11A, and the released modulators probably reach concentrations only of the order of 100 nM (Brezina et al. 2003a). Furthermore, the analysis in Fig. 11 was done with a constant, regular motor neuron firing pattern in the steady state, whereas the firing patterns recorded *in vivo* are extremely variable and irregular (Horn et al. 2004; Zhurov et al. 2004) and the modulated neuromuscular system generally operates far from the steady state (Brezina et al. 2003a,b,2005). Is the temperature compensation still effective under these circumstances?

To answer this question, we ran our model with the motor neuron firing patterns recorded *in vivo*. Specifically, we used the firing patterns of the motor neurons B15 and B16 from an entire ~2½-h-long meal, comprising 749 cycles of the feeding behavior, recorded in an intact, freely feeding animal by Horn et al. (2004) and analyzed by Brezina et al. (2005). Fig. 12 shows the results. The two traces at the top of each of A and B are the 2½-h-long firing patterns of the two motor neurons, the inputs to the model. The traces below then show the resulting trajectories of the most relevant downstream variables of the model, namely the concentrations of SCP and MM, the modulation of the relaxation rate, and the final modulated contraction amplitude, at 15° C (A) and at 25° C (B). Clearly, increasing the temperature from 15° C to 25° C decreased the modulator concentrations so as to make them essentially invisible on the scale used in Fig. 12. Yet the modulation of the relaxation rate decreased hardly at all, and neither did the modulated contraction amplitude. (Note again that the model incorporated no intrinsic temperature-dependence for any component of the modulation of contraction

⁷The dose-response relation (Eqs. 9, 10 in Fig. 11 legend) is strictly hyperbolic only if the Hill coefficient h , here $h_{\mathbf{R}}$, is equal to 1; here $h_{\mathbf{R}} = 0.7$. The concave-down curvature of the dose-response relation, hence the consequences discussed here, holds for $h \leq 1$; for $h > 1$, it holds only over part of the domain of the dose-response relation.

amplitude; with such incorporation, the modulated contraction amplitude would probably have been as large at 25° C as at 15° C or even larger.) Fig. 12C summarizes measurements, comparable to those made in Figs. 3, 4 and 10, of the absolute relaxation rate of the simulated contraction in each of the 749 cycles of the meal. This final, most realistic measure of the relaxation rate was likewise well maintained at 25° C at a high level (*red*) not appreciably different from that at 15° C (*blue*). Furthermore, this maintenance was the result of the modulation: when the modulation of the relaxation rate was disabled in the model, the absolute relaxation rate decreased several-fold, at both 15° C and 25° C (*empty circles*).⁸ It appeared, therefore, that the temperature-compensating mechanisms operating in the model, and presumably in the real system, are indeed very effective even with the frequencies and patterns in which the motor neurons actually fire in real behavior.

Discussion

Temperature compensation in the B15/B16-ARC neuromuscular system of Aplysia

The basic experimental result of this paper is that, while modulator release from the ARC motor neurons decreases dramatically with increasing temperature (Fig. 2), the modulation of ARC muscle contraction amplitude and relaxation rate that is brought about by the released modulators does not decrease at all, and may even increase (Figs. 3–5).

The apparent paradox of this result requires us to reexamine our assumptions. Two potential straightforward explanations must be considered. First, it could be that, after all, modulator release does *not* decrease with increasing temperature. The data in Fig. 2 are sparse. However, they are part of an extensive, solid body of work by Vilim et al. (1996a,b,2000) in which modulator release was directly measured by radioimmunoassay rather than merely inferred from the subsequent effects of the released modulators. Furthermore, one of the early effects of the released modulators, elevation of cAMP in the ARC muscle, has likewise been reported to decrease with increasing temperature (Whim and Lloyd 1990; see further below).

Second, the possibility has to be considered that the observed changes in contraction amplitude and relaxation rate are not really due to the released modulators. A very extensive body of evidence (reviewed by Weiss et al. 1993; Brezina et al. 2003a; see Introduction) argues, however, that they are. The modulatory peptide genes are expressed by the motor neurons; the peptides are processed and are contained in dense-core vesicles in the motor neuron terminals within the ARC muscle; the modulators are released from these terminals onto the muscle fibers with physiological frequencies and patterns of firing of the motor neurons; exogenous application of the modulators, at the concentrations likely be achieved by the motor neuron firing, mimics the changes in contraction shape observed with the motor neuron firing. It has not yet been possible to carry out in a completely satisfactory manner the final demonstration, that blocking one of the earlier steps in this sequence—release of the modulators, ideally—blocks also the observed changes in contraction shape, due to the lack of specific pharmacological or other tools. (Indeed, our initial motivation for studying temperature-dependence was to develop such a tool, in the expectation that, if increased temperature so dramatically decreases the release of the modulators, it should of course decrease all of their effects too, indeed for practical purposes eliminate them to reveal just the basal, unmodulated contractions of the muscle. Evidently, this simple logic can fail.) However, the gap has to a large extent been bridged by modeling of the sort that we have continued in this paper, which has previously shown that, when the intrinsic characteristics of the individual steps in the modulatory sequence are modeled individually, joined together, and activated by the patterns

⁸Even without the modulation of the relaxation rate, the relaxation rates were slightly different at 15° C and 25° C in Fig. 12C because the modulation of contraction amplitude, still present, altered also to some extent the relaxation rate, in the model as in reality: see footnote 5.

of motor neuron firing that are used in experiments or recorded *in vivo*, the cumulative changes in contraction shape predicted by the model largely reproduce those that are actually observed (Brezina et al. 2003a,b, 2005). As a last point, we note that it would be difficult to explain how the two motor neuron cross-paradigm in Fig. 4 works if it is not through the release from each motor neuron of some modulatory substance that has long-lasting, general effects on contractions, even those elicited by the other motor neuron.

We believe therefore that we can rule out these two essentially artifactual explanations for our results. We can rule them out all the more easily because we have in this paper developed what we believe is the correct resolution of the apparent paradox. Experimentally, we have found that the final modulatory effects have very considerable intrinsic temperature-dependencies of their own, opposite to that of the modulator release (Figs. 7–9), so that as the modulator release decreases with increasing temperature, the modulatory effects intrinsically increase. At the same time, our modeling has revealed another, very different mechanism of temperature compensation that is built into the structure of the system. Because of the curvature of the intrinsic dose-response relations of the modulatory effects, a given decrease in modulator release and concentration decreases the modulatory effects much less (Fig. 11). The model incorporating both of these intrinsic mechanisms of temperature compensation is well able to reproduce the cumulative temperature-dependence of the modulation that is brought about, through the entire sequence of modulatory steps, by the motor neuron firing (Fig. 10). That is, even though one of the steps in the sequence, namely the release of the modulators, undergoes a dramatic decrease as the temperature increases, the final modulation that is observed does not decrease at all. Even though there are large intrinsic temperature-dependencies within the sequence, the cumulative final temperature-dependence is small.

We have focused here principally on the modulation of the relaxation rate and neglected that of contraction amplitude, largely for technical reasons: the modulation of the relaxation rate is, to all appearances, a single effect whereas that of contraction amplitude is distinctly composite. However, it is likely that the modulation of contraction amplitude is temperature-compensated by the same two mechanisms. There are indications in our data that the opposing effects that combine to give the net modulation of contraction amplitude—in particular, the enhancement of Ca current that potentiates contraction amplitude and the activation of K current that depresses it—each have their own intrinsic temperature-dependence, increasing, like the modulation of the relaxation rate, with increasing temperature (Fig. 7B). The Ca- and K-current effects' intrinsic dose-response relations, too, are shaped like that of the modulation of the relaxation rate (Brezina et al. 1996, 2003a).

Clearly, each of the “elementary” steps into which we have analytically decomposed the modulatory sequence here is itself a black box containing lower-level processes, each with its own intrinsic temperature-dependence. This is implied already by the fact that the rate of one of our “elementary” processes, namely the modulator release, *decreases* with increasing temperature. On thermodynamic grounds, we should expect all truly elementary reaction rates to increase with temperature. The temperature-dependence of the modulator release is therefore presumably the resultant of the temperature-dependencies of opposing lower-level processes, each increasing with temperature but with the “backward” rate—itsself probably the resultant of still lower-level processes—increasing more steeply than the “forward” rate. A similar explanation can be given for our attribution of temperature-dependence to the magnitude of the modulation (Figs. 7C, 9A; Fig. 10 legend), which is not a rate at all but presumably is the resultant of the rates of lower-level processes.

For the modulation of the relaxation rate, some of the lower-level detail can be sketched in. The step Modulator concentration → Relaxation rate modulation probably proceeds, in its early stages, through a conventional sequence of binding of the modulators, the SCPs and MMs, to

their respective receptors in the membrane of the ARC muscle fibers, activation of a G protein such as G_s , activation of adenylyl cyclase, and elevation of cAMP in the muscle fibers (Lloyd et al. 1984; Whim and Lloyd 1989, 1990; Brezina et al. 1994a; Hooper et al. 1994b). Whim and Lloyd (1990) have reported that, when a fixed concentration of SCP is exogenously applied, the resulting elevation of cAMP is identical at 16° C and 22° C. If so, then this entire early sequence of events, up to the elevation of cAMP, has no intrinsic temperature-dependence and cannot be the locus of the temperature-dependence that we have discovered here in Figs. 7–9. cAMP then activates cAMP-dependent protein kinase (PKA; Hooper et al. 1994a,b), which then phosphorylates the giant muscle protein twitchin (Probst et al. 1994; Heierhorst et al. 1994, 1995, 1996; see also Siegman et al. 1997, 1998; Yamada et al. 2001). The phosphorylation/dephosphorylation state of twitchin in the ARC muscle has been implicated in the control of the relaxation rate (Probst et al. 1994), although the precise mechanism remains unclear. Twitchin is a structural protein in the muscle, but it is also a kinase that, under the control of signals such as the cAMP-dependent phosphorylation as well as Ca^{2+} acting through Ca^{2+} -binding proteins such as calmodulin and S100 (Heierhorst et al. 1996), will phosphorylate, directly or indirectly by phosphorylating other kinases, other muscle proteins. With regard to the temperature-dependence, the activity of some molluscan PKAs has been reported to be temperature-dependent (e.g., Bardales et al. 2004), but probably much of the temperature-dependence that we have discovered will be found to originate somewhere within the late, complex steps involving twitchin. Certainly the very slow reversal of the modulation of the relaxation rate, which is strongly temperature-dependent (Fig. 9C), is not likely to be rate-limited by any such early process as a decay in free Ca^{2+} concentration, cAMP level, or even PKA activity, all of which decay much faster (Whim and Lloyd 1990; Probst et al. 1994; see Brezina et al. 2003a), but may well reflect a subsequent slow process such as dephosphorylation.

Temperature affects all biological processes, and a full appreciation of how the B15/B16-ARC neuromuscular system functions throughout the temperature range that the animal may encounter will require knowledge of the temperature-dependence also of all of the other important components of the system. For example, how does temperature affect the shape of the basal, unmodulated contractions? [There are indications that increasing temperature decreases their amplitude (Vilim et al. 1996a) and perhaps increases their relaxation rate (Figs. 7C, 8, 9A here).] How does temperature affect the firing frequencies and patterns of the motor neurons? Nothing is furthermore known about how the animal may, in the wild, acclimatize to different temperatures. Nevertheless, we believe that our results here have demonstrated, in basic outline at least, how the combination of the temperature-compensating mechanisms that are built into the structure of the system can maintain its output—the strength of the modulatory tuning and, as a consequence, the modulated contraction shape—relatively constant despite large changes in temperature. In our simulation with realistic motor neuron firing patterns (Fig. 12), the overall strength of the modulatory tuning varies hardly at all from 15° C to 25° C and perhaps even higher (Fig. 11A, *inset*). Since the different dimensions of the contraction shape—amplitude and relaxation rate, for instance—are the result of different processes with somewhat different cumulative temperature-dependence, the shape will not remain *exactly* identical at all temperatures, and the extent of its change will furthermore depend on the motor neuron firing frequency and pattern. However, these small residual changes in contraction shape are unlikely to matter functionally. In this neuromuscular system, the real motor neuron firing patterns and resulting contraction shapes do not appear to be precisely tailored to the specific behavioral demands in any particular cycle of the feeding behavior. Rather, they exhibit large quasi-random variability—much larger than the residual changes with temperature—from one cycle to the next (as can be seen in Fig. 12) so as to diversify, it is conjectured, the feeding movements in the face of environmental uncertainty (Horn et al. 2004; Brezina et al. 2005).

Temperature compensation in other systems

Since temperature affects all biological reactions, all animals that are active over a range of temperatures, unless they regulate their internal temperature, face this same problem with respect to each of their physiological systems, large and small: how to make the overall output of the system built from individual temperature-sensitive reactions sufficiently temperature-insensitive to maintain function throughout the temperature range. A well-known example is that of circadian oscillators, which cannot allow the period of the clock to change if the temperature changes (see, e.g., Roenneberg and Merrow 1999). The fact that temperature affects all reactions, and furthermore has—unlike, say, a neuromodulator—universal access to them all, at the same time provides plenty of material from which temperature compensation can be built. In the systems that have been investigated, it is interesting to note the extreme heterogeneity of the types of processes whose intrinsic temperature-dependencies complement each other to maintain the overall output. On different time scales, the temperature-dependencies of gene expression, biochemical and signal-transduction pathways, biophysical processes in neurons, synaptic physiology, muscle contractility, neuronal firing patterns and higher-order control strategies of the nervous system, and behavior can all play complementary roles (e.g., Montgomery and Macdonald 1990; Rall and Woledge 1990; Rome 1990; Heitler and Edwards 1998; Feder and Hofmann 1999; Neumeister et al. 2000; Pörtner 2002; Podrabsky and Somero 2004). In addition to this relatively self-evident mechanism of complementation of intrinsic temperature-dependencies, it is recognized (e.g., Montgomery and Macdonald 1990; see below) that the way in which the processes are coupled together—the structure of the network—also plays a major role. Temperature-compensatory mechanisms are thus of two types, an instance of each of which we have analyzed in this paper.

General lessons

Changing temperature is just one kind of stress that biological networks must resist (Roenneberg and Merrow 1999), and the mechanisms of temperature compensation have much in common with the general mechanisms that have been identified in many networks that make their output robust against dynamic noise, phenotypic parameter variation, and environmental perturbations of all kinds. Both the intrinsic characteristics of the processes within the network and the dynamic nonlinear properties that emerge from the way in which the processes are connected together are important. For example, recent work has particularly emphasized the role of negative feedback in the stabilization of circadian oscillators (e.g., Cheng et al. 2001; Smolen et al. 2001; Gonze et al. 2002; Vilar et al. 2002). However, as we have shown here, even in the absence of feedback merely the typical saturating shapes of the dose-response relations in the network exert a stabilizing influence, and this influence will grow as more such steps are connected in sequential cascades (see, e.g., Kenakin 1997).

At the same time, the dynamic interconnectedness of such networks makes it difficult to use simple linear logic to predict the outcome of their perturbation. Fox and Lloyd (2001) and Koh and Weiss (2005), citing the fact that modulator release decreases dramatically with increasing temperature in the ARC system, have used temperature to try to separate, in another buccal neuromuscular system and in the central nervous system of *Aplysia*, the subsequent effects of modulators released there from basal, modulator-independent effects. Effects that likewise decreased with increasing temperature were identified as potential effects of the released modulators; effects that did not decrease were not. We, too, initially wished to use this logic in the ARC system itself. There, however, the logic clearly fails: by it, we would have had to conclude that the effects on contraction amplitude and relaxation rate, very well established as due to the modulators by numerous other lines of evidence, were not modulator-dependent. Instead, our studies have led us to discover the temperature-compensating mechanisms that we have described in this paper. In view of the existence of such mechanisms, the use of

temperature to separate modulator-dependent from modulator-independent processes requires reconsideration.

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References

- Bardales JR, Díaz-Enrich MJ, Ibarguren I, Villamarín JA. Isoforms of cAMP-dependent protein kinase in the bivalve mollusk *Mytilus galloprovincialis*: activation by cyclic nucleotides and effect of temperature. *Arch Biochem Biophys* 2004;432:71–78. [PubMed: 15519298]
- Brezina V, Bank B, Cropper EC, Rosen S, Vilim FS, Kupfermann I, Weiss KR. Nine members of the myomodulin family of peptide cotransmitters at the B16-ARC neuromuscular junction of *Aplysia*. *J Neurophysiol* 1995;74:54–72. [PubMed: 7472354]
- Brezina V, Church PJ, Weiss KR. Temporal pattern dependence of neuronal peptide transmitter release: models and experiments. *J Neurosci* 2000a;20:6760–6772. [PubMed: 10995819]
- Brezina V, Evans CG, Weiss KR. Enhancement of Ca current in the accessory radula closer muscle of *Aplysia californica* by neuromodulators that potentiate its contractions. *J Neurosci* 1994a;14:4393–4411. [PubMed: 7913122]
- Brezina V, Evans CG, Weiss KR. Activation of K current in the accessory radula closer muscle of *Aplysia californica* by neuromodulators that depress its contractions. *J Neurosci* 1994b;14:4412–4432. [PubMed: 7913123]
- Brezina V, Horn CC, Weiss KR. Modeling neuromuscular modulation in *Aplysia*. III. Interaction of central motor commands and peripheral modulatory state for optimal behavior. *J Neurophysiol* 2005;93:1523–1556. [PubMed: 15469963]
- Brezina V, Orekhova IV, Weiss KR. Functional uncoupling of linked neurotransmitter effects by combinatorial convergence. *Science* 1996;273:806–810. [PubMed: 8670423]
- Brezina V, Orekhova IV, Weiss KR. Optimization of rhythmic behaviors by modulation of the neuromuscular transform. *J Neurophysiol* 2000b;83:260–279. [PubMed: 10634870]
- Brezina V, Orekhova IV, Weiss KR. Neuromuscular modulation in *Aplysia*. I. Dynamic model. *J Neurophysiol* 2003a;90:2592–2612. [PubMed: 12853443]
- Brezina V, Orekhova IV, Weiss KR. Neuromuscular modulation in *Aplysia*. II. Modulation of the neuromuscular transform in behavior. *J Neurophysiol* 2003b;90:2613–2628. [PubMed: 12853444]
- Brezina V, Weiss KR. Ca²⁺-activated K current in the ARC muscle of *Aplysia*. *J Neurophysiol* 1995;73:993–1004. [PubMed: 7608784]
- Carefoot TH. *Aplysia*: its biology and ecology. *Oceanogr Mar Biol Ann Rev* 1987;25:167–284.
- Cheng P, Yang Y, Liu Y. Interlocked feedback loops contribute to the robustness of the *Neurospora* circadian clock. *Proc Natl Acad Sci USA* 2001;98:7408–7413. [PubMed: 11416214]
- Church PJ, Whim MD, Lloyd PE. Modulation of neuromuscular transmission by conventional and peptide transmitters released from excitatory and inhibitory motor neurons in *Aplysia*. *J Neurosci* 1993;13:2790–2800. [PubMed: 8392535]
- Cohen JL, Weiss KR, Kupfermann I. Motor control of buccal muscles in *Aplysia*. *J Neurophysiol* 1978;41:157–180. [PubMed: 202682]
- Cropper EC, Kupfermann I, Weiss KR. Differential firing patterns of the peptide-containing cholinergic motor neurons B15 and B16 during feeding behavior in *Aplysia*. *Brain Res* 1990a;522:176–179. [PubMed: 2224515]
- Cropper EC, Lloyd PE, Reed W, Tenenbaum R, Kupfermann I, Weiss KR. Multiple neuropeptides in cholinergic motor neurons of *Aplysia*: evidence for modulation intrinsic to the motor circuit. *Proc Natl Acad Sci USA* 1987a;84:3486–3490. [PubMed: 3472218]
- Cropper EC, Miller MW, Tenenbaum R, Kolks MAG, Kupfermann I, Weiss KR. Structure and action of buccalin: a modulatory neuropeptide localized to an identified small cardioactive peptide-containing cholinergic motor neuron of *Aplysia californica*. *Proc Natl Acad Sci USA* 1988;85:6177–6181. [PubMed: 3413086]

- Cropper EC, Miller MW, Vilim FS, Tenenbaum R, Kupfermann I, Weiss KR. Buccalin is present in the cholinergic motor neuron B16 of *Aplysia* and it depresses accessory radula closer muscle contractions evoked by stimulation of B16. *Brain Res* 1990b;512:175–179. [PubMed: 2337805]
- Cropper EC, Price D, Tenenbaum R, Kupfermann I, Weiss KR. Release of peptide cotransmitters from a cholinergic motor neuron under physiological conditions. *Proc Natl Acad Sci USA* 1990c;87:933–937. [PubMed: 2153979]
- Cropper EC, Tenenbaum R, Kolks MAG, Kupfermann I, Weiss KR. Myomodulin: a bioactive neuropeptide present in an identified cholinergic buccal motor neuron of *Aplysia*. *Proc Natl Acad Sci USA* 1987b;84:5483–5486. [PubMed: 3474664]
- Cropper EC, Vilim FS, Alevizos A, Tenenbaum R, Kolks MAG, Rosen S, Kupfermann I, Weiss KR. Structure, bioactivity, and cellular localization of myomodulin B: a novel *Aplysia* peptide. *Peptides* 1991;12:683–690. [PubMed: 1788132]
- Evans CG, Vilim FS, Harish O, Kupfermann I, Weiss KR, Cropper EC. Modulation of radula opener muscles in *Aplysia*. *J Neurophysiol* 1999;82:1339–1351. [PubMed: 10482753]
- Feder ME, Hofmann GE. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annu Rev Physiol* 1999;61:243–282. [PubMed: 10099689]
- Fox LE, Lloyd PE. Evidence that post-tetanic potentiation is mediated by neuropeptide release in *Aplysia*. *J Neurophysiol* 2001;86:2845–2855. [PubMed: 11731541]
- Gev S, Achituv Y, Susswein AJ. Seasonal determinants of the life cycle in two species of *Aplysia* found in shallow waters along the Mediterranean coasts of Israel. *J Exp Mar Biol Ecol* 1984;74:67–73.
- Gomez D, Halloy J, Goldbeter A. Robustness of circadian rhythms with respect to molecular noise. *Proc Natl Acad Sci USA* 2002;99:673–678. [PubMed: 11792856]
- Heierhorst J, Kobe B, Feil SC, Parker MW, Benian BM, Weiss KR, Kemp BE. Ca^{2+} /S100 regulation of giant protein kinases. *Nature* 1996;380:636–639. [PubMed: 8602266]
- Heierhorst J, Probst WC, Kohanski RA, Buku A, Weiss KR. Phosphorylation of myosin regulatory light chains by the molluscan twitchin kinase. *Eur J Biochem* 1995;233:426–431. [PubMed: 7588784]
- Heierhorst J, Probst WC, Vilim FS, Buku A, Weiss KR. Autophosphorylation of molluscan twitchin and interaction of its kinase domain with calcium/calmodulin. *J Biol Chem* 1994;269:21086–21093. [PubMed: 8063728]
- Heitler WJ, Edwards DH. Effect of temperature on a voltage-sensitive electrical synapse in crayfish. *J Exp Biol* 1998;201:503–513. [PubMed: 9438826]
- Hooper SL, Brezina V, Cropper EC, and Weiss KR.** Flexibility of muscle control by modulation of muscle properties. In: *Beyond Neurotransmission: Neuromodulation and Its Importance for Information Processing*, edited by Katz PS. Oxford, UK: Oxford University Press, 1999, p. 241–274.
- Hooper SL, Probst WC, Cropper EC, Kupfermann I, Weiss KR. SCP application or B15 stimulation activates cAPK in the ARC muscle of *Aplysia*. *Brain Res* 1994a;657:337–341. [PubMed: 7820639]
- Hooper SL, Probst WC, Cropper EC, Kupfermann I, Weiss KR. Myomodulin application increases cAMP and activates cAMP-dependent protein kinase in the accessory radula closer muscle of *Aplysia*. *Neurosci Lett* 1994b;179:167–170. [PubMed: 7845614]
- Horn CC, Zhurov Y, Orekhova IV, Proekt A, Kupfermann I, Weiss KR, Brezina V. Cycle-to-cycle variability of neuromuscular activity in *Aplysia* feeding behavior. *J Neurophysiol* 2004;92:157–180. [PubMed: 14985412]
- Hurwitz I, Cropper EC, Vilim FS, Alexeeva V, Susswein AJ, Kupfermann I, Weiss KR. Serotonergic and peptidergic modulation of the buccal mass protractor muscle (I2) in *Aplysia*. *J Neurophysiol* 2000;84:2810–2820. [PubMed: 11110811]
- Keating C, Lloyd PE. Differential modulation of motor neurons that innervate the same muscle but use different excitatory transmitters in *Aplysia*. *J Neurophysiol* 1999;82:1759–1767. [PubMed: 10515965]
- Kenakin T.** *Pharmacologic Analysis of Drug-Receptor Interaction* (3rd ed). Philadelphia, PA: Lippincott-Raven, 1997.
- Koh H-Y and Weiss KR.** Peptidergic contribution to posttetanic potentiation at a central synapse of *Aplysia*. *J Neurophysiol* 10.1152/jn.00073.2005 (April 7, 2005), online, 2005.
- Kupfermann I. Feeding behavior in *Aplysia*: a simple system for the study of motivation. *Behav Biol* 1974;10:1–26. [PubMed: 4815142]

- Kupfermann I, Brezina V, Cropper EC, Deodhar D, Probst WC, Rosen SC, Vilim FS, and Weiss KR.** Reconfiguration of the peripheral plant during various forms of feeding behaviors in the mollusc *Aplysia*. In: *Neurons, Networks, and Motor Behavior*, edited by Stein PSG, Grillner S, Selverston AI, and Stuart DG. Cambridge, MA: MIT Press, 1997, p. 217–222.
- Kupfermann I, Carew TJ. Behavior patterns of *Aplysia californica* in its natural environment. *Behav Biol* 1974;12:317–337. [PubMed: 4447559]
- Lloyd PE, Kupfermann I, Weiss KR. Evidence for parallel actions of a molluscan neuropeptide and serotonin in mediating arousal in *Aplysia*. *Proc Natl Acad Sci USA* 1984;81:2934–2937. [PubMed: 6326155]
- Montgomery JC, Macdonald JA. Effects of temperature on nervous system: implications for behavioral performance. *Am J Physiol* 1990;259:R191–R196. [PubMed: 2201212]
- Neumeister H, Ripley B, Preuss T, Gilly WF. Effects of temperature on escape jetting in the squid *Loligo opalescens*. *J Exp Biol* 2000;203:547–557. [PubMed: 10637183]
- Orehkova IV, Alexeeva V, Church PJ, Weiss KR, Brezina V. Multiple presynaptic and postsynaptic sites of inhibitory modulation by myomodulin at ARC neuromuscular junctions of *Aplysia*. *J Neurophysiol* 2003;89:1488–1502. [PubMed: 12626624]
- Podrabsky JE, Somero GN. Changes in gene expression associated with acclimation to constant temperatures and fluctuating daily temperatures in an annual killifish *Austrofundulus limnaeus*. *J Exp Biol* 2004;207:2237–2254. [PubMed: 15159429]
- Pörtner HO. Physiological basis of temperature-dependent biogeography: trade-offs in muscle design and performance in polar ectotherms. *J Exp Biol* 2002;205:2217–2230. [PubMed: 12110656]
- Probst WC, Cropper EC, Heierhorst J, Hooper SL, Jaffe H, Vilim F, Beushausen S, Kupfermann I, Weiss KR. cAMP-dependent phosphorylation of *Aplysia* twitchin may mediate modulation of muscle contractions by neuropeptide cotransmitters. *Proc Natl Acad Sci USA* 1994;91:8487–8491. [PubMed: 8078908]
- Rall JA, Woledge RC. Influence of temperature on mechanics and energetics of muscle contraction. *Am J Physiol* 1990;259:R197–R203. [PubMed: 2201213]
- Roenneberg T, Merrow M. Circadian systems and metabolism. *J Biol Rhythms* 1999;14:449–459. [PubMed: 10643741]
- Rome LC. Influence of temperature on muscle recruitment and muscle function in vivo. *Am J Physiol* 1990;259:R210–R222. [PubMed: 2201215]
- Siegmán MJ, Funabara D, Kinoshita S, Watabe S, Hartshorne DJ, Butler TM. Phosphorylation of a twitchin-related protein controls catch and calcium sensitivity of force production in invertebrate smooth muscle. *Proc Natl Acad Sci USA* 1998;95:5383–5388. [PubMed: 9560285]
- Siegmán MJ, Mooers SU, Li C, Narayan S, Trinkle-Mulcahy L, Watabe S, Hartshorne DJ, Butler TM. Phosphorylation of a high molecular weight (~600 kDa) protein regulates catch in invertebrate smooth muscle. *J Muscle Res Cell Motil* 1997;18:655–670. [PubMed: 9429159]
- Smolen P, Baxter DA, Byrne JH. Modeling circadian oscillations with interlocking positive and negative feedback loops. *J Neurosci* 2001;21:6644–6656. [PubMed: 11517254]
- Vilar JMG, Kueh HY, Barkai N, Leibler S. Mechanisms of noise-resistance in genetic oscillators. *Proc Natl Acad Sci USA* 2002;99:5988–5992. [PubMed: 11972055]
- Vilim FS, Cropper EC, Price DA, Kupfermann I, Weiss KR. Release of peptide cotransmitters in *Aplysia*: regulation and functional implications. *J Neurosci* 1996a;16:8105–8114. [PubMed: 8987835]
- Vilim FS, Cropper EC, Price DA, Kupfermann I, Weiss KR. Peptide cotransmitter release from motoneuron B16 in *Aplysia californica*: costorage, corelease, and functional implications. *J Neurosci* 2000;20:2036–2042. [PubMed: 10684904]
- Vilim FS, Cropper EC, Rosen SC, Tenenbaum R, Kupfermann I, Weiss KR. Structure, localization, and action of buccalin B: a bioactive peptide from *Aplysia*. *Peptides* 1994;15:959–969. [PubMed: 7991459]
- Vilim FS, Price DA, Lesser W, Kupfermann I, Weiss KR. Costorage and corelease of modulatory peptide cotransmitters with partially antagonistic actions on the accessory radula closer muscle of *Aplysia californica*. *J Neurosci* 1996b;16:8092–8104. [PubMed: 8987834]

- Weiss KR, Brezina V, Cropper EC, Heierhorst J, Hooper SL, Probst WC, Rosen SC, Vilim FS, Kupfermann I. Physiology and biochemistry of peptidergic cotransmission in *Aplysia*. *J Physiol (Paris)* 1993;87:141–151. [PubMed: 7907908]
- Weiss KR, Cohen JL, Kupfermann I. Modulatory control of buccal musculature by a serotonergic neuron (metacerebral cell) in *Aplysia*. *J Neurophysiol* 1978;41:181–203. [PubMed: 621542]
- Whim MD, Lloyd PE. Frequency-dependent release of peptide cotransmitters from identified cholinergic motor neurons in *Aplysia*. *Proc Natl Acad Sci USA* 1989;86:9034–9038. [PubMed: 2554338]
- Whim MD, Lloyd PE. Neuropeptide cotransmitters released from an identified cholinergic motor neuron modulate neuromuscular efficacy in *Aplysia*. *J Neurosci* 1990;10:3313–3322. [PubMed: 1976768]
- Yamada A, Yoshio M, Kojima H, Oiwa K. An *in vitro* assay reveals essential protein components for the “catch” state of invertebrate smooth muscle. *Proc Natl Acad Sci USA* 2001;98:6635–6640. [PubMed: 11371623]
- Zhurov Y, Weiss KR, and Brezina V.** Intraburst variability of motor neuron spike timing shapes contractions of the ARC muscle of *Aplysia* Abstract 537.5, *2004 Abstract Viewer* Washington, DC: Society for Neuroscience, online, 2004.

Figure 1.

Schematic summary of the structure of the B15/B16-ARC neuromuscular system. The variables $X(t)$ are the variables of the dynamical model of the system from Brezina et al. (2003a,b) that we use here in Figs. 2 and 10–12. For details see Introduction, Methods, and *Reproduction of the observed temperature-dependence in a mathematical model of the system* in Results.

Figure 2.

Temperature-dependence of modulatory peptide cotransmitter release from motor neuron B15. The *points* replot the experimental data from Fig. 6B of Vilim et al. (1996a). Vilim et al. used radioimmunoassay to directly measure the total amounts of SCP (*filled points*) and buccalin (*open points*) appearing in the perfusate of the ARC muscle when motor neuron B15 was intracellularly stimulated to fire in a physiologically realistic bursting pattern, with intraburst firing frequency $f_{\text{intra}} = 12$ Hz, burst duration $d_{\text{intra}} = 3.5$ s, and interburst interval $d_{\text{inter}} = 3.5$ s, for a total length of firing $L = 10$ min, at 15°C, 17°C, and 19°C. Each point is a mean of $n = 4$ preparations, with SE smaller than the mean symbol size, normalized however within each preparation as follows. Because different preparations can release quite different absolute amounts of peptides (Vilim et al. 1996a,b; Brezina et al. 2000a), Vilim et al. expressed all of the data as percent of the grand total amount of peptide released in the entire series of repetitions of the motor neuron firing pattern at the three temperatures within each preparation. Here we have simply rescaled the plot of Vilim et al. to absolute peptide amounts by multiplying each percentage by the mean grand total amount of peptide released in the 4 preparations (F.S. Vilim, personal communication). Thus the small errors seen here reflect just the variance of the effect of temperature; the much larger variance between preparations has been normalized out. The *continuous curves* are best fits of our model of the release process (Brezina et al. 2000a, 2003a). To model the temperature-dependence, we use the standard concept of the temperature coefficient, Q_{10} , relating the value of variable X at temperature T to its value at 15°C by

$$X(T) = X(15^\circ\text{C}) Q_{10}^{(T-15)/10}. \quad (1)$$

With such a coefficient, $Q_{10,r}$, incorporated into the release equations to scale the instantaneous release, r (see Brezina et al. 2003a, Eq. 7 in Fig. 11 legend below), analysis of the model (see

Brezina et al. 2000a, analysis of Model II) shows that R , the total amount of peptide released by the entire block of firing of length L , as measured in these experiments by Vilim et al., is described by the equation (the equivalent of Eq. 15 of Brezina et al. 2000a)

$$R(L) = S_0(1 - \exp\{-Q_{10,r}^{(T-15)/10} p_{\infty} \Phi \langle f \rangle^y [L - \tau_p + \tau_p \exp(-L / \tau_p)]\}), \quad (2)$$

where S_0 is the initial size of the releasable peptide pool, $p_{\infty} = \langle f \rangle k_{p+} / k_{p-}$ and $\tau_p = 1/k_{p-}$ are respectively the steady-state value and the time constant of a slow reaction governing the probability of release, $\Phi = D^{1-y}$ is the pattern-dependence of the release, $D = d_{\text{intra}} / (d_{\text{intra}} + d_{\text{inter}})$ is the duty cycle of the firing pattern, and $\langle f \rangle = f_{\text{intra}} D$ is the mean firing frequency, with the parameter values [determined from other data of Vilim et al. (1996a,b) by Brezina et al. (2000a, 2003a)] $k_{p+} = 4.0 \times 10^{-10}$, $k_{p-} = 3.4 \times 10^{-3} \text{ s}^{-1}$, $y = 3$, and f_{intra} , d_{intra} , and d_{inter} as above. This leaves just $Q_{10,r}$, $S_{0,\text{SCP}}$, and $S_{0,\text{Buc}}$ as free parameters. Fitting Eq. 2 simultaneously to both the SCP and buccalin data yielded $Q_{10,r} = 5.05 \times 10^{-2}$ ($\approx 1/20$), $S_{0,\text{SCP}} = 628 \text{ fmol}$, and $S_{0,\text{Buc}} = 245 \text{ fmol}$, and the curves shown. [The value of $S_{0,\text{SCP}}$ found here is 16% larger, and the value of $S_{0,\text{Buc}}$ is 24% larger, than the consensus values of 541 and 198 fmol that were determined by Brezina et al. (2000a), presumably again because the different preparations used released different absolute amounts of peptides.]

Figure 3.

Single motor neuron self-modulation: representative experiment. Motor neuron B16 was fired at 13 Hz for 2 s every 5 s for 3 min, at $15^{\circ}C$ (A) and $\sim 22^{\circ}C$ (B). The *continuous waveform* in each case is the instantaneous length of the ARC muscle relative to the baseline length at the beginning of the motor neuron firing (axis at left; decreasing length, hence increasing contraction amplitude, is plotted upward); the *large black points* are measurements of the relaxation rate of the contraction elicited by each motor neuron burst (axis at right; see Fig. 1 and Methods). Left and right ARC muscles of the same preparation.

Figure 4.

Two motor neuron cross-modulation: representative experiment. Motor neuron B15 was fired at 25 Hz for 1 s every 100 s to elicit monitoring contractions, motor neuron B16 at 25 Hz for 4 s every 10 s for 60 s to release modulators, at 15°C (*A*) and 25°C (*B*). The *continuous waveform* in each of *A1* and *B1* is the instantaneous length of the ARC muscle (axis at left), the *large black points* are measurements of the relaxation rate of each contraction (axis at right). *A2* and *B2* expand the contractions 1–3 from *A1* and *B1*. Left and right ARC muscles of the same preparation.

Figure 5.

Two motor neuron cross-modulation: summary of measurements of the modulation of relaxation rate. *A*: the modulated relaxation rate, of the monitoring contraction ~2 min after the end of the modulator-releasing firing (i.e., contraction 2 in Fig. 4), is plotted against the control relaxation rate, of the monitoring contraction before the modulator-releasing firing (contraction 1 in Fig. 4). Motor neuron B16 was fired to elicit the monitoring contractions and motor neuron B15 to release the modulators, at 15°C (filled blue circles, $n = 15$) or 25°C (red circles, $n = 5$), or, conversely, B15 was fired to elicit the monitoring contractions and B16 to release the modulators, at 15°C (blue squares, $n = 8$) or 25°C (red squares, $n = 6$). The green lines join matched pairs of measurements, at 15°C and 25°C, in the two ARC muscles of the same preparation. The open blue circles are measurements from experiments in which motor neuron B16 was fired to elicit the monitoring contractions and motor neuron B15 to release the modulators, at 15°C, but in the presence of 10⁻⁵ M exogenous SCP_B ($n = 6$). *B*: the same measurements of control and modulated relaxation rate as in *A* plotted along one dimension for statistical comparison. The same symbols are used as in *A*; the motor neurons B15 and B16 are pooled. The box in each column indicates the mean $\pm$ SE. The thin black lines join the corresponding control and modulated values that are plotted against each other in *A*; the thick black lines join the means. Statistical significance was tested with two-way ANOVA followed by pairwise multiple comparisons using the Holm-Sidak test. Comparing the control with the

modulated mean under each of the three conditions, there were highly significant differences ($p < 0.001$) at 15°C and at 25°C, but no significant difference ($p > 0.05$) at 15°C in the presence of exogenous SCP_B. Comparing between conditions, there was no significant difference between the control means ($p > 0.05$, “n.s.”), but a highly significant difference between the modulated means ($p < 0.001$, “***”), at 15°C and 25°C. For further explanation see *Quantification of the modulation of relaxation rate* in Results.

Figure 6.

Two motor neuron cross-modulation: reversal of the modulation of relaxation rate. In each of the experiments in Fig. 5 (without exogenous SCP), the time series of the relaxation rates measured from each of the monitoring contractions from before the modulator-releasing firing to ~16 min after it (i.e., the span shown in Fig. 4, *A1* and *B1*) was normalized to between 0% and 100%, the smallest and largest values measured in that particular experiment. Plotted here are the means $\pm$ SE of the normalized time series at 15°C (blue; $n = 23$) and 25°C (red; $n = 11$). Statistical significance was tested with two-way ANOVA followed by pairwise multiple comparisons using the Holm-Sidak test. The overall difference between the 15°C and 25°C conditions was highly significant ($p < 0.001$); *** then indicates $p < 0.001$, and *, $p < 0.05$, for the difference between the 15°C and 25°C means at a particular timepoint.

Figure 7.

Modulation by exogenous modulator: dose-response relations for exogenous SCP_B. Motor neuron B15 was fired at 20 Hz for 1 s every 100 s to elicit monitoring contractions while increasing concentrations of SCP_B were applied to the ARC muscle. The concentrations were applied cumulatively, without intervening wash; each concentration was allowed to work for at least 8 min before the parameters of the final contraction were measured. Five preparations were used, with one of the muscles in each preparation tested at 15°C and the other at 25°C, but for statistical purposes they are here treated as independent experiments. **A**: representative contractions at 15°C and 25°C, in the two muscles of the same preparation. Shown are the final contractions at 10⁻⁹, 10⁻⁸, 10⁻⁷, 10⁻⁶, and 10⁻⁵ M SCP_B (“-9” ... “-5”), all superimposed on

the same control contraction for comparison. For simplicity, only the motor neuron B15 burst that elicited the control contraction, at 25°C, is shown in each case. *B*: mean $\pm$ SE of the peak contraction amplitude, normalized to the peak amplitude of the control contraction in each muscle, as a function of the SCP_B concentration, at 15°C (*blue*; $n = 5$) and 25°C (*red*; $n = 5$). *C*: mean $\pm$ SE of the relaxation rate as a function of the SCP_B concentration, at 15°C (*solid blue*; $n = 5$) and 25°C (*red*; $n = 5$). The *dashed blue curve* is the 15°C dose-response relation projected into the range spanned by the 25°C dose-response relation and multiplied by 5. For further explanation see *Modulation of the relaxation rate by exogenous modulator is intrinsically stronger at higher temperature* in Results.

Figure 8.

Modulation by exogenous modulator: magnitude of the modulation of relaxation rate by 10^{-7} M SCP_B . Figure constructed along the lines of Fig. 5. *A*: the modulated relaxation rate, of the final monitoring contraction elicited by motor neuron B15 after the SCP_B had been applied to the muscle and the modulation had fully developed (>16 min after the SCP_B application: see Fig. 9A), is plotted against the control relaxation rate. The *green lines* join matched pairs of measurements, made in the same muscle at 15°C (*blue circle*; $n = 10$) and 25°C (*red circle*; $n = 10$) (tested in either order in different muscles) with two applications of SCP_B separated by wash. *B*: the same measurements of control and modulated relaxation rate as in *A* plotted along one dimension for statistical comparison. Statistical significance was tested with two-way ANOVA followed by pairwise multiple comparisons using the Holm-

Sidak test. There were highly significant differences ($p < 0.001$) between the control and modulated means at each temperature as well as between temperatures (“***”).

Figure 9.

Modulation by exogenous modulator: time course of the modulation of relaxation rate by 10^{-7} M SCP_B . **A**: unnormalized time course of the development of the modulation. Means $\pm$ SE of the absolute relaxation rates of successive monitoring contractions elicited by motor neuron B15 before and during the application of SCP_B (grey block), at 15°C (blue, $n=10$) and 25°C (red, $n=10$). The labels “Fig. 8, control value” and “Fig. 8, modulated value” indicate where the measurements were taken for Fig. 8. The solid black curves are the best single-exponential fits, constrained in each case to have the time constant $\tau = 179$ s obtained from **B**, to the data at the two temperatures, yielding the magnitudes of the fully developed modulation, 3.24-fold larger at 25°C than at 15°C. **B**: normalized time course of the

development of the modulation. Same data as in *A* but first normalized to between 0% and 100%, the smallest and largest values measured within each experiment, as in Fig. 6. Tested with two-way ANOVA, the overall difference between the 15°C and 25°C conditions was not significant ($p > 0.05$). The *solid black curve* is the best single-exponential fit simultaneously to the data at both temperatures, with $\tau = 179$ s. *C*: normalized time course of the reversal of the modulation upon washout of the SCP_B. Means $\pm$ SE of the relaxation rates of monitoring contractions continued after the end of those measured in *A* and *B* in a subset of those experiments ($n = 3$ each at 15°C and 25°C), normalized as in *B*. Statistical significance was tested with two-way ANOVA followed by pairwise multiple comparisons using the Holm-Sidak test. The overall difference between the 15°C and 25°C conditions was highly significant ($p < 0.001$); *** then indicates $p < 0.001$, **, $p < 0.01$, and *, $p < 0.05$, for the difference between the 15°C and 25°C means at a particular timepoint. The *solid black curves* are the best single-exponential fits, over the range of times indicated, to the data at the two temperatures, with $\tau = 3,995$ s at 15° C and $\tau = 462$ s at 25° C.

Figure 10.

Two motor neuron cross-modulation reproduced with a mathematical model incorporating temperature-dependence. Here an experiment like that in Fig. 4 was simulated, with motor neuron B16 fired to elicit the monitoring contractions and motor neuron B15 to release the modulator (SCP). f , firing frequency; c , contraction (dimensionless). The model was taken from Brezina et al. (2003a,b) and used without modification except for the incorporation of temperature-dependence for two of its steps. The intrinsic temperature-dependence of modulator release was modeled from the experimental data in Fig. 2 as described in Fig. 2 legend. The intrinsic temperature-dependence of the modulation of the relaxation rate was modeled from the data in Figs. 7–9 as follows. In the model the modulator-induced increase in relaxation rate is described by the variable $\mathbf{R}$ (Fig. 1), which ranges from 0 to a maximal value, $\mathbf{R}_{\max}$ (Brezina et al. 2003a). $\mathbf{R}$ is then converted to an increase in the absolute relaxation rate of the muscle, in units of s^{-1} , through a coupling factor $\delta_{\mathbf{R}}$ (see Eq. 1h of Brezina et al. 2003b; here $\delta_{\mathbf{R}} = 0.05$). The dynamics of $\mathbf{R}$, when controlled by a single modulator such as SCP, are governed by the first-order differential equation

$$\frac{d\mathbf{R}(t)}{dt} = k_{\mathbf{R}^+} C'_{\text{SCP}}(t) [\mathbf{R}_{\max} - \mathbf{R}(t)] - k_{\mathbf{R}^-} \mathbf{R}(t) \quad (3)$$

where $C'_{\text{SCP}} \equiv C_{\text{SCP}}^{h_{\mathbf{R}}}$ is the concentration of SCP, C_{SCP} , modified by the Hill coefficient $h_{\mathbf{R}} = 0.7$, $k_{\mathbf{R}^+}$ and $k_{\mathbf{R}^-}$ are rate constants, and t is time. After a step at $t = 0$ to a new, maintained $C_{\text{SCP}}(t \geq 0)$ [with an intrinsic time constant of <30 s (Brezina et al. 2003a), the step in C_{SCP}

can be considered quasi-instantaneous on the time scale of the experiments in Fig. 9], Eq. 3 predicts a single-exponential rise (or fall) in R ,

$$R(t) = R_{\infty} - [R_{\infty} - R(t=0)]\exp(-t/\tau_R), \quad (4)$$

with steady-state value

$$R_{\infty} = \frac{C_{SCP} R_{\max}}{C_{SCP} + K_R}, \quad (5)$$

where $K_R \equiv k_{R^-}/k_{R^+}$, and time constant

$$\tau_R = \frac{1}{k_{R^+} C_{SCP} + k_{R^-}} \quad (6)$$

(see Eqs. 5 of Brezina et al. 2003a). To preserve the shape of the steady-state dose-response relation (Eq. 5) as modeled by Brezina et al. (2003a), furthermore apparently at all temperatures (Fig. 7C), the value $K_R = 3.64 \times 10^{-6} M^{h_R}$ of Brezina et al. (2003a) must be preserved at all temperatures. This restricts the choice of k_{R^+} and k_{R^-} and requires both to have the same temperature coefficient, Q_{10,k_R} . When $C_{SCP}(t \geq 0) = 0$, as in Fig. 9C, Eq. 6 yields $\tau_R = 1/k_{R^-}$. Thus the values of k_{R^-} at 15°C and 25°C, and so Q_{10,k_R} as well as, through the relationship $K_R = k_{R^-}/k_{R^+} = 3.64 \times 10^{-6} M^{h_R}$, the corresponding values of k_{R^+} , can in principle be immediately obtained from the time constants of the fitted exponentials in Fig. 9C. The ratio of these time constants implies $Q_{10,k_R} \approx 8.6$. However, the absolute values of the time constants are not entirely consistent with some of the previous values compiled by Brezina et al. (2003a), and in general not all of the available data can be completely accommodated within the simplifying framework of Eqs. 4–6 as discussed by Brezina et al. (2003a). We therefore selected a consensus set of values of k_{R^+} , k_{R^-} , and Q_{10,k_R} that appeared to represent the best compromise between the previous data of Brezina et al. (2003a) and the time constants in Fig. 9C, as well as Fig. 9, A and B, here. We set $k_{R^+}(15^\circ\text{C}) = 152.5 \text{ s}^{-1} M^{-h_R}$, $k_{R^-}(15^\circ\text{C}) = 5.55 \times 10^{-4} \text{ s}^{-1}$ (so that, still, $K_R = 3.64 \times 10^{-6} M^{h_R}$), and, conservatively, $Q_{10,k_R} = 5$. Finally, the temperature-dependent scaling of the magnitude of the modulation seen in Figs. 7C and 9A requires an additional temperature coefficient of $R_{\max}$, $Q_{10,R_{\max}}$. Fig. 7C suggests $Q_{10,R_{\max}} \approx 5$ and Fig. 9A $Q_{10,R_{\max}} \approx 3.2$; we set, conservatively, $Q_{10,R_{\max}} = 3$, with $R_{\max}(15^\circ\text{C}) = 100\%$.

Figure 11.

Second, structural mechanism of temperature compensation: analysis with the model. A: cumulative steady-state temperature-dependence at successive levels in the sequence Motor neuron firing → Modulator release → Modulator concentration → Relaxation rate modulation, illustrated with motor neuron B15 and SCP. (Motor neuron B16 and MM would present a qualitatively identical picture, although quantitatively the curves would be slightly different.) With input firing of motor neuron B15 at a steady frequency $f_{B15} = 10$ Hz, the model was solved analytically for the steady states of r_{SCP} , C_{SCP} , and R , the relevant successive variables in the sequence (Fig. 1). These steady states are given by the equations [taken from Brezina et al. (2003a) or derived from the master dynamical equations therein]

$$r_{SCP,\infty} = Q_{10,r}^{(T-15)/10} S_{0,SCP} P_{\infty} f_{B15}^Y \quad (7)$$

[S_{SCP} , the size of the releasable pool of SCP, was fixed at the initial size $S_{0,SCP}$ to eliminate the progressive depletion of the pool which would otherwise prevent the system from reaching any nonzero steady state (Brezina et al. 2003a)],

$$C_{SCP,\infty} = r_{SCP,\infty} / v k_{C,SCP} \quad (8)$$

where $v = 10 \mu\text{l}$ is the effective volume into which the SCP is released and $k_{C,SCP} = 0.1 \text{ s}^{-1}$ is the rate constant of SCP removal from v , and either

$$R_{\infty} = \frac{C_{SCP,\infty} R_{\max}}{C_{SCP,\infty} + K_R} \quad (9)$$

without any intrinsic temperature-dependence, or

$$R_{\infty} = \frac{Q_{10, R_{\max}}^{(T-15)/10} C'_{SCP, \infty} R_{\max}}{C'_{SCP, \infty} + K_R}, \quad (10)$$

incorporating the intrinsic temperature-dependence of R that was modeled in Fig. 10 legend. (Variables and parameters not defined here have already been introduced, and the specific parameter values used here have been given, in Fig. 2 and 10 legends.) Eqs. 7–10 are plotted here as a function of the temperature T , normalized in each case to the magnitude at 15°C; Eqs. 7 and 8, when normalized both yielding the same curve, are shown by the *light grey curve*; Eq. 9, by the *dark grey curve*; and Eq. 10, by the *black curve*. The *inset* extends the temperature range of the main plot to 40°C. *B*: intrinsic steady-state dose-response relation of the step Modulator concentration $\rightarrow$ Relaxation rate modulation, i.e., plot of R_{∞} , as % of $R_{\max}$ or the temperature-scaled $R_{\max}$ (either Eq. 9 or 10), as a function of C_{SCP} [or C_{MM} : the intrinsic dose-response relations of the two modulators are identical (Brezina et al. 1996,2003a)]. For further explanation see *Second, structural mechanism of temperature compensation: saturating curvature of dose-response relations* in Results.

Figure 12.

Temperature compensation during realistic feeding behavior, simulated with the model. The model was run at 15°C (A) or 25°C (B). The two traces at the *top* of each of A and B, identical at the two temperatures, are the inputs to the model, the instantaneous firing frequencies of the motor neurons B15 and B16 extracted by Brezina et al. (2005) from the electrical activity recorded with an electrode chronically implanted in the ARC muscle by Horn et al. (2004) during a ~2½-h-long meal, comprising 749 cycles of the feeding behavior, in an intact, freely feeding animal. The traces *below* show the resulting trajectories of the most relevant downstream variables of the model (see Fig. 1): the SCP and MM concentrations C_{SCP} and C_{MM} , the modulation of the relaxation rate R , and the modulated contraction amplitude c . C: mean $\pm$ SD of the absolute relaxation rate measured from each of the 749 cycles of the meal, at 15°C (blue) and 25°C (red), with the full modulation (filled circles) or with the modulation of the relaxation rate disabled (e.g., with $R(t) = 0$; empty circles). The intrinsic temperature-dependencies of MM release and of the modulation of the relaxation rate by MM were modeled to be the same as for SCP; all other aspects of the model not already given in Fig. 2, 10, and 11 legends were as described by Brezina et al. (2003a,b).