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J Neurophysiol 78:2951-2965, 1997.

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Neural Circuit Mediating Tentacle Withdrawal in *Helix aspersa*, With Specific Reference to the Competence of the Motor Neuron C3

STEVEN A. PRESCOTT, NISHI GILL, AND RONALD CHASE
Department of Biology, McGill University, Montreal, Quebec H3A 1B1, Canada

Prescott, Steven A., Nishi Gill, and Ronald Chase. Neural circuit mediating tentacle withdrawal in *Helix aspersa*, with specific reference to the competence of the motor neuron C3. *J. Neurophysiol.* 78: 2951–2965, 1997. The tentacle withdrawal reflex in the terrestrial snail *Helix aspersa* involves bending and retraction of the tentacles. When elicited by mechanical stimulation of the tentacle, the reflex is mediated by the conjoint action of the central and peripheral nervous systems. The neural circuit underlying the stimulus-response pathways was studied in vitro using a combination of morphological and physiological techniques. Sensory input caused by stimulation of the nose (situated at the superior tentacle's tip) first passes into the tentacle ganglion. Motor fibers are likely excited in the tentacle ganglion to form a peripheral stimulus-response pathway. While still in the tentacle ganglion, the excitation caused by a brief stimulus is transformed into a prolonged neuronal discharge. This modified signal travels, via the olfactory nerve, to the cerebral ganglion where it excites the giant motor neuron C3 along with numerous smaller motor neurons. Afferent input to C3 also arrives from several other sources. The afferent convergence is followed by a marked divergence of C3's output. C3 innervates the muscles mediating both tentacle retraction and tentacle bending through multiple cerebral nerves. Thus C3's pattern of effector innervation allows this single cell to elicit and coordinate both components of the tentacle withdrawal reflex. Lesion experiments indicate that C3 is responsible for 85% of the central contribution to tentacle retraction, though C3 is actually sufficient to mediate maximal muscle contraction as evidenced by intracellular stimulation. In addition to C3, three groups of putative central motor neurons were identified through nerve backfills and nerve recordings. The additional motor neurons mediating tentacle retraction are important for maximizing the rate of muscle contraction, whereas those mediating tentacle bending are likely more important for nondefensive behaviors. These neurons are arranged in parallel with C3, but unlike C3, each of these neurons innervates only a single effector or portion thereof. Given C3's direct innervation of multiple effectors and its sufficiency to evoke strong responses in those effectors, we conclude that C3 is paramount in eliciting and coordinating tentacle withdrawal.

INTRODUCTION

The terrestrial snail *Helix aspersa* has two pairs of tentacles. An olfactory epithelium (nose) subserving the snail's principal sense for distant perception (Chase 1986) is situated at the tip of each superior tentacle. With the nose thus exposed, it is important for the snail to be able to rapidly withdraw its tentacles. Withdrawal is effected by tentacle retraction and, to a lesser degree, by tentacle bending. When stimulated repeatedly, the reflex exhibits a complex combination of habituation and sensitization (Balaban 1993; Christoffersen et al. 1981; Prescott and Chase 1996). The relative simplicity of the neural circuit mediating tentacle withdrawal should allow investigation of the interaction between these opposing learning processes (Prescott and Chase 1997). However, the motor control of tentacle movements has not yet been described in detail, and without such a description, an explanation of the reflex's plastic changes is impossible.

The focus of this paper is on reflexes evoked by tentacular stimulation (i.e., local reflexes). As with other mollusks such as *Aplysia* (Kupfermann et al. 1971; Peretz 1970; Perlman 1979), *Spisula* (Prior 1972), and *Tritonia* (Hoyle and Willows 1973), the peripheral nervous system (PNS) of *H. aspersa* is well developed and capable of mediating local reflexes in conjoint action with the CNS (Nonne 1925). Lesion experiments in *Helix* showed that the PNS is capable of mediating between 45 and 75% (depending on response magnitude) of the total tentacle withdrawal reflex (Prescott and Chase 1996). Evidence from the present experiments indicates that the CNS is also very effective in mediating muscle contraction (more than one would have expected from the aforementioned lesion experiments) and acts to increase the rate and duration of the response. Furthermore, the CNS is important for mediating more generalized head and foot retractions, which occur with sufficiently noxious stimulation (Balaban 1993; Zakharov 1992).

The peripheral stimulus-response (S-R) pathway mediating tentacle retraction remains poorly understood. Peripheral motor neurons have not been identified and are believed to be absent (Hanström 1925; Rogers 1968). The only innervation of the distal musculature is thought to be by axons of central motor neurons arriving via the olfactory nerve (OlN) and the tentacle ganglion (Hanström 1925; Zaitseva 1991). These fibers probably receive synaptic input in the tentacle ganglion and thereby constitute the final elements of the peripheral S-R pathway (see also Bullock 1965).

The central S-R pathway is studied more easily. A single motor neuron, named C3, has been identified (Cottrell et al. 1983; Zakharov et al. 1982). C3 has a broad receptive field and is particularly sensitive to chemical and mechanical stimulation of the nose (Chase and Hall 1996). C3 is very effective at mediating muscle contraction (Bewick et al. 1990; Cottrell et al. 1983; Zakharov et al. 1982) and does so using a divergent efferent signal to multiple muscles via multiple nerves. In the present study, we have further elucidated the motor competence and quantified its contribution to the withdrawal reflex. Bewick et al. (1990) showed that C3's motor control of the tentacle retractor muscle (TRM) is through a monosynaptic circuit, independent of other central motor neurons. However, putative motor neurons additional to C3 have been labeled by tentacle retractor nerve (TRN)

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backfills (Zakharov et al. 1982). The present experiments investigate the possible roles of these cells.

Although tentacle retraction is the most robust component of tentacle withdrawal, it is accompanied by tentacle bending (Lemaire and Chase 1997; Zakharov 1992). Retraction and bending are mediated by different muscles, the TRM and the tegumental muscle, respectively (tegumental muscle = skin muscle, Zakharov 1992; tentacle musculature, Peschel et al. 1996). The TRM and the tegumental muscle are separate except where they converge at the tentacle tip, thus allowing tentacle inversion during retraction (Wondrak 1977). Although the innervation of these muscles is via different nerves (Schmalz 1914), both muscles are innervated by C3, suggesting that C3 may be important for the coordinated excitation of these muscles. As with the TRM, the tegumental muscle also is innervated by central motor neurons additional to C3. The role of these central motor neurons and their relationship with C3 and with non-C3 motor neurons mediating tentacle retraction were investigated.

METHODS

The experiments were performed on mature specimens of the common garden snail *H. aspersa* (>3 g including shell and with curled shell margins) originating from Santa Barbara, CA. Exceptions occurred in some morphology preparations where immature snails (<0.5 g) were used to reduce the distance between C3's soma and its axon terminals. The CNS was removed from the animal, partially desheathed, and pinned to a silicone elastomer (Sylgard)-coated dish. At least one superior tentacle was left attached via the OlN and the TRN. All other nerves were cut except as noted in RESULTS. The tissues were bathed in a saline solution that contained (in mM) 80 NaCl, 4 KCl, 7 $CaCl_2$, 5 $MgCl_2$, 1.5 glucose, and 5 tris(hydroxymethyl)aminomethane (tris)-HCl buffer, at pH 7.8. The start of physiological experimentation was delayed ≥30 min after the end of the dissection.

Morphology

For intracellular labeling of C3, its soma was penetrated with a micropipette (40–100 MΩ) filled with either 8% Neurobiotin (Vector Labs) in 0.1 M tris-HCl buffer (pH 7.4) or 5% biocytin (Sigma) in 0.5 M potassium acetate (pH 7.4). Injection was achieved by iontophoresis and processing followed standard methods (details in Gill 1996). Preparations were viewed as whole mounts. For counting axon profiles, nerves were embedded in Spurr, cut in 1-μm-thick cross sections, stained with toluidine blue, and mounted in Permount. Nerve backfills were performed by sucking the cut end of the nerve into a glass pipette containing 5% biocytin in 0.5 M potassium acetate. The pipette was left in place for 6–16 h.

Physiology

The tentacle was pinned at its distal end to immobilize the nose. Mechanical stimulation of the nose was effected by directing a jet of saline using a three-way solenoid valve (Prescott and Chase 1996). Four different stimulus strengths were achieved by adjusting the pump flow rate to 0.23, 0.32, 0.41, or 0.65 ml/s. Very light stimulation was achieved by switching the solenoid without through flow. In all cases, the duration of stimulation was 1 s and the interstimulus interval was 4 min.

The proximal end of the TRM was attached to a force transducer (Grass FT0.03C) using a bent hook. The TRM was stretched to a length of 5 mm to standardize resting tension, and contractile force was measured isometrically. For intracellular recordings, pipettes contained 2 M potassium acetate (5–35 MΩ). For extracellular recordings from nerves, pipette tips were broken and fire polished. Suction was used to take up the nerve either en passant or at the cut end. The physiological data were digitized and stored on computer (Digidata 1200 A/D converter and Axotape 2.0.2 software, both from Axon Instruments).

Direct driving (intracellular stimulation)

Direct driving of C3 was achieved by intracellular current injection in such a way as to recreate a natural spiking pattern. A cell's response to mechanical stimulation of the nose was recorded on FM tape. This was played back through a low-pass filter to remove the action potentials, leaving only the synaptic depolarization. The signal was injected into the micropipette with a variable gain. In some cases, to increase the initial firing frequency, an arbitrarily modulated DC signal was recorded and played back. It should be pointed out that the natural firing frequency was too high to allow for replication by triggering each spike individually with a depolarizing pulse. The spike train generated in C3 was considered a sufficiently accurate recreation of the sensory evoked response if two criteria were satisfied: first, the total spike count was within 10% of that produced by mechanical stimulation; second, the firing frequency during the first second of activity was within 25% of the sensory evoked response. The above criteria were applied by comparison to a spike train produced by mechanical stimulation immediately before the direct driving. For direct driving after a TRN lesion, the stimulus waveform for all trials was the same as that used just before the lesion.

Lesions

Lesions to nerves were produced by cutting. Previously described controls demonstrate that this technique does not cause any appreciable plasticity in the reflex (Prescott and Chase 1996). Lesions to C3 were achieved through two different methods. In the first, large hyperpolarizing currents (30–45 nA) were injected into the cell body. Although this method eliminated spikes from the somatic recording site, the status of spikes initiated and conducted in distal neurites was uncertain (later nerve recordings verified the efficacy of lesions produced by this method). Therefore, a photoinactivation procedure was used alternatively (adapted from Elliott and Kleindienst 1990). Cells were injected with a 3% solution of Lucifer yellow (Molecular Probes) for ≥15 min or until the cell body and the main axon fluoresced brightly. Photoinactivation was effected using blue light of 442 nm from a helium-cadmium laser (Model 456-10 s, Omnichrome). The soma and axon loop were illuminated until the cell depolarized >30 mV above the baseline resting potential and all spontaneous activity was eliminated (~20 min).

Because hyperpolarization lesions are reversible, three pairs of reflex responses were recorded by alternating between the intact state and the lesion state. The percent reflex loss was calculated for each pair of reflex responses and then averaged across the three pairs. For the irreversible photoinactivation lesions and lesions to nerves, averages were calculated for three responses recorded before the lesion and for three responses recorded after the lesion, and the percent reflex loss was calculated from these averages.

Statistical analysis

Statistical results are presented as means ± SE. Nonlinear curve fitting with logistic functions was done using Sigmastat 1.03 (Jandel Scientific), whereas other regressions and *t*-tests were per-

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formed using Sigmaplot 2.01 (Jandel Scientific). A level of insignificance refers to $P > 0.05$.

RESULTS

Gross anatomy of tentacular muscles and cerebral nerves

A schematic representation of the left cerebral ganglion and its innervation of muscles associated with the superior tentacle is shown in Fig. 1. The olfactory epithelium (nose) and the eye are located at the tip of the tentacle together with the tentacle ganglion. The tentacle tip is pulled into the body when the TRM contracts. The proximal portion of the TRM eventually connects with the columellar muscle. Rapid contractions are characteristic of the darkly colored distal fibers, whereas slower, more tonic contractions are characteristic of the lightly colored proximal fibers (ten Cate and Verleur 1952). Innervation of these regions is also different: the most distal part is innervated by motor fibers from the tentacle ganglion (see INTRODUCTION); the middle portion is innervated by the TRN and, to a lesser degree, the olfactory nerve; the most proximal part is innervated by the columellar muscle nerve originating from the pleural ganglion (Zakharov 1992). The tegumental muscle is a thin sheet of muscle underlying the skin of the tentacle that mediates tentacle bending (Peschel et al. 1996) as well as controlling tentacle length without inversion (Wondrak 1977). The tegumental muscle is innervated, on its medial aspect, by the internal peritentacular nerve (PtNi), and on its lateral aspect, by the external peritentacular nerve (PtNe) (Peschel et al. 1996).

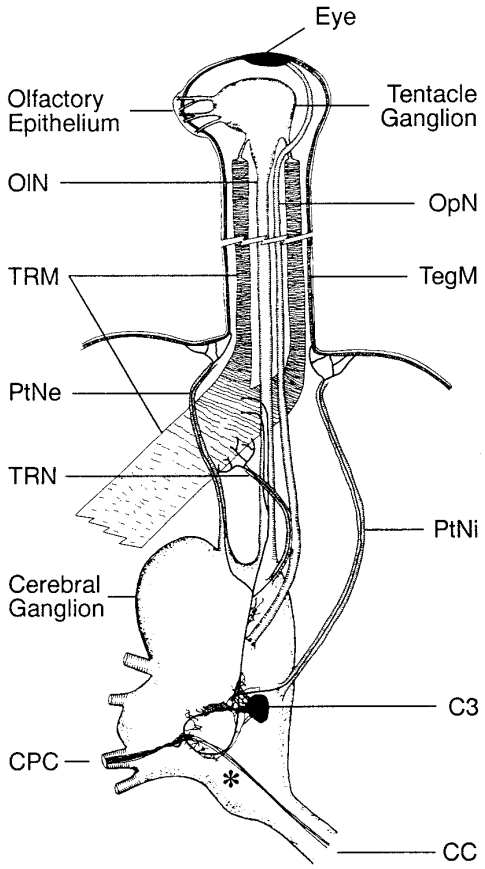

FIG. 1. Anatomy relevant to the tentacle withdrawal reflex. Left superior tentacle is shown with its innervation from the ipsilateral cerebral ganglion. An idealized representation of the motor neuron C3 is also shown. Tentacle retractor muscle (TRM) is innervated by the tentacle retractor nerve (TRN) and the olfactory nerve (OlN); the tegumental muscle (TegM) is innervated by the internal peritentacular nerve (PtNi) and the external peritentacular nerve (PtNe) on the medial and lateral sides of the tentacle, respectively. * General position of the cell bodies of non-C3 motor neurons involved in tentacle withdrawal. Structures are not drawn to scale. CC, cerebral commissure; CPC, cerebropedal connective; OpN, optic nerve.

Previous descriptions of the cerebral nerves (Ierusalimsky et al. 1992; Zakharov et al. 1982) have recognized that the optic nerve consists of two branches, sensory and motor, which innervate the eye spot and the TRM, respectively. Careful observations made here indicate that the ''branches'' not only innervate different sites, but also exit the procerebrum at different positions, and though the branches may sometimes run together in close apposition, analysis of nerve cross sections indicates that they remain as separate nerves. The optic nerve motor branch is clearly a misnomer, and we refer to it in this paper as the tentacle retractor nerve.

Morphology of the motor neuron C3

C3's morphology has been briefly described by Chase and Hall (1996), Cottrell et al. (1983), and Zakharov et al. (1982); those descriptions are supplemented herein (see also Gill 1996). C3 is the second largest cell in the cerebral ganglion, with a soma diameter of ~110 μm. The cell has a distinguishing looped main process along which dense dendritic branching occurs (Fig. 1). The extensive dendritic arborization is consistent with C3's high level of synaptic input (Winlow and Kandel 1976), and the widespread distribution of these dendrites lends morphological validity to the fact that there is a high degree of afferent signal convergence. Zakharov et al. (1982) reported that C3 receives input from the olfactory nerve, the internal lip nerve, the external lip nerve, the statocyst nerve, the cerebropedal connective, and the cerebropleural connective on the ipsilateral side. From observations of synaptic depolarization in response to nerve stimulation, we now can add to the above list, input from the following: the medial lip nerve, the external peritentacular nerve, and the internal peritentacular nerve on the ipsilateral side. C3 also receives weak input from the contralateral tentacles, but the two bilaterally located C3 neurons are not connected (Cottrell et al. 1983; B. Hall and R. Chase, unpublished data).

C3's axonal projections are also impressive (Fig. 1). Tentacle withdrawal involves the coordinated contraction of retractor and tegumental muscles, each of which is innervated by different nerves. C3 itself innervates both of these muscles, the TRM via the TRN and the OlN and the tegumental muscle via the PtNe and PtNi (all 4 projections were reliably present in 14 labeled cells). C3's axons branch proximally, in or near the CNS (Fig. 1), as well as distally, near the muscles (Figs. 1 and 2). It is assumed that the degree of distal branching reflects the degree to which the cell is able to excite a muscle; therefore, it is interesting to note that the pattern of branching varies consistently between different nerves. C3 axons exit the OlN at multiple places along its length and innervate the TRM without branching (Fig. 2*A*). In contrast, C3 axons traveling in the TRN (Fig. 2*B*), the

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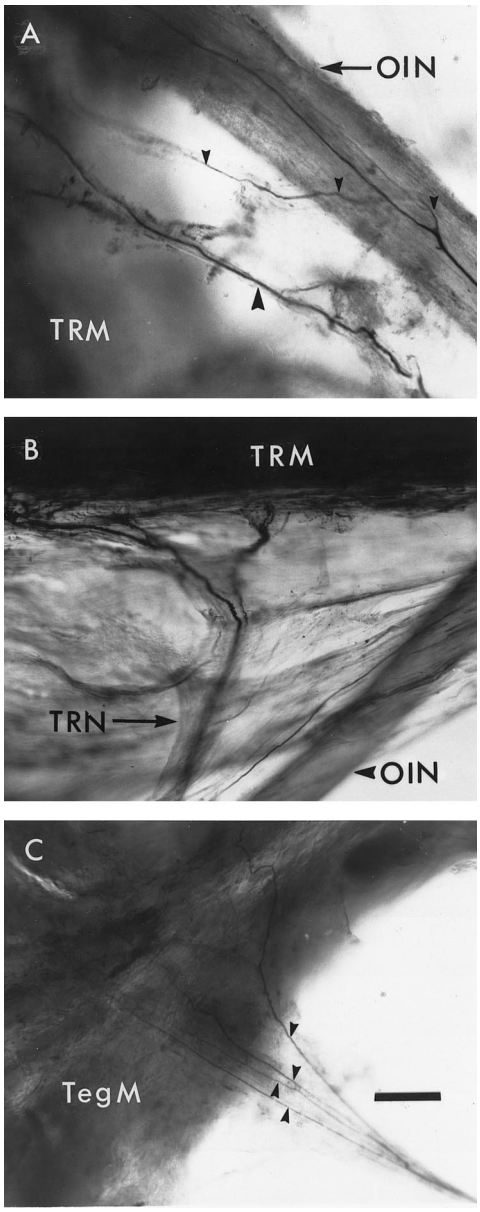

FIG. 2. Innervation of tentacle muscles by the neuron C3 via cerebral nerves. *A*: OlN. Fine branches emerge from the main C3 axon (small arrowheads), exit the OlN, and innervate the TRM without extensive branching at the muscle. Large arrowhead, a C3 axon that exits the OlN outside the frame of the micrograph. Scale bar = 100 μm. *B*: TRN. At the terminus of the TRN, the C3 axon branches extensively onto the TRM. OlN is out of the plane of focus. Scale bar = 150 μm. *C*: PtNe. As in *B*, the C3 axon branches extensively at the nerve terminus (arrowheads) and innervates the tegumental muscle. Scale bar = 100 μm.

PtNe (Fig. 2*C*), and the PtNi (data not shown) branch extensively at the nerve terminus.

In addition to axonal projections into multiple ipsilateral nerves important for tentacle withdrawal, fibers in the cerebral commissure and the cerebropedal connective were labeled by intracellular C3 fills. These central projections have not been previously reported, and they were not consistently observed in our preparations (cerebral commissure, 50%; cerebropedal connective, 90%). When present, however, both projections were characterized by multiple branchings near the main C3 axon (Fig. 1). The fibers' ultimate destinations could not be determined, and though one may speculate that they could be important for generalized head and foot retraction, they are not involved in tentacle withdrawal per se so they are not discussed further (see Gill 1996). In addition to the commissural projections and the cerebropedal connective projections, there may be outputs from C3 that remain in the ipsilateral cerebral ganglion. Because these are difficult to study morphologically, physiological methods were employed to test the possibility that C3 may synapse on other central motor neurons involved in tentacle withdrawal (see following text).

Morphology of non-C3 central motor neurons

Zakharov et al. (1982) reported a cluster of neurons located in the postcerebrum, roughly posterior to C3, that were labeled by TRN backfills; those results are replicated herein and reported in finer detail. Soma counts in five backfilled preparations yielded 21.4 ± 3.5 cell bodies in the ipsilateral postcerebrum (Fig. 1, *). In three other preparations, the TRN was examined histologically in semi-thin cross-sections. The counts of axon profiles in these preparations (22.0 ± 0.6) closely match the counts of somata. The size of these cells is variable: about half the cells have cell bodies ~40 μm in diameter and tend to be clustered together, whereas the remaining cells measure roughly half that size and are scattered more widely. Despite this, all the fibers tend to enter the TRN through a common fascicle, which also contains C3's axon.

Cells in the same region also were labeled through backfills of the PtNe (n = 4) and the PtNi (n = 3). In the former case, 22.3 ± 4.5 cells bodies were labeled; in the latter, 27.0 ± 10.5 cells bodies were labeled. These cell bodies were not tightly clustered, but they still tended to be localized to the postcerebrum. In some cases, there were one or two cells located conspicuously anterior. Cells labeled through peritentacular nerve backfills varied within the same size range as those labeled through TRN backfills.

Backfills of the OlN (n = 6) also labeled putative motor neurons in the postcerebrum. We counted 15.2 ± 3.2 cell bodies with diameters of ~20 μm.

Motor competence of central and peripheral pathways mediating tentacle retraction

To relate C3's activity to the behavioral reflex, C3 was driven by intracellular current injection to produce a spike train equivalent to that recorded during sensory stimulation (Fig. 3*A*). This procedure elicited a peak muscle tension that was 76.2 ± 8.8% of tension generated during a normal reflex response (Fig. 3*B*). After a TRN lesion, direct driving of C3 could generate only 23.9 ± 6.4% of the reflex muscle tension. The substantial reduction in the efficacy of direct driving after the lesion indicates that the TRN carries the majority of the efferent signal from C3 to the TRM. An analysis of reflex responses after lesions confirms this view. The reflex response amplitude was 70.9 ± 10.0% of the intact reflex after a TRN lesion and hardly was altered (68.0 ± 13.2%) after a subsequent OlN lesion (Fig. 3*B*). This result is consistent with the morphological data that show a more elaborate innervation of the TRM via the TRN

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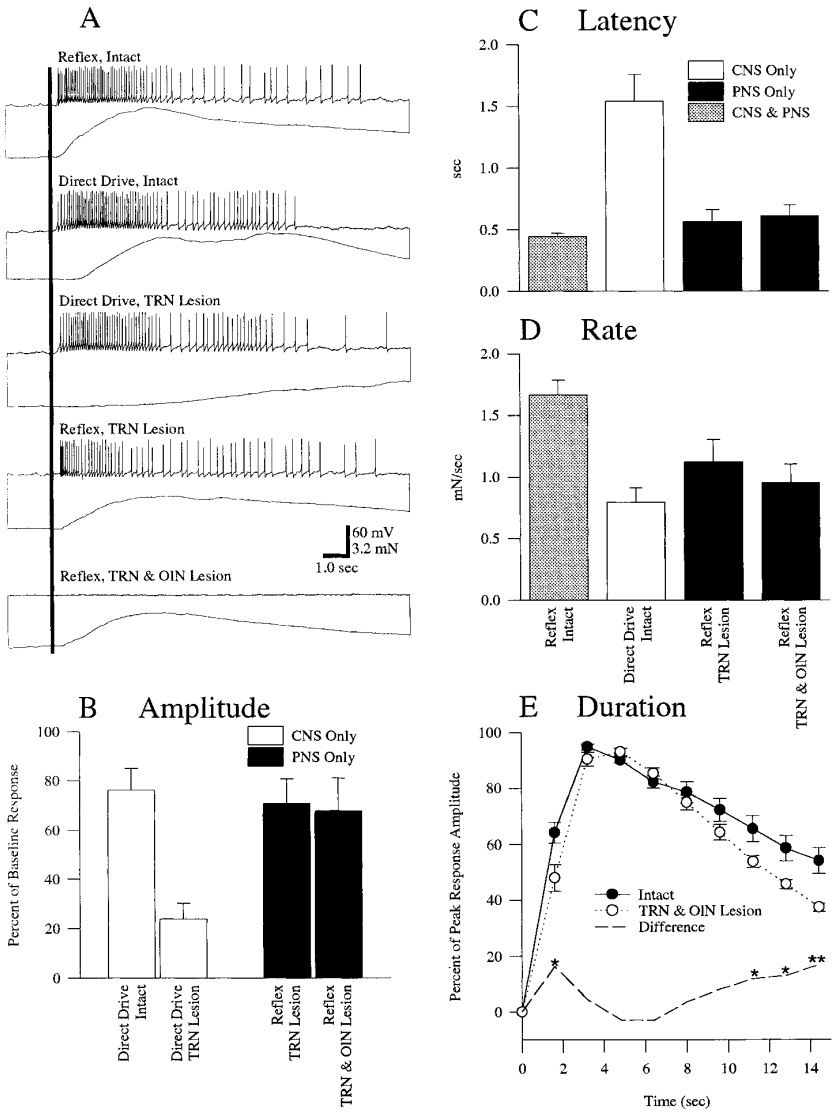

FIG. 3. Motor competence of the central and peripheral pathways mediating tentacle retraction. *A*: sample traces of reflex responses and muscle contractions induced by direct driving of C3 under various conditions. *Top*: C3; *bottom*: TRM. Thick vertical line marks the onset of stimulation. All traces are from the same preparation. Mechanical stimulus strength in all cases was 0.32 ml/s. Each condition shown here was repeated 3 times and averaged in each animal tested ($n = 5$). *B*: amplitude of muscle response. Peak tensions are expressed as a percentage of the reflex response amplitude in the same, intact, preparation. Bar shading shown here, and in the following parts of the figure, is interpretative. *C*: latency of muscle response to develop 0.5 mN tension. Bar labels correspond to those in *D*. Time was measured from the first action potential in the C3 response. Mean latency from mechanical stimulus onset to the first action potential was 277 ± 72 ms. *D*: rate of muscle response measured as the slope of the muscle trace between 0.1 and 1.1 mN tension. *E*: duration of muscle response. Muscle tension was measured at 1.6-s intervals, normalized to the peak tension in that response, and averaged across the 5 animals for each of the conditions. *Bottom curve* shows the difference between the 2 conditions. Unpaired *t*-tests: $*P < 0.05$; $**P < 0.02$.

than via the OlN (Figs. 1 and 2, *A* and *B*). In contrast to these efferent projections, the OlN is necessary to carry the afferent signal from the nose to C3 (Fig. 3*A*), whereas neither the TRN nor the optic nerve carry any detectable amount of afferent information to C3 (data not shown).

Although peak contraction amplitude is a useful way of reporting the efficacy of S-R pathway components (Fig. 3*B*), the shape of the muscle trace also changes depending on the status of the circuitry, as quantified in Fig. 3, *C–E*. For instance, the integrity of the nerve pathways affects the latency of TRM contraction (Fig. 3*C*). The latency from the onset of spiking in C3 to a reflex contraction of 0.5 mN in an intact preparation is 0.444 ± 0.027 s, whereas the latency for the same contraction increases significantly to 1.544 ± 0.217 s when elicited by direct driving of C3 ($P < 0.001$; unpaired *t*-test). Lesions of the TRN cause a further, large increase in latency with direct driving (7.046 ± 0.937 s; example shown in Fig. 3*A*) but no significant increase for the reflex contraction (0.568 ± 0.097 s). As expected, lesions of the OlN likewise did not significantly increase the latency of the reflex contraction (0.614 ± 0.091 s). The analysis of response latency thus suggests that the earliest phase of the muscle contraction is mediated solely by peripheral pathways, whereas the later phase is influenced by the CNS. However, response rate data (described later) are not wholly consistent with this conclusion.

It is not obvious why the latency of C3's action on the muscle should be as high as 1.5 s, given that action potentials are conducted to the muscle in ~80 ms (Bewick et al. 1990), but data consistent with our own observations have been reported by Zakharov et al. (1982), who suggested that C3 exerts an early occult action on the muscle. Another explanation is to attribute an early central contribution to motor neurons other than C3. This would account for the fact that the initial slope of the muscle trace (before 1.5 s) is greater when the CNS is intact than after it is lesioned (Fig. 3*A*). We undertook the measurement of contraction rate to investigate this issue.

The rate of contraction is defined as the slope of the muscle trace between 0.1 and 1.1 mN (Fig. 3*D*). The rate of reflex contraction in the intact preparation was 1.67 ± 0.12 mN/s. Direct driving of C3 elicited a lower rate (0.80 ±

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0.12 mN/s); nonetheless, this rate is substantial and reflects the fact that C3's motor effect is delayed in onset but not necessarily slow to act once started. Rates of reflex contraction after a TRN lesion or a combined TRN and OlN lesion were intermediate (1.12 ± 0.18 and 0.96 ± 0.15 mN/s, respectively), but significantly lower than the rate in intact preparations ($P < 0.05$; unpaired *t*-test). Contractions under these conditions occur at the normal, relatively short latencies. This is taken to indicate that the central S-R pathway is necessary to maximize the rate of muscle contraction, and non-C3 central motor neurons may be largely responsible.

As for the later stages of muscle contraction, it is noteworthy that tension can be maintained long after the end of stimulation, either in the presence or the absence of the CNS (Fig. 3*A*). This implies either that a prolonged response is characteristic of the muscle fibers or that peripheral pathways are sufficient to supply continued excitation. However, because C3 continues to fire long after the stimulus (Fig. 3*A*), it also may contribute to the maintenance of tension. The physiological basis for prolonged activity in C3 and other neurons in the S-R pathway is considered later.

To identify subtle differences in the central and peripheral influences on duration, contraction amplitude was normalized to peak tension, measured at 1.6-s time intervals, and then averaged for each time interval measurement to give a muscle response profile for each condition. The response profiles for reflexes with and without the CNS are compared in Fig. 3*E* to identify the phases of the response in which there were significant differences in tension. Tension is significantly different ($P < 0.05$; unpaired *t*-test) at 1.6 s, which is consistent with the contraction rate data presented above. Tensions are also significantly different at the last three measured times ($P < 0.05$ and $P < 0.02$; unpaired *t*-tests). This late divergence of the two curves in Fig. 3*E* indicates that the central S-R pathway increases the duration of the muscle response above what the peripheral S-R pathway is capable of mediating.

Additivity of central and peripheral pathways mediating tentacle retraction

To investigate some of the issues raised by the results presented in Fig. 3, experiments were performed to see whether the loss in reflexive muscle response caused by the selective removal of C3 from the circuit was equivalent to the muscle response caused by the selective stimulation of C3. Figure 4*A* shows representative responses at two stimulus strengths, 0.23 and 0.41 ml/s. Statistical comparison of the *left* and *right* bars of each pair shown in Fig. 4*B* indicates that the only significant difference between the Reflex and the sum of Reflex, C3 Lesion plus Direct Drive occurs when the CNS is intact, and then, only in response to strong stimulation ($P < 0.001$, paired *t*-test shown on Fig. 4*B*). The lack of linear additivity (i.e., the significant difference between *left* and *right* bars) at high stimulus strengths is explained by saturation of the muscle response (Prescott and Chase 1996). When the TRN is lesioned, peak muscle tension is reduced (see following text) and therefore response saturation is eliminated; under these conditions, additivity is linear.

Lesion experiments again show that the TRN carries most of the information from C3 to the TRM. Comparing the

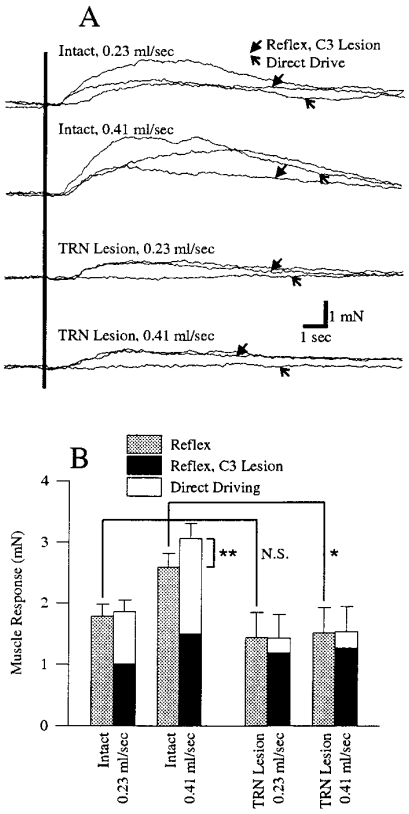

FIG. 4. Additivity of the central and peripheral pathways mediating tentacle retraction. *A*: sample traces of muscle responses under various conditions. In each group of 3 traces, the unmarked trace reaching the greatest amplitude shows the reflex with C3 intact; the reflex response after a C3 lesion and the direct drive response are marked (→). Stimulus flow rates are indicated. All traces are from the same preparation. Thick line marks the onset of stimulation. *B*: comparison of the mean reflex response (*left bar* in each pair) and the mean sum of Reflex, C3 Lesion response and Direct Driving response (*right bar* in each pair) under conditions shown in *A*. Sums greater than the Reflex response (*right bar* > *left bar* within a pair) are indicative of response saturation. Results of paired *t*-tests comparing bars within each pair are insignificant unless marked otherwise on the graph. $n = 5$ for intact preparations; $n = 3$ for TRN lesion preparations. Results of unpaired *t*-tests between intact and TRN lesion conditions shown on the graph are indicative of the increased importance of central mediation for larger responses. *t*-tests: NS, $P > 0.05$; $*P < 0.05$; $**P < 0.001$.

effects of TRN lesions at the two stimulus strengths, the reduction in the Reflex response is only significant at the higher stimulus strength ($P < 0.05$, unpaired *t*-tests shown on Fig. 4*B*). Similarly, the Direct Driving response is significantly decreased by the TRN lesion only at the higher stimulus strength ($P < 0.02$, unpaired *t*-test). These results suggest that the central S-R pathway becomes increasingly important in the mediation of the response as the stimulus strength is increased. This conclusion is supported by comparing the two pairs of bars for intact preparations on Fig. 4*B*, where the Direct Driving increases more than does the Reflex, C3 Lesion. Thus although mediation of the reflex by the PNS increases with increasing stimulus strength, mediation by the CNS increases to a greater extent.

Contribution of C3 to the central pathway mediating tentacle retraction

To quantify C3's contribution to the central component of the reflex, its contribution to the total reflex was first

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determined by hyperpolarization or photoinactivation procedures, and then, in the same preparation, the central component was determined by the loss of response after removing the CNS (Fig. 5*A*). C3's contribution to the central component is the ratio of these two reflex losses. Because response amplitudes can saturate due to mechanical factors (see preceding text), it was necessary to take saturation into account so as not to underestimate C3's contribution. This was accomplished by adding, to the Intact response amplitudes (Fig. 5*B*), a value equal to the difference between the mean Intact; Reflex amplitude and the mean Intact; Reflex, C3 Lesion + Direct Driving amplitude as shown in the *left* and *right* bars of each pair, respectively, of Fig. 4*B*. The correction factors determined in this way were 0.07 and 0.48 mN for 0.23 and 0.41 ml/s, respectively.

Using hyperpolarization lesions, it was found that C3 contributes 83.9 ± 5.8% of the central component of the reflex when tested with a stimulus of 0.41 ml/s (Fig. 5*B*). With photoinactivation lesions, C3 contributes 84.7 ± 4.4%. Because the difference between these two values is not significant (unpaired *t*-test), the results were combined to calculate an estimate of 84.3 ± 3.4% for C3's contribution to the central component. Tests were also conducted with a weaker stimulus to determine whether C3's contribution remains constant even as the central component declines relative to the peripheral component (Prescott and Chase 1996). Using a stimulus of 0.23 ml/s, and only hyperpolarization lesions, C3's contribution to the central component was found to be 88.9 ± 5.0% (Fig. 5*B*), which is not significantly different from 84.3% (unpaired *t*-test). Thus taking the mean of these two percentages, we calculate a final estimate of 85.3 ± 2.8% for C3's contribution to the central component of the reflex.

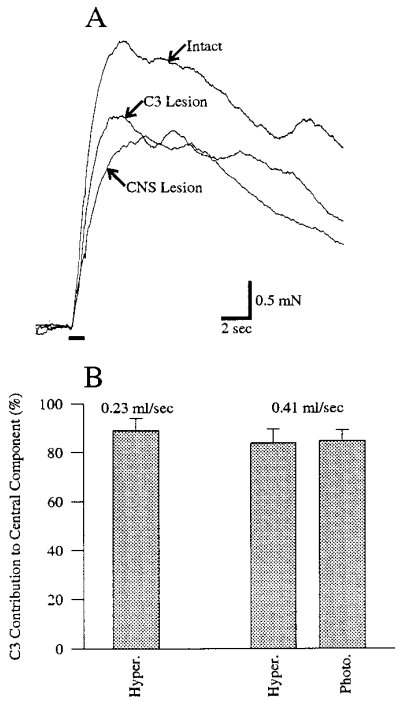

FIG. 5. Contribution of C3 to the central pathway mediating tentacle retraction. *A*: sample traces of muscle responses showing the effects of C3 photoinactivation and CNS removal. All traces are from the same preparation. Thick line under the muscle traces indicates the stimulus duration. *B*: percentage of the central component mediated by C3. Amount of tension lost after a C3 lesion is expressed relative to the amount of tension lost after a complete CNS lesion. Percentages were calculated separately for each preparation before averaging. $n = 3$ for stimulus strength of 0.23 ml/s; $n = 10$ for 0.41 ml/s (5 for each lesion type). Hyper., lesion by hyperpolarization; photo., lesion by photoinactivation. Results are corrected for saturation as described in RESULTS. Uncorrected values are 87.4 ± 5.6% for 0.23 ml/s and 76.4 ± 5.5% for 0.41 ml/s.

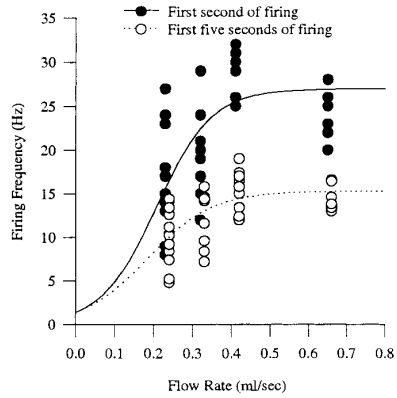

FIG. 6. Correlation of stimulus strength and C3 firing frequency. Mechanical stimuli were applied to the nose using a 1-s pulse of saline, the strength of which is expressed as flow rate. Points represent individual responses from naive animals ($n = 39$). Firing frequency was calculated based on the spike number in either the 1st 1 s of firing (solid circle) or the 1st 5 s of firing (open circle); the points are fit with solid curves $\{y = 26.96/[1 + 1.50 \exp(-14.13x + 2.52)]\}$ and dotted curves $\{y = 15.29/[1 + 1.73 \exp(-12.18x + 1.72)]\}$, respectively. Asymptotic rise of the curves reflects the response saturation at high stimulus strengths. Vertical separation of the curves, which is greatest at high spiking frequencies, indicates the reduction in spiking frequency over time.

Relationships among stimulus strength, C3 activity, and the TRM response

The tentacle withdrawal response is both sensitive and rapid (by snail standards). As illustrated in Fig. 3, muscle tension sufficient to withdraw the tentacle is developed quickly and spiking in C3 is robust. To further quantify C3's contribution to the withdrawal reflex over a range of stimulus strengths, we analyzed C3's response to sensory stimulation and related the intensity of C3's response to the strength of muscle contraction.

The correlation between C3 firing frequency and stimulus strength is shown in Fig. 6. C3's mechanical sensitivity is evident in the fact that firing frequency is quite high even for the lowest stimulus strength that could be reliably applied and measured (0.23 ml/s). Although the rate of firing slows after the initial second (compare solid and dotted lines on Fig. 6), both early and late components of the response quickly reach a maximum for stimulus strengths higher than 0.23 ml/s. Both sets of data are fit by sigmoid curves that rise rapidly to different maxima, though the two curves are virtually indistinguishable after the maxima are normalized. The flow rates to produce a half-maximal response are 0.21 and 0.19 ml/s for 1- and 5-s intervals, respectively. A similar steepness characterizes the S-R curves for C3's chemical sensitivity (Chase and Hall 1996) and for the reflex's mechanical sensitivity (Prescott and Chase 1996).

Comparing the scatter of the open and solid circles on Fig.

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6, it is evident that the variability is reduced considerably by calculating firing frequency during a 5-s time interval. In light of this, and given the similarity in the shape of the S-R curves, subsequent reporting of C3 activity is based on firing frequency calculated over 5 s.

To relate C3's activity with muscle response amplitude, correlations between the two were derived over a range of C3 spiking frequencies for different conditions of interest (Fig. 7). In each case, the data are fit with a sigmoid curve, the logistic function for which is written at the *bottom* of each graph. In the Reflex, Intact condition (Fig. 7*A*), the data are nicely fit by the sigmoid curve ($r^2 = 0.90$). The correlation is even greater for the Direct Drive, Intact condition ($r^2 = 0.95$), shown in Fig. 7*B*; as before, a natural spiking pattern was recreated in C3. Note that in both conditions, the muscle response amplitude saturates at ~3 mN. This restriction is due to mechanical limitations of muscle contraction rather than to limitations on the excitation of C3 because even when C3 is driven to fire at unnaturally high frequencies, the muscle response still saturates at 3 mN. Under natural conditions, C3 activity saturates at roughly the same frequency as that which causes saturated muscle contraction (dotted curve, Fig. 6). Figure 7*B* also shows C3's sufficiency to mediate maximal TRM contraction at natural firing frequencies.

After a TRN lesion (Fig. 7*C*), C3 firing reflects the strength of reflex excitation ($r^2 = 0.78$), but it has only a negligible influence on muscle contraction (see Fig. 3*B*). The muscle response again saturates, but now the saturation level is at ~2 mN rather than 3 mN. Saturation at this level of tension is not caused by mechanical limitations but instead reflects limits on excitation through the peripheral S-R pathway that can be overcome through sensitization (Prescott and Chase 1996).

To illustrate how the theoretical, linear summation of PNS and CNS components compares with the observed reflex, the empirically derived regressions (Fig. 7, *A–C*) are replotted in Fig. 7*D* together with the theoretical prediction. Given that C3 accounts for 85% of the central component, the CNS contribution to the reflex is approximated by the direct drive condition; a correction factor for the remaining 15% was not applied because, at low muscle tensions, the effect is negligible, and at high muscle tensions, the effect is precluded by response saturation. The PNS contribution to the reflex is given by the response after a TRN lesion because most of the efference from the CNS has been eliminated. Under this lesion condition, because afference to the CNS is still intact, the firing frequency of C3 permits a calibration of the reflex strength whereby the PNS and CNS contributions can be added theoretically at the same level of reflex excitation. As shown in Fig. 7*D*, the regressions for the observed reflex and the theoretical reflex are fairly close at low muscle tension, but they diverge as the muscle response increases >2 mN. In other words, the central and peripheral contributions are additive until the muscle response starts to saturate, indicating redundant motor control to ensure

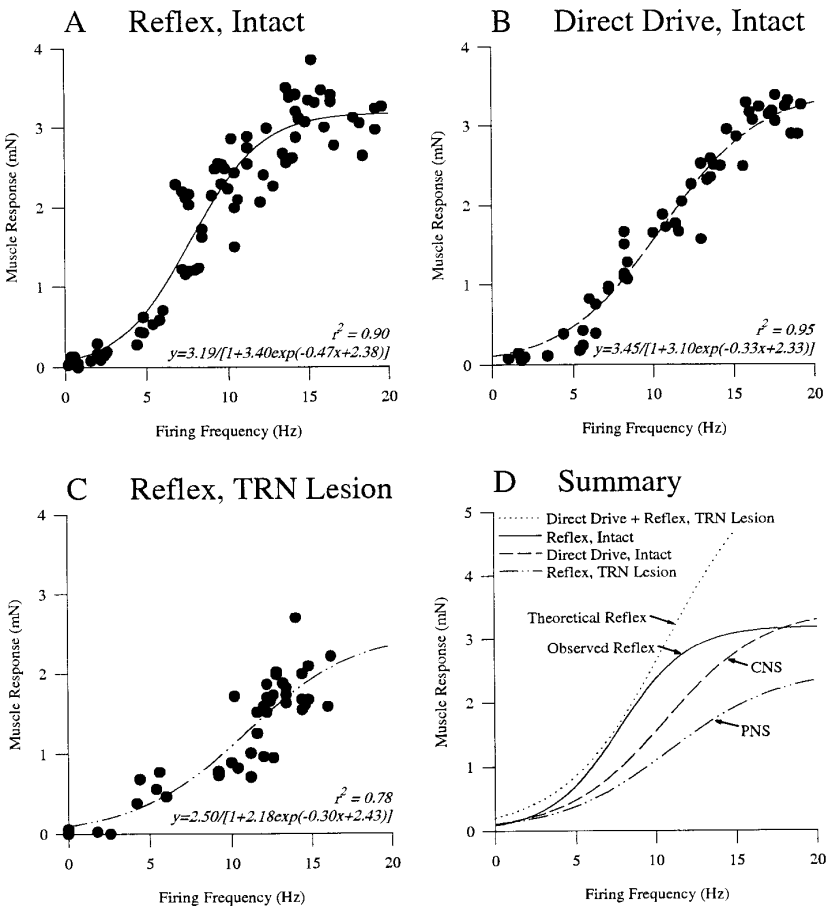

FIG. 7. Correlation of C3 firing frequency and the TRM response. $n = 3$ for each condition; each preparation was used for only a single condition. Order of stimulus strengths was randomized. Data for each condition (*A–C*) are fit with a sigmoid curve described by the logistic function written on each graph. *A*: reflex responses in intact preparations. *B*: responses elicited by direct driving of C3 in intact preparations. *C*: reflex responses after tentacle retractor nerve lesions. *D*: summary of relationships shown in the previous parts. Curves shown in *B* and *C* are assumed to reflect the CNS and peripheral nerve system (PNS) components, respectively. The CNS and PNS curves are added together to give the theoretical reflex, which may be compared with the observed reflex (from *A*). Divergence of these latter 2 curves is taken to indicate saturation of the muscle response.

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maximal response when required. The results also indicate the necessity and sufficiency of C3 to elicit maximal TRM contraction.

Relationships among non-C3 motor neurons, C3, and the TRM response

Evidence from TRN backfills and cross-sections indicates the presence of ~22 cerebral neurons, each with an axon in the TRN. Attempts at recording intracellularly from these neurons were unsuccessful but non-C3 spiking could be discerned clearly in TRN recordings. As shown on the sample trace (Fig. 8*A*), spikes recorded extracellularly from the TRN can be distinguished on the basis of amplitude and shape; one type corresponds to C3 as evidenced by simultaneous intracellular C3 recordings. All of these spikes are conducted away from the CNS to the TRM (data not shown). In spike amplitude histograms (Fig. 8, *B* and *C*), C3 spikes are represented by a single sharp peak, whereas

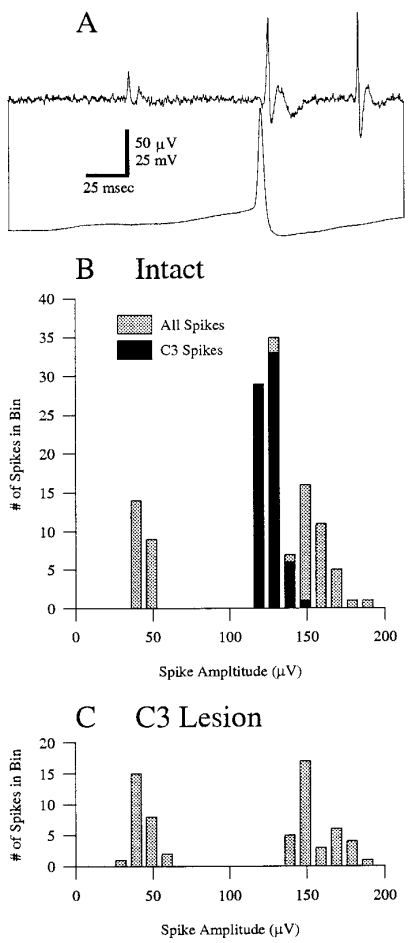

FIG. 8. Non-C3 central motor neurons involved in tentacle retraction and their relationship with C3. *A*: sample of simultaneous recordings from the tentacle retractor nerve and C3. *Top*: TRN extracellular; *bottom*: C3 intracellular. Direction of spike conduction was verified as being away from the CNS, consistent with a motor function. *B* and *C*: histograms of spike sizes before and after C3 lesion, respectively. Amplitudes of spikes occurring in the 1st 10 s of a typical reflex response were measured peak to peak. Hyperpolarization effectively blocked all C3 activity, but this had no effect on the activity of other motor neurons as indicated by the lack of change in the remaining 2 peaks (compare *B* and *C*).

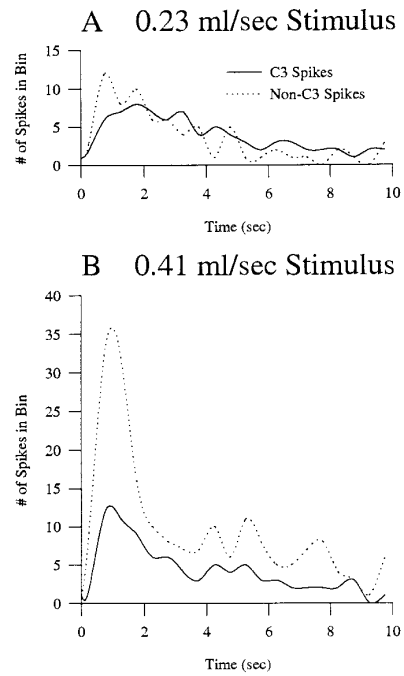

FIG. 9. Response profiles in the tentacle retractor nerve. *A* and *B*: each response profile is based on a typical response; both responses were recorded from the same preparation. Spikes recorded during the 1st 10 s of the response were identified as C3 or non-C3 and counted in 0.5-s time bins to create the response profiles. Stimulus begins at *time 0* and lasts 1 s. Spiking continues long after the end of stimulation but is greatest in the early phase of the response, which is also the phase during which the greatest separation of the 2 curves occurs. These data are consistent with non-C3 neurons contributing to the early phase of tentacle retraction, which cannot be fully accounted for by C3 activity (see Fig. 3).

spikes corresponding to non-C3 neurons are represented by two peaks separated by a threefold difference in spike amplitude. Despite the dichotomy in spike amplitudes, there is no concomitant dichotomy in axon diameters as measured from TRN cross-sections (data not shown), although there might be some correspondence between non-C3 spike types and the large and small cell bodies visualized through TRN backfills.

The next step was to investigate whether these cells had a motor function, and, if so, how the presence of so many additional motor neurons might be reconciled with the data (Fig. 5) indicating that activity in C3 is sufficient to account for virtually the entire central component. The data in Fig. 3 indicate that although a central contribution to muscle contraction is important to maximize contraction rate, this contribution is not attributable to C3. It therefore seemed plausible that other motor neurons in the CNS could be influential in mediating the early phase of muscle contraction. Such a function also could explain why stimulation of the TRN can elicit more rapid TRM contraction than stimulation of C3 alone (Zakharov 1982). To investigate the mediation of specific phases of the muscle response, the rate of spiking in C3 and non-C3 neurons was analyzed throughout the first 10 s of responses. Representative response profiles from 0.23- and 0.41-ml/s stimulation are shown in Fig. 9, *A* and *B*, respectively. Both spike types occur at their highest frequency within the first 2 s after stimulation regardless of stimulus strength. Despite this, the separation of the two curves is greatest during the first 2 s

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with non-C3 spikes consistently exhibiting the higher firing rate. The separation is exaggerated at the higher stimulus strength primarily because of an increase in non-C3 spikes; firing frequency in C3 is nearly saturated during this time and has little capacity to increase (see Fig. 6). The time when the neurons are firing at their highest rates corresponds to the earliest phase of the muscle response when contraction rate is high. These data are consistent with non-C3 neurons playing a predominant role in controlling the initial rate of muscle contraction, especially at high stimulus strengths. Given these data, we assume that at least some of the spikes reported in Fig. 9 are attributable to motor neurons; however, we cannot rule out nonmotor functions for non-C3 neurons. To summarize, central motor neurons other than C3 appear to act in concert with the peripheral S-R pathway to ensure a rapid withdrawal reflex, and although C3 may contribute to this initial contraction (Fig. 3), its most powerful influence is delayed.

We also investigated whether C3 might recruit other motor neurons centrally, although previous evidence suggested that this was not the case (Bewick et al. 1990). We tried to replicate Bewick et al.'s experiments by blocking synaptic transmission with low Ca^{2+}/high Mg^{2+} saline in the CNS bath. However, following their protocol, we did not achieve a successful block, and when we increased Mg^{2+} levels, normal C3 functioning was impaired. As an alternative, we tested whether intracellular stimulation of C3 would excite the other motor neurons (monitored by extracellular electrodes on the TRN). Even with strong stimulation of C3, the other cells remained totally silent, indicating no direct excitatory connection. To test whether C3 might have a modulatory effect on the other motor neurons, the response of the non-C3 motor neurons to mechanical stimulation of the nose was monitored with C3 intact or lesioned (Fig. 8, *B* and *C*, respectively). Apart from the presence or absence of C3 spikes, there are no significant differences between the two histograms, indicating a lack of modulatory effect by C3. The conclusion, consistent with that of Bewick et al. (1990), is that C3 has neither direct excitatory nor modulatory effects on other motor neurons in the CNS.

Morphological evidence indicates that a separate set of putative motor neurons innervates the TRM via the OlN (Hanström 1925). Contrary to Zakharov (1992), we observed that stimulation of the peripheral nerve stump of a cut OlN elicits robust TRM contraction at a short latency (data not shown). This is consistent with the existence of motor neurons in the OlN, although an alternative (or additional) explanation that we cannot rule out is peripheral excitation of motor neurons via centripetal sensory neurons or interneurons. Lesion experiments (Fig. 3) indicate that the OlN carries only a minimal efferent signal to the TRM. The lesion results thus seem to contradict the morphological and stimulation data. However, the distal neurites of the motor neurons might remain functional after surgical separation from their cell bodies in the CNS. Synapses in the tentacle ganglion could excite these neurites, which would in turn excite the muscle, thereby completing a peripheral S-R pathway (Hanström 1925; Zaitseva 1991; see also Bullock 1965). The lack of these cells' involvement in the central S-R pathway could be attributed to either a lack of central excitation or a collision of this excitation with antidromically conducted activity from the periphery.

Motor competence of pathway components mediating tentacle bending

Peschel et al. (1996) have reported that the peritentacular nerves are responsible for the central control of tentacle bending. C3's axons enter both these nerves and branch extensively at the nerve termini where they innervate the tegumental muscle (Figs. 1 and 2*C*). Direct driving of C3 causes contraction of the tegumental muscle (Fig. 10*A*). The relative weakness of this contraction reflects the size and structure of the tegumental muscle.

As in the case of the TRM, there are non-C3 central neurons that innervate the tegumental muscle. We investigated these cells and their relationship with C3. Based on similar arguments used for TRM motor neurons, we have assumed that at least some of these cells are motor neurons. A sample recording from the PtNe, the PtNi, and C3 is shown in Fig. 10*B*. C3 spikes can be identified in both nerves along with a variety of other spikes the amplitudes of which were analyzed in the same manner as in Fig. 8, *B* and *C* (data not shown). The data suggest that non-C3 neurons innervating the tegumental muscle may be divided into two subpopulations as was done for non-C3 cells innervating the TRM. Simultaneous recordings from pairs of nerves (PtNe, PtNi, and TRN) indicate that, other than C3 spikes, time-locked spikes in multiple nerves are rare (<20% of non-C3 spikes). On the basis of their consistent amplitude and shape, the non-C3 spikes time-locked between nerves seem to be attributable to a single neuron. Thus neurons excited by tentacle stimulation appear to project into only one nerve, with the exception of C3 and one other neuron, possibly CV1 or CV2 (Fuss and Teyke 1996; S. Fuss, personal communication). As before, C3 had no direct excitatory or modulatory effect on the other central cells (data not shown).

Neurons projecting into the peritentacular nerves are distinguished by their high level of spontaneous activity relative to C3 and to neurons projecting into the TRN. Although the level of activity may fluctuate, spontaneous spiking in both the PtNe and PtNi is usually >5 Hz. This is represented at *time 0* on the response profiles shown in Fig. 10, *C* and *D*, which can be compared with Fig. 9 for the analogous case in the TRN. Besides the difference in level, the source of the spontaneous activity is also different. When the nose is removed from the CNS, spontaneous activity in C3 and in the TRN is eliminated, whereas it remains in the peritentacular nerves (excluding C3 spikes).

Non-C3 central neurons projecting into the peritentacular nerves increase their firing rates in response to mechanical stimulation of the nose (Fig. 10, *C* and *D*), but this increase is not great relative to their level of spontaneous activity; C3 on the other hand, shows its usual large increase in firing frequency (>50% of all spikes recorded in the PtNe and the PtNi are attributable to C3). This differential increase in activity, together with the morphological evidence for extensive branching of C3's axon terminals in the tegumental muscle (Fig. 2*C*), suggests that C3 plays a predominant role in mediating tentacle bending in response to noxious stimulation of the nose.

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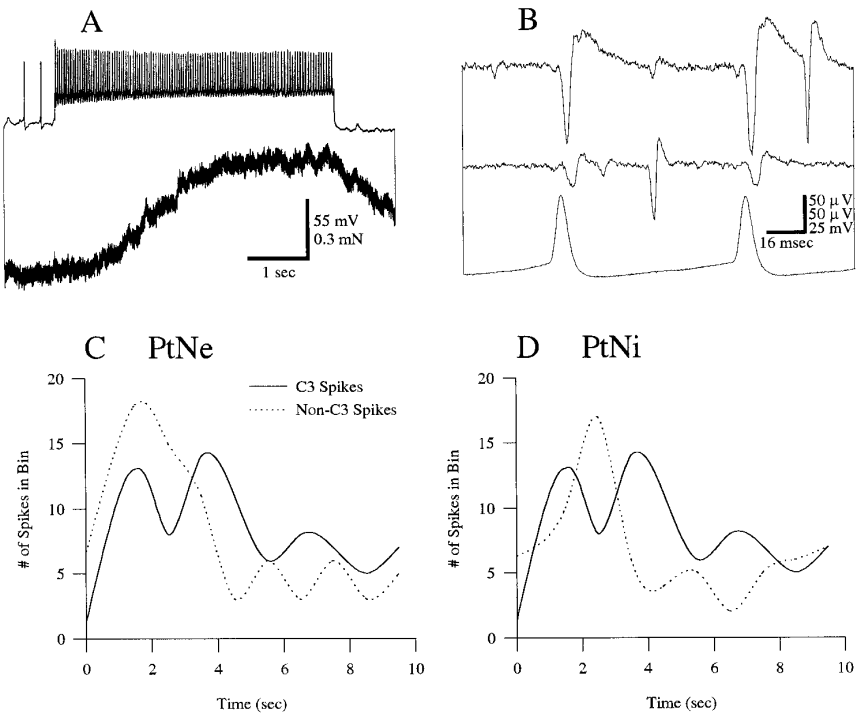

FIG. 10. Motor neurons involved in tentacle bending. *A*: TegM response caused by C3. *Top*: C3; *bottom*: TegM. Peritentacular nerves were left attached to the muscle during dissection. Distal tip of the tentacle was immobilized and the proximal portion of the inverted tentacle's tegument was hooked to a force transducer to monitor TegM contraction in response to C3 stimulation (2 nA depolarizing square wave). *B*: sample nerve recordings with simultaneous intracellular C3 recording. *Top*: PtNe; *middle*: PtNi; *bottom*: C3. Direction of spike conduction was verified as being away from the CNS. Note that C3 spikes and the spike in the *center* of the trace occur nearly simultaneously in both nerves; otherwise, the vast majority of spikes (>80%) are restricted to one or the other nerve. *C* and *D*: response profiles in PtNe and PtNi, respectively. Spikes in the 1st 10 s of a typical reflex response to 0.41 m/s stimulation were counted in 1-s time bins for each nerve. Although the C3 response profiles are identical in the 2 peritentacular nerves, the non-C3 profiles are similar but not identical, indicating that most non-C3 spikes do not occur simultaneously in both nerves. Note that spontaneous activity, represented at *time 0*, is much higher in the peritentacular nerves than in the tentacle retractor nerve (Fig. 9) and furthermore, that C3's activity increases more so than does non-C3 activity in response to stimulation. These data are consistent with C3 playing an important role in stimulus-elicited tentacle bending, whereas the non-C3 cells predominantly subserve other roles.

Prolongation of neuronal activity after stimulation of the tentacle

Evident in all the cell responses seen thus far, and most notably in Fig. 9, is the fact that motor neurons continue to fire long after the end of tentacle stimulation, consequently causing prolonged muscle contraction. The prolongation is not, however, unique to mechanical stimuli because responses to chemical stimuli (Chase and Hall 1996) and electrical stimuli (data not shown), applied to the nose, elicit comparable prolonged firing in C3. The generality of prolongation among different stimulus modalities suggests that prolongation is attributable to some process other than sensory transduction. In contrast to nose stimulation, nerve stimulation or direct intracellular stimulation of C3 (with a depolarizing pulse) causes only a phasic cell response. It is therefore reasonable to suppose that a locus for neural signal prolongation exists in the periphery, early in the circuit but after sensory transduction, and therefore probably in the tentacle ganglion. Given a transformation at this location, one would predict that the afferent signal to the CNS would exhibit the effects of this peripheral transformation, and assuming no further transformation in the CNS, correspond closely to the efferent signal from the CNS. To investigate this, spikes were counted during a 30-s time interval and divided into 1-s time bins to create response profiles. Note that the afferent (Fig. 11*A*) and efferent (Fig. 11*B*) response profiles are similarly shaped. In all cases, the maximum firing frequency occurs at 2 s and the falling phase of the response decays exponentially back to resting levels.

Comparison of the afferent and efferent signals at each stimulus strength (Fig. 11*C*) indicates that prolongation of the efferent signal is accounted for by prolongation of the afferent signal. Therefore, prolongation occurs at a peripheral location in the central S-R pathway. The efferent signal is in fact slightly shorter than the afferent signal; this may be caused by depression in the CNS during the course of the response.

Comparison among stimulus strengths (Fig. 11*C*) indicates that the degree of signal prolongation is related to signal intensity. C3's responses to stronger stimulation were also analyzed and fit with exponential curves. The results are expressed here as response half-lives ($n = 3$ responses for each stimulus strength): disturbance, 1.8 s; 0.23 ml/s, 3.0 s; 0.32 ml/s, 3.0 s; 0.41 ml/s, 5.0 s. The tendency toward longer half-lives with increasing stimulus strength suggests that C3's output is not only amplitude-coded (Fig. 6), but is also duration-coded, i.e., stimulus intensity is reflected in both the intensity and duration of C3's response.

DISCUSSION

The tentacle withdrawal reflex is a crucial defensive behavior for terrestrial snails. Its importance is reflected in the sensitivity, rapidity, and strength of the reflex. Until now, investigations of the cellular basis for the behavior have focused solely on the giant C3 neuron (Bewick et al. 1990; Cottrell et al. 1983; Zakharov et al. 1982). We too have studied C3, but we have also extended the investigation to include other cells involved in the reflex (Fig. 12).

The tentacle withdrawal reflex, when elicited by stimulation of the tentacle, is mediated by the combined actions of central and peripheral S-R pathways. The competence of the peripheral S-R pathway originally was shown by Nonne (1925) and more recently quantified by us (Prescott and Chase 1996). Peripheral motor neurons have not been identified and are believed to be absent (Hanström 1925; Rogers 1968), but it has been suggested that the axons of central motor neurons, running through the olfactory nerve and the tentacle ganglion into the musculature at the tentacle's tip, might be excited in the tentacle ganglion and thereby form

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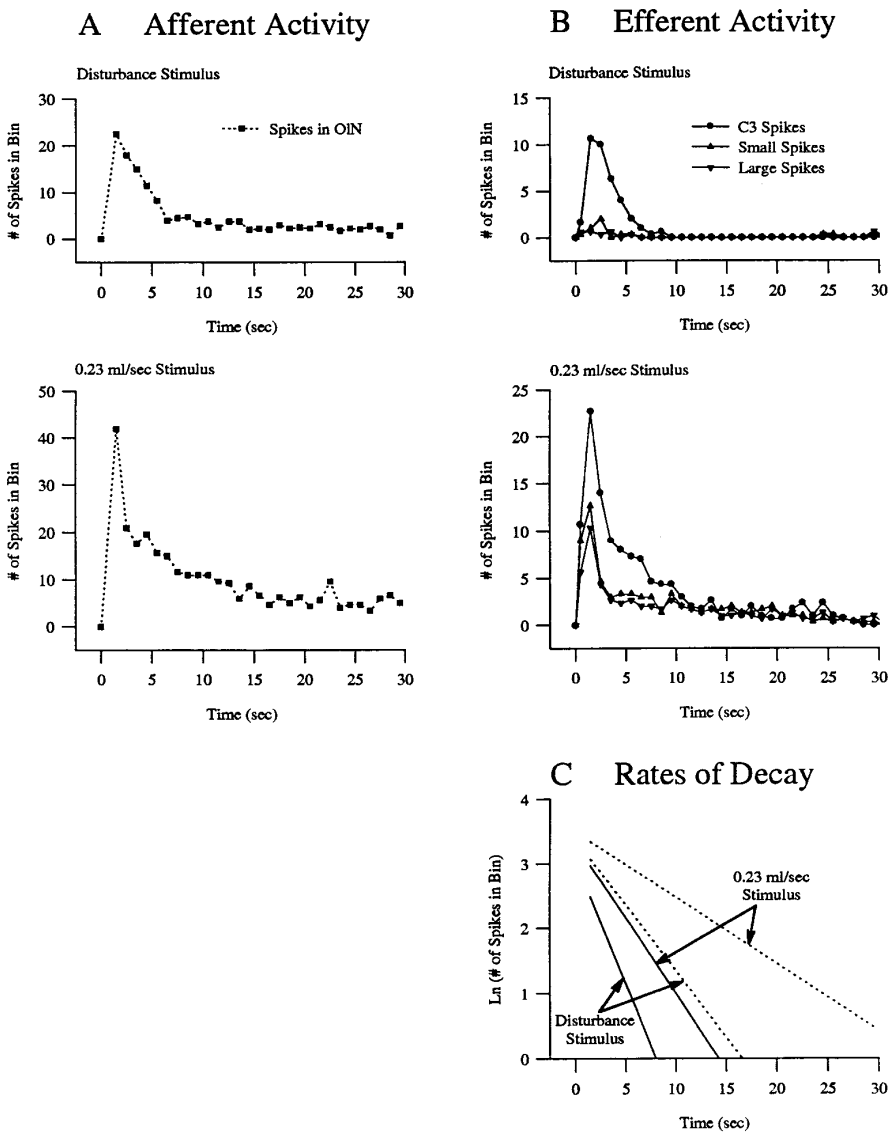

FIG. 11. Analysis of prolonged activity in the central pathway mediating tentacle retraction. *A*: afferent activity recorded from the olfactory nerve. CNS was lesioned for *A* to remove any efferent activity that otherwise might be recorded in the olfactory nerve. Having done this, any possible centrifugal modulation that normally might occur was removed. Evidently centrifugal influences are not necessary for prolongation to occur, but they may modify prolongation in, as yet, unknown ways. *B*: efferent activity recorded from the tentacle retractor nerve. Disturbance stimulus refers to very light stimulation caused by the switching of a solenoid without through flow (see METHODS). For each stimulus strength, 3 responses were recorded from the same animal and averaged; a single, different preparation was used for each part, *A* and *B*. Each response profile was created in the same way as in Fig. 10, but spikes were counted over 30 s and put in 1-s time bins. *C*: rates of signal decay. Olfactory nerve's afferent signal (from *A;* · · ·) and C3's efferent signal (from *B;* ——) were fit with single exponential decay curves shown on a ln-transformed *y*-axis. Equations for the curves (before transformation) are as follows: afferent signal, disturbance stimulus, $y = 29\ \exp(-0.20x)$, $r^2 = 0.92$; afferent signal, 23 ml/s stimulus, $y = 33\ \exp(-0.10x)$, $r^2 = 0.81$; efferent signal, disturbance stimulus, $y = 21\ \exp(-0.38x)$, $r^2 = 0.97$; efferent signal, 23 ml/s stimulus, $y = 28\ \exp(-0.23x)$, $r^2 = 0.94$. Note that slope is correlated inversely with stimulus strength, whereas the *y* intercept is correlated positively with stimulus strength consistent with both a duration and amplitude code, respectively (see text). Also, the efferent signal has a smaller *y* intercept and a steeper slope than the afferent signal for an equivalent stimulus strength.

the peripheral S-R pathway (Hanström 1925; Zaitseva 1991; see also Bullock 1965). Lesion experiments (Fig. 3) indicate that these motor neurons do not contribute significantly to central mediation of the withdrawal reflex elicited by tentacle stimulation. Figure 12 shows the hypothesized connectivity of cells in the tentacle ganglion.

Consistent with other reflexes that are mediated both centrally and peripherally, for example, the siphon closure and withdrawal response of *Spisula* (Prior 1972), central mediation becomes increasingly important as the stimulus, and consequently the response magnitude, increases (Fig. 4) (Balaban 1993; Prescott and Chase 1996; Zakharov 1992). The recruitment of central pathways not only enhances local reflexes, but it also allows the elicitation of more generalized withdrawal behaviors.

Another function of the central S-R pathway is that of increasing the duration of tentacle retraction (Fig. 3). This can be explained partially by the fact that the central S-R pathway innervates the TRM approximately midway along its length, in a region that characteristically contracts more slowly and more tonically than the distal TRM, which is innervated by the peripheral S-R pathway (ten Cate and Verleur 1952). Furthermore, although contraction is, for the most part, mediated by acetylcholine (Bewick et al. 1990), Falconer et al. (1993) have described a mechanism by which the neuropeptide FMRFamide, released by C3, may cause slower, more prolonged rhythmic contraction. Modulatory effects of FMRFamide on the TRM have not been demonstrated, although some evidence is suggestive (Cottrell 1989).

An additional mechanism that increases the duration of tentacle withdrawal is the prolonged neuronal excitation that persists after the end of stimulation (Figs. 9–11). Although this phenomenon was observed in the central S-R pathway, it may be equally true of the peripheral circuit. Chase (1981) described interneurons in the tentacle ganglion of *Achatina* that respond with prolonged firing to brief stimulation; similar responses occur in the ''higher order sensory neurons'' of *Lymnaea* (Janse 1974). This persistent activity surely contributes to the prolonged TRM contraction. Although we cannot offer a mechanistic explanation for the prolongation of the neural signal, we can localize the transformation to

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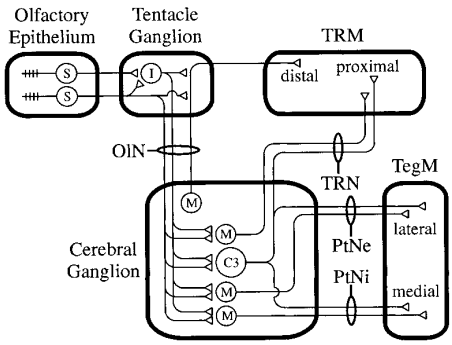

FIG. 12. Schematic of the neural circuit mediating tentacle withdrawal. Excluding the neuron C3, single circles represent groups of cells. Flow of neural information is from *left to right,* starting with stimulation of the olfactory epithelium (nose). Sensory neurons (S) are shown to synapse on interneurons (I) and motor neuronal fibers in the tentacle ganglion as well as projecting centrally. At least some of the connections from centripetal sensory neurons and interneurons to C3 and other motor neurons (M) are monosynaptic, although polysynaptic connections also may exist. Non-C3 neurons, with 1 exception not shown on the schematic, each project into a single nerve, whereas C3 has divergent projections into multiple nerves. C3's projection into the OlN is not shown because its functional influence is minimal.

the tentacle ganglion (Fig. 11) and presumably to interneurons like those described by Janse (1974) and Chase (1981). Given this localization, prolonged activation also may be a characteristic of the motor elements in the peripheral S-R pathway. Similar prolongations of neural activity have been reported for circuits mediating withdrawal behaviors in *Aplysia* (Cleary and Byrne 1993; Frost and Kandel 1995; White et al. 1993). Such a transformation would help explain what White et al. (1993) refer to as a duration-coded output. A duration code exists for C3's output in vitro (Fig. 11) and for the withdrawal reflex, as reported in behavioral experiments (Chase and Hall 1996).

Figure 12 shows our current understanding of the central S-R pathway mediating tentacle withdrawal in *H. aspersa.* Sensory input reaches the cerebral ganglion via the tentacle ganglion and the olfactory nerve. It is very likely that at least part of this pathway is polysynaptic with connections made in the tentacle ganglion (Bullock 1965; Chase et al. 1981; Chase and Tolloczko 1993; Hanström 1925). As seen in *Aplysia* (Bailey et al. 1979), sensory neuron collaterals may make connections in the periphery while the main axon projects centrally. Once these projections enter the CNS, they synapse directly onto the neuron C3 (Chase and Hall 1996). These fibers also synapse onto other motor neurons, and although these latter connections have not been shown to be monosynaptic, it is a reasonable assumption for at least some of the connections given that the motor neurons' latency to spiking in response to tentacle stimulation is similar to C3's latency (250–300 ms).

C3 innervates the TRM via the TRN and, to a lesser degree, via the olfactory nerve (Figs. 1 and 2, *A* and *B*). Lesion experiments indicate that C3 accounts for 85% of the central component (Fig. 5) or ~50% of tentacle retraction. This ranks C3 among the most powerful motor neurons mediating comparable reflexes. In *Aplysia* for example, the motor neurons L7 and LDG1 each mediate ~35% of the gill withdrawal reflex (Kupfermann et al. 1971, 1974), although these values depend on the preparation and the response measure. For instance, Cohen et al. (1997) showed that LDG1, in a modified preparation, can mediate 84% of the efferent vein contraction, a measure of gill withdrawal. In the case of *Helix,* lesion experiments actually underestimate the contribution of C3 given that direct stimulation of C3 can elicit maximal muscle contraction (Figs. 3 and 7). The discrepancy between the lesion and stimulation results is explained by saturation of the muscle response and redundancy in the motor control under conditions of strong stimulation (Figs. 4 and 7). However, there would be less redundancy when tentacle retraction is elicited by stimulation elsewhere than the tentacle, under which conditions the peripheral S-R pathway would not be directly recruited.

Other putative central motor neurons contributing to the central mediation of tentacle retraction also innervate the TRM via the TRN. The main function of these cells is to maximize the rate of contraction (Fig. 9), the importance of which is paramount in the case of defensive behaviors. These motor neurons are arranged in parallel with C3 (Figs. 8 and 12), but unlike C3, their axonal projections are restricted to the TRN.

C3 not only innervates the TRM, but it also innervates the tegumental muscle through both the external and the internal peritentacular nerves, and it is capable of causing muscle contraction (Fig. 10*A*). Peschel et al. (1996) have shown that stimulation of these two nerves elicits bending of the tentacle. Tentacle bending is concomitant with tentacle retraction during reflexive withdrawal (Lemaire and Chase 1997; Zakharov 1992). In addition to C3, other putative central motor neurons innervate the tegumental muscle. Like the previously described TRM motor neurons, the cells responsive to mechanical stimulation of the tentacle are arranged in parallel with C3 (Fig. 12) and have axonal projections restricted to a single nerve (with 1 exception). Although these neurons increase their activity in response to tentacle stimulation, the increase in C3's activity is much greater (Fig. 10, *C* and *D*). Given this differential increase in activity, and C3's rich innervation of the tegumental muscle (Fig. 2*C*), we postulate that C3 plays a predominant role in control of tentacle bending. As for the non-C3 motor neurons, they contribute to defensive tentacle bending but are likely more important for controlling tentacle angle (Peschel et al. 1996) and tentacle length (Wondrak 1977). These roles would be more consistent with the high level of spontaneous activity observed in the cells.

C3 fits the general description of giant molluscan neurons (Gillette 1991) not only in its soma size but also in the extent of its peripheral innervation (Fig. 1). Its divergent output to separate effectors responsible for different aspects of tentacle withdrawal allows it to coordinate retraction and bending. A similar pattern of innervation was described in the *Ariolimax* L4 motor neuron, which also mediates tentacle withdrawal (Chan and Moffett 1982). C3's coordination of the reflex is made possible by the cell's innervation of effectors rather than by electronic coupling (Ferguson and Benjamin 1991) or synaptic interactions (Fredman and Jahan-Parwar 1977) among a group of motor neurons or by higher-order interneurons (Balaban 1979; Cleary and Byrne 1993). Presumably the smaller motor neurons also subserving tentacle withdrawal in *Helix* allow some variability or

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fine tuning of the reflex because they innervate only a single effector or portion thereof. Despite these cells being arranged in parallel with C3, the convergent input to C3 and the subsequent divergent output represent a funneling of the neural signal responsible for reflex elicitation. On the basis of this architecture, C3 is an important control point in the central S-R pathway, and thus, likely a key locus for plasticity (Balaban 1993; Christoffersen et al. 1981; Prescott and Chase 1996) and for modulation such as occurs during mating (Balaban and Chase 1990; Zakharov 1992). It is hoped that the increased understanding of C3 and the neural circuit of which it is a part will benefit future investigations into the plasticity and modulation exhibited by this withdrawal reflex.

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Address for reprint requests: R. Chase, Dept. of Biology, McGill University, 1205 Ave. Docteur Penfield, Montreal, Quebec H3A 1B1, Canada.

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