

## Functions of the subesophageal ganglion in the medicinal leech revealed by ablation of neuromeres in embryos

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### Summary

Two general trends in the evolution of the nervous system have been toward centralization of neuronal somata and cephalization of the central nervous system (CNS). These organizational trends are apparent in the nervous system of annelid worms, including leeches. To determine if the anterior brain of the leech serves functions similar to those of the brains of more complex organisms, including vertebrates, we ablated one of the two major regions of the cephalic brain – the subesophageal ganglion (SubEG). For anatomical reasons, ablations were performed in embryos, rather than in adults. At the end of embryonic development, we observed the leeches' spontaneous behaviour and their responses to moderate touch. We observed that, although the midbody ganglia of the leech CNS display a high degree of local

autonomy, the cephalic brain provides generalized excitation to the rest of the CNS, is a source of selective inhibition that modulates behaviour, integrates sensory information from the head with signals from the rest of the body, and plays an important role in organizing at least some complicated whole-body behaviours. These roles of the leech cephalic brain are common features of brain function in many organisms, and our results are consistent with the hypothesis that they arose early in evolution and have been conserved in complex nervous systems.

Key words: annelid, anterior brain, central nervous system, cephalic brain, *Hirudo medicinalis*, leech, subesophageal ganglion.

### Introduction

A centralized nervous system with a cephalic concentration of neurons (i.e. a brain) is common among complex animals. The number and variety of taxa with nervous systems that include a brain and a longitudinal nerve cord (Bullock and Horridge, 1965) suggest that this pattern evolved early and persisted because it was so successful. The more recent discovery that homologous molecules are expressed in very similar patterns during the development of the nervous system in *Drosophila* and in mice reinforces the notion that the fundamental organization of the nervous system evolved early and was then conserved (Kammermeier and Reichert, 2001; Reichert and Simeone, 2001), as does the homology in structure observed in many taxa (Ghysen, 2003). Although the term 'brain' has proven to be somewhat difficult to define, one characteristic that distinguishes a brain from a ganglion is that a brain typically serves the entire organism, not simply a particular segment or body part (Sarnat and Netsky, 2002), and that it is organized into functionally separate regions (Delcomyn, 1998).

In leeches, which are annelid worms, the central nervous system (CNS) includes two brains, one at the anterior end of the ventral nerve cord and the other at the posterior end. The anterior and posterior brains are compacted collections of several segmental ganglionic equivalents of neurons (Muller et al., 1981). Although their nervous system is relatively simple (a large fraction of the neurons in the CNS are located in ganglia strung along the ventral nerve cord, with one ganglion in each of the 21 midbody segments), leeches display a wide variety of behaviours including shortening, locomotion, probing, neuronally controlled heartbeat and a variety of segmental behaviours; the neuronal basis of many of these behaviours is well understood (Kristan et al., 2005; Mazzoni et al., 2005). This richness of background information, coupled with the relative simplicity of the annelid nervous system, makes leeches a particularly useful group in which to explore the effects of centralization and cephalization in the nervous system.

The anterior 'head brain' of *Hirudo medicinalis* L. (the European medicinal leech) is composed of a supraesophageal

ganglion (SupraEG) and a subesophageal ganglion (SubEG). This brain lacks large and well-defined sensory projection areas, such as the olfactory antennal lobe or the complex visual processing areas in the brains of insects (Bullock and Horridge, 1965; Burrows, 1996; Gupta, 1987). Instead, the SupraEG is undivided, and the SubEG consists of four regions called neuromeres. The SupraEG, which arises from the anteriormost part of the embryo called the prostomium (Stent et al., 1992), contains a variety of peptidergic neurons (Crisp et al., 2002), and its function remains relatively unexplored. The SubEG arises embryonically from four ganglion-like clusters of neurons at the anterior of the ventral nerve cord (Stent et al., 1992), and in adults this embryonic origin is reflected in the four bilaterally symmetric neuromeres (Fig. 1A,B). In adults, the cells of the SubEG are tightly compacted and the neuromere boundaries are somewhat obscured, making well-defined ablations with clean boundaries difficult to produce. To explore the function of the brain in adults, previous workers entirely removed the SupraEG or the SubEG. Bullock (Bullock and Horridge, 1965) reported that removing the entire SupraEG caused increased activity, heightened excitability and changes in the pattern of swimming. By contrast, removing the SubEG

depressed spontaneous activity, although the leeches could still crawl and swim if prodded sufficiently.

At the opposite end of the experimental spectrum, the function of the SubEG has been explored one neuron at a time. Many neurons that are found in the segmental ganglia [e.g. mechanosensory neurons (Yau, 1976) and the serotonergic neuromodulatory Retzius cells (Lent, 1977)] are present in the SubEG. In addition, previous work has revealed that many neurons with distinctive functions are found only in the SubEG. For example, cell Tr1 can initiate swimming (Broduehrer and Friesen, 1986; Kristan and Weeks, 1983), and cells SIN-1 and Tr2 can terminate or suppress swimming (Broduehrer et al., 1995a; Taylor et al., 2003). Cell R3b1 participates in the choice of whether a leech will swim or crawl (Esch et al., 2002). These identified neurons occupy predictable positions and are distributed among the neuromeres of the SubEG, rather than being clustered together. This pattern suggests that the SubEG is not organized into circumscribed regions, each of which controls a particular behaviour (e.g. swimming or locomotion), but individual neuromeres might still have distinctive functions. Ablating defined subregions of the brain has been widely used in studying the functional anatomy of the vertebrate brain

(Mogensen et al., 2005), but the small and highly compacted brain of many invertebrates, including the leech (Fig. 1A), makes clean ablation of subsections difficult. This problem can be circumvented during leech embryogenesis, because in the middle of development the precursors of the SubEG are separate from one another (Fig. 1B) and can be ablated independently.

To explore the function of the SubEG of *Hirudo* as a whole and to ask if it includes functionally distinct regions, we ablated one or more neuromeres about half-way through development, and when each leech completed development we evaluated its behaviour. Some of our results have been previously presented in abstract form (French et al., 2004).

## Materials and methods

### *Embryo production and maintenance*

*Hirudo* embryos were obtained from a breeding colony maintained in the laboratory. Embryos were released from their cocoons no earlier than 9 days after egg deposition and were then held at 20–24°C in 'embryo water', i.e. sterile-filtered Arrowhead spring water to which we added 38 µmoles of MgCl<sub>2</sub> and 53 µmoles of CaCl<sub>2</sub> per litre.

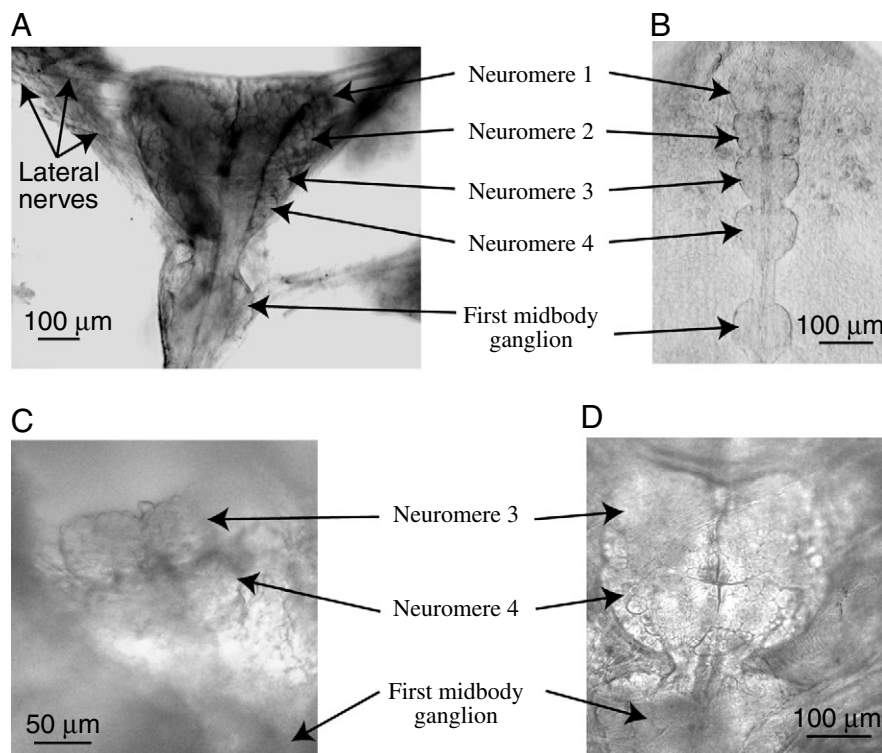


Fig. 1. Structure of subesophageal ganglion (SubEG) from embryonic and juvenile leeches. (A) SubEG and first midbody ganglion from a juvenile leech. Surrounding tissue was removed to reveal the structure, and connections with the supraesophageal ganglion were severed. (B) SubEG in embryo at 47% of embryonic development (% ED; Reynolds et al., 1998b), which is about the stage at which we performed surgeries. The ganglionic precursors to each of the neuromeres are still separate at this stage. (C) The head of an embryo at 50% ED (ventral view) following ablation of presumptive neuromeres 1 and 2. (D) SubEG of juvenile leech in which neuromeres 1 and 2 were ablated at 50% ED.

*Surgical manipulations*

Surgery was performed on embryos at 48–50% of embryonic development (ED; Reynolds et al., 1998b) in ice-cold normal leech saline (in mmol l<sup>-1</sup>: 115 NaCl, 4 KCl, 1.8 CaCl<sub>2</sub>·2H<sub>2</sub>O, 1.5 MgCl<sub>2</sub>·6H<sub>2</sub>O, 10 glucose, 4.6 Tris-maleate, 5.4 Tris-base, pH 7.4,) containing 8% ethanol. After surgery, leeches were transferred to ice-cold embryo water that was allowed to warm up to room temperature.

In one type of surgery, all or part of the SubEG was removed using electrolytically sharpened tungsten pins and very fine scissors. We refer to these leeches as ‘ablated’. Removing posterior neuromeres effectively disconnects any remaining anterior neuromeres from the ventral nerve cord, so we usually started all ablations at the anterior of the SubEG.

All SubEG ablations also severed the sensory and motor connections between the CNS and peripheral structures in the head. To determine how the behaviours were modified by disconnecting the SubEG from the periphery, we performed the same tests on animals in which the SubEG was intact and still connected with the ventral nerve cord, but all nerves to the periphery in the head had been cut. We call this group of embryos ‘nerve cuts’ or just ‘cut’. For sham-operated controls,

we exposed the neuromere precursors and nerves in the head but did not cut any neuronal structures. Throughout the study, embryos were maintained individually in embryo water in 35 mm Petri dishes, and their water was changed every other day.

*Behavioural observations*

Many behaviours of *Hirudo medicinalis* have been well described in adults (Kristan et al., 2005) and in embryos (French et al., 2005; Reynolds et al., 1998a). In the present study, we observed and characterized additional behaviours in juvenile leeches shortly after hatching (Table 1 summarizes all of the behaviours observed). After they reached 100% ED, leeches were observed in their Petri dishes every other day for 6 days, for a total of three observations per leech. Embryonic leeches heal rapidly and completely, so it was typically impossible to tell from visual inspection which leeches had been treated surgically, and after surgery all embryos were coded so the observer was unaware of each embryo’s surgical treatment.

We observed both spontaneous and elicited behaviours. For ‘spontaneous behaviour’ we watched each individual for a

Table 1. *Behaviours of juvenile leeches*

Behaviour name <sup>a</sup>	Abbreviation	Description
Avoid	<i>avoid</i>	Withdraws from point of stimulation without bending or whole-body shortening
Crawling (S, E)	<i>crawl</i>	Elongates the body, attaches front sucker, releases rear sucker, and shortens to execute well-coordinated steps
Detaching (E)	<i>det</i>	Simultaneously releases both front and rear sucker
Dorso-ventral flexions (E)	<i>dvf</i>	Unattached, bends the whole body longitudinally around the middle segments either dorsally or ventrally
Elementary movements (S)	<i>elm</i>	Unattached, bends part of the body around the midbody segments either dorsally or ventrally, shortens or elongates
Elongating (E)	<i>e</i>	Increases length of body from head to tail
Freezing (E)	<i>freeze</i>	Startle response; stops all movement and holds body rigid
Front sucker release (E)	<i>fsr</i>	Lifts front sucker off the substrate
Inactive (S)	<i>inact</i>	Body is relaxed; leech executes no movements; suckers may or may not be attached
Incomplete crawling (S, E)	<i>ic</i>	Makes movements that resemble parts of crawling behaviour, but fails to complete any steps
Local bending (E)	<i>lb</i>	Contracts longitudinal muscles on one side of body while relaxing on the other side, forming a bend; behaviour is confined to one or a few segments
Pivoting (E)	<i>pivot</i>	Swings the anterior of body in an arc, while keeping rear sucker attached
Probing (S, E)	<i>probe</i>	With the rear sucker attached, leech elongates and explores surrounding area by moving its anterior end, then shortens
Shortening (E)	<i>sh</i>	A withdrawal response; leech contracts its whole body longitudinally, making it shorter
Slow probing (S)	<i>slow probe</i>	Probing (see above) but very lethargic
Swimming (S, E)	<i>swim</i>	Sinusoidal undulations that traverse the body from anterior to posterior, propelling the animal through the water
Whole-body contractions (E)	<i>wbc</i>	Waves of contraction run along the body away from point of stimulation

<sup>a</sup>E, elicited behaviour; S, spontaneous behaviour.

6-min period after moving its Petri plate to the stage of a dissecting microscope and illuminating it with bright light. We recorded the time the leech spent in each behaviour and expressed the times as a percentage of the total time. For most groups of leeches we then observed 'elicited behaviour' by gently prodding the leech with round-ended forceps on the dorsal surface at each of three locations: anterior (very close to the head), midbody (about half-way between the head and tail) and posterior (just anterior to the rear sucker). Leeches typically responded to this stimulation; the notation 'no response' was rare. During each trial the animal was allowed to complete its response and return to baseline behaviour prior to the next stimulation, and a minimum of 20 s separated stimuli to minimize habituation or sensitization. Leeches were stimulated in each location three times during each observation period for a total of nine stimuli per session, and the order of stimulation was randomized. If a leech executed more than one behaviour successively in response to a single stimulus, all behaviours were recorded. (Most responses consisted of a single behaviour, and it was rare to observe more than two.)

We observed a total of 95 individuals from 10 cocoons (fertilized *Hirudo* eggs develop encased in a cocoon secreted by the parent leech; Fernandez and Stent, 1982). The spontaneous behaviour of all leeches was recorded. The elicited locomotory behaviour of most of the leeches was observed, but many leeches never moved around spontaneously, so the total number of animals in which we observed spontaneous locomotion was 84. In a subset of the embryos (41 embryos from four cocoons), the full behavioural repertoire in response to touch at the anterior, middle and posterior of the body was observed.

#### *Evaluation of surgery*

The extent of each ablation or cut was recorded immediately after surgery, and it was confirmed in each individual by *post-mortem* dissection (Fig. 1C,D). Leeches were transferred to ice-cold normal leech saline containing 16% ethanol, the SubEG was exposed, and the animal was incubated for 1–3 h in 0.1% Neutral red (Sigma, St Louis, MO, USA) dissolved in standard leech saline to label the monoaminergic neurons (Stuart et al., 1974). The number and position of labelled Retzius neuron somata indicated the number of neuromeres remaining following ablation. The general morphology of the SubEG and its associated nerves was noted. In some cases, a solution of 0.2% methylene blue dye (Sigma) was applied and then washed off to reduce ambiguity in the structure of the neuromeres and of the nerves connecting the SubEG to the periphery.

#### *Statistical analyses*

All juvenile leeches executed a wide variety of behaviours, and to determine whether surgery had produced changes in their behavioural repertoires we used a direct ordination method called canonical correspondence analysis (CCA) (Ter Braak, 1986). CCA is a multivariate technique that has been used in zoology, botany and ecology but, to our knowledge,

has not previously been applied to neurobiological data. It generates a reduced number of synthetic variables that summarize information about groups of several raw variables (in this case, behaviours) that were measured in the observed 'experimental units' (in this case, individual animals). The synthetic variables (called CCA axes) can then be used to evaluate patterns of responses across multiple raw variables simultaneously.

CCA is designed for discrete – rather than continuous – variables and it constrains axes to correlate maximally with pre-assigned sets of independent variables, in this case the treatment group of the animal (i.e. control, nerve cut or neuromere ablation). The method imposed no particular ordering with respect to the experimental treatment.

Although CCA will detect linear responses, the data do not have to be linear. CCA is applicable to unimodal patterns in which the discrete variables (in this study, the behaviours) change in frequency from uncommon to common and then back to uncommon again in a non-linear fashion. For example, in our study, treatments that differed in severity could produce this pattern of change if unique behaviours were seen in leeches with moderately severe ablations, but not in control leeches or in leeches with very severe ablations. A CCA 'individual score' is a numerical representation of the entire suite of behaviours that an individual exhibited (e.g. Fig. 2C); individuals with similar scores exhibited similar behavioural repertoires. These individual scores can be interpreted by comparing them with 'behaviour scores' (e.g. Fig. 2B), which are calculated simultaneously with the individual scores. Just as leeches with similar individual scores displayed a similar suite of behaviours, constellations of behaviours with similar scores were typically performed by individual leeches. This property of the analytic method effectively emphasized the behaviours that were most different among the groups. Behavioural scores furthest from zero contributed most to distinctions among the groups. (The sign of the scores is arbitrary; if all signs were reversed, the conclusions would be the same.)

We evaluated both spontaneous and elicited behaviour using CCA. We conducted a CCA to determine how elicited behaviour varied with the stimulus location in control leeches (this group included untreated and sham-operated animals, which were indistinguishable). Then, with experimental treatments (control, nerve cut or neuromere ablation) considered as three independent variables, we conducted separate CCA analyses for (1) spontaneous behaviours, and for elicited behaviours observed when leeches were stimulated in the (2) anterior, (3) middle or (4) posterior of the body. Significance of each CCA was assessed with a randomization test (Legendre and Legendre, 1998). Briefly, the test is based on randomly reshuffling the observed behaviours without constraining them into experimental groups. The test statistic in this method is a 'pseudo-F', or the ratio of the constrained (by the surgical treatments) to the unconstrained (ignoring surgical treatment) 'inertia', which is a multivariate measure of variance. Because this pseudo-F does not follow the usual

F-distribution, the behavioural data were repeatedly and randomly reshuffled, and a new CCA was calculated each time (we used 999 repeats). A pseudo-F was calculated for each reshuffled CCA, and the pseudo-F calculated for the data was compared with the entire distribution of reshuffled pseudo-Fs. If the observed pseudo-F was larger than all of the 999 randomly reshuffled values, then the probability of obtaining such an observation by chance is estimated to be 1/1000, or  $P \leq 0.001$ . Individual scores from significant CCAs were subjected to a one-way analysis of variance (ANOVA), followed by a Student's *t*-test *post-hoc* procedure, to assess for significance differences among treatment groups.

CCA produces as many axes as there are raw behavioural variables, but preliminary analysis confirmed that the first CCA axis captured the major effects of surgical treatment or touch location. Thus, rather than presenting results by plotting CCA1 against CCA2, we have confined our presentation to CCA1 individual and behaviour scores for all analyses.

## Results

### Spontaneous behaviour

All groups of juvenile leeches spontaneously produced a variety of behaviours (Table 1). By comparing the overall patterns of spontaneous behaviours we found strong differences among the three experimental groups in the general level of activity (Fig. 2A). Control leeches were very active, spending 55% of their time actively exploring their environment, typically with the rear sucker anchored (*probe*). By contrast, leeches with ablations spent approximately half of the observation time doing nothing at all (*inact*). They probed less than controls, and the motions were lethargic (*slow probe*), and if they performed any locomotion they typically swam (*swim*) or made incomplete crawling movements (*ic*). Leeches with nerve cuts produced a behavioural pattern that was intermediate between the control group and the group with ablations: they swam and probed slowly more than control leeches and were less active than controls but were not totally inactive. They probed and crawled more often than leeches with ablations but less often than controls.

The results of the CCA are shown in panels B and C in Figs 2–6. Panels labelled B show CCA behaviour scores; in these figures, the behaviours with scores farthest from zero contributed most to distinguishing among experimental treatments. Behaviours common to all groups are located near the centre of the axis. Panels labelled C show binned CCA1 individual scores. Differences

among groups can be interpreted by comparing the position of individuals in each treatment group along CCA1 (panel C) and then matching these positions to the behaviour scores (panel B). For example, in the analysis of spontaneous behaviour (Fig. 2B), crawling and probing lie at the far negative end of the CCA behaviour score axis, whereas incomplete crawling and swimming lie at the far positive end. Comparing these scores with the individual CCA scores (Fig. 2C), control leeches had individual CCA scores with the largest negative values, indicating that crawling and probing best distinguished

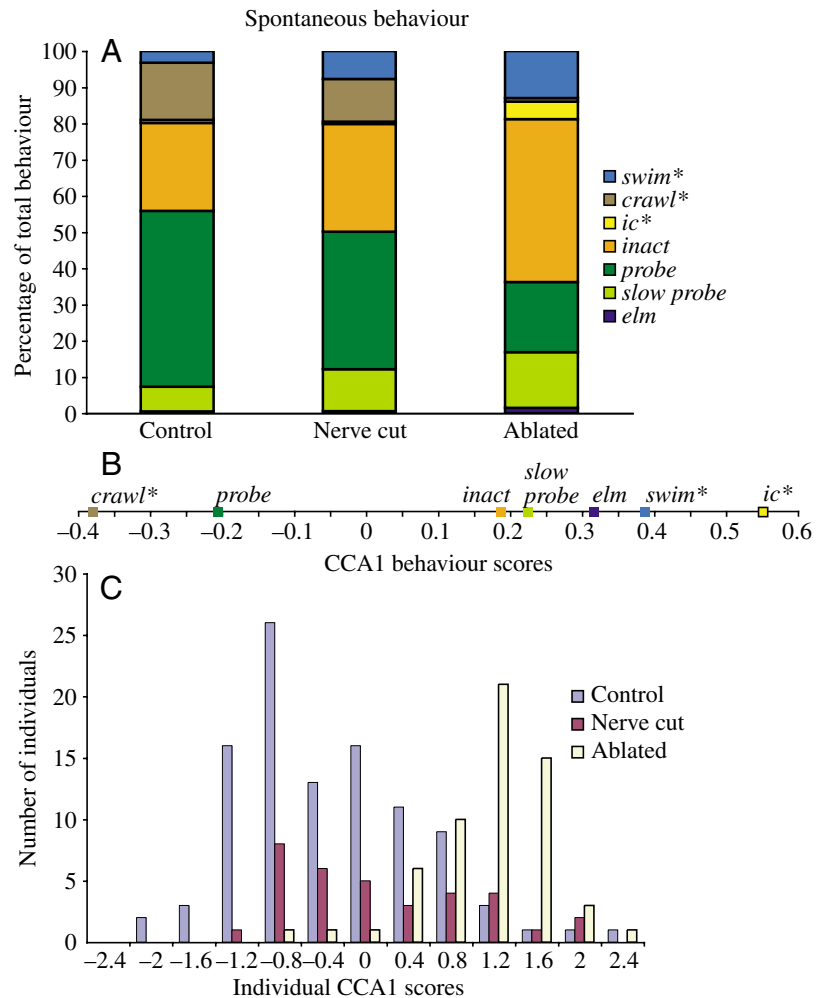


Fig. 2. Effects of surgery on spontaneous behaviour. (A) All spontaneous behaviours observed, divided into experimental groups. See Materials and methods for a description of the surgical treatments and Table 1 for descriptions of the behaviours. The control group ( $N=40$ ) included both intact leeches and sham-operated controls; we found no differences between the two groups. Nerve cut,  $N=9$ . Ablated some or all of the SubEG,  $N=33$ . In Figs 2–6, \* indicates locomotory behaviours, which are discussed in Fig. 7. (B) Distribution of behaviours along the CCA1 behaviour score axis. The behaviours that lie furthest to the left or right along this axis most strongly distinguished among treatment groups. Behaviours located in the centre of the axis were seen in all groups. (C) Distributions of individual CCA1 scores in the three experimental groups. Individual scores within each group were binned and arrayed along the CCA1 axis. Crawling and probing distinctively characterized the control group, whereas swimming and incomplete crawling (*ic*) characterized leeches with ablations.

this group from the others. By contrast, leeches with SubEG ablations had the largest positive individual CCA scores, indicating that incomplete crawling and swimming were the most distinguishing behaviours of these leeches. The distribution of scores for leeches with nerve cuts was intermediate between the distributions of control leeches and leeches with ablations, indicating that deafferentation caused behavioural changes similar to ablation, but the effects were significantly less pronounced. Note that the behaviours that most strongly distinguished the groups were not necessarily the most common behaviours within each group but rather the ones that were least likely to be shared with other groups. The distributions of all three experimental groups (cuts, ablations

and control) are significantly different from one another (ANOVA,  $F_{2,192}=64.2$ ,  $P<0.001$ ,  $N=195$ ).

#### Elicited behaviour: the effect of stimulus location

To determine how elicited behaviours varied with the touch location, we touched each leech with moderate pressure (enough to indent the skin but not enough to do damage) on the dorsal side of the body at the anterior, midbody or posterior of the animal (Fig. 3A). Control leeches stimulated anteriorly most often shortened (*sh*), i.e. they withdrew from the touch by contracting the entire body longitudinally while keeping the tail sucker attached to the substrate. A much less common response to anterior touch was to release the front sucker (*fsr*). Although two other behaviours, detaching (*det*) and freezing (*freeze*), were more common than *fsr*, CCA indicated that *sh* and *fsr* together best distinguished the control response to anterior touch from touches elsewhere on the body (Fig. 3B,C). When control leeches were touched in the midbody (Fig. 3A), their most common response was local bending (*lb*), in which the body wall at the site of the touch contracts while the body wall on the side opposite to the touch relaxes, causing the body to move away from the site of the touch (Kristan, 1982). Touching control leeches at the posterior typically elicited crawling (*crawl*).

In general, locomotory responses (i.e. *crawl*, *swim* and *ic*) were more common in response to posterior touch than in response to touch at other locations, and, in control animals, *crawl* was the most likely response (Fig. 3A). Whole-body contraction (*wbc*) was elicited by posterior touch, but it was typically produced in conjunction with other behaviours, and this tendency to combine with other behaviours reduced the absolute value of its CCA behaviour score. A similar trend was seen in the behaviour score for *lb* in response to posterior touch; it was generally seen along with other behaviours that were more strongly associated with posterior stimulation. Distributions of the individual scores (Fig. 3C) revealed that when the scores for the behaviours in control animals were grouped by touch location, all groups were significantly different from one other (ANOVA,  $F_{2,192}=64.2$ ,  $P<0.001$ ,  $N=195$ ).

#### Elicited behaviour: the effect of surgical manipulation

Anterior stimulation elicited *sh* in all groups of leeches, regardless of treatment (Fig. 4A), so failed to distinguish among treatment groups. CCA showed that anterior touch characteristically also elicited *probe*, *fsr* and *crawl* in control animals (Fig. 4B,C), whereas following ablation, pivoting (*pivot*) and *lb* were more common. Leeches with nerve cuts displayed a behavioural profile in which there was less probing than in control leeches but more probing than in leeches with ablations. CCA scores for individual leeches with nerve cuts lay between the scores of controls and those of

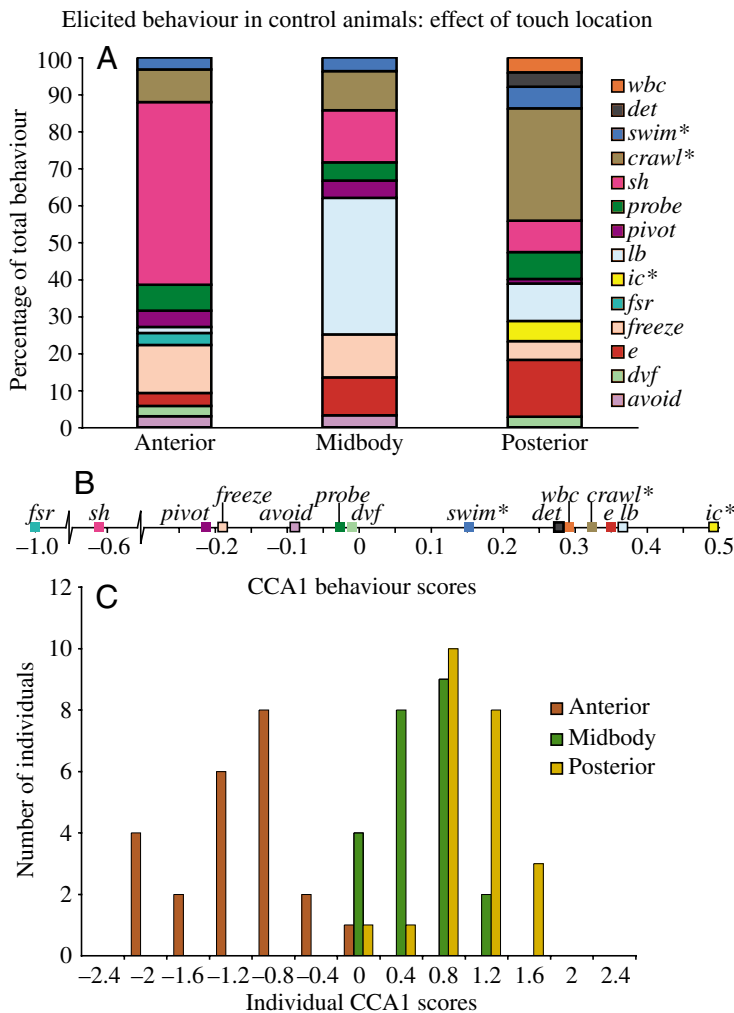


Fig. 3. Behaviours elicited in control animals by touching three locations on the body. (A) Distribution of behaviours elicited by touching the anterior, the midbody or the posterior of the leech ( $N=22$ ). The most common response varied with touch location: shortening at the anterior, local bending in the midbody, and crawling at the posterior. (B) Distribution of behaviours along the CCA1 behaviour score axis. (C) Distribution of individual CCA1 scores. The distribution of behavioural responses elicited by touch at the anterior are farthest from the distribution of behaviours elicited by touch at the posterior; the distribution of responses to midbody stimulation lies between the other two. See Table 1 for a description of the behaviours.

leeches with ablations (Fig. 4C). The overall distribution effect of surgery was significant (ANOVA,  $F_{2,38}=6.86$ ,  $P<0.001$ ,  $N=41$ ), but the only pair of groups that were significantly different from one another were control and ablated leeches.

Because midbody stimulation most often elicited *lb* in all treatment groups (Fig. 5), this behaviour did not distinguish among the groups, whereas less common behaviours did (Fig. 5B). *Probe* and *crawl* were the most distinctive responses seen in control leeches, whereas *swim* and *pivot* behaviours distinguished leeches with ablations of the SubEG (Fig. 5C). Leeches with nerve cuts were once again intermediate between the control leeches and the leeches with ablations. The overall effect of surgery was significant (ANOVA,  $F_{2,38}=12.33$ ,  $P<0.001$ ,  $N=41$ ), which was due to significant differences between control and ablated leeches and between nerve cut and ablated leeches.

Posterior stimulation elicited locomotion in all three groups, but the form of the locomotion varied among the groups (Fig. 6A), so swimming and crawling effectively distinguished among the treatments (Fig. 6B). The most common response of control leeches to posterior touch was crawling, whereas leeches with SubEG ablations swam more often than they crawled (Fig. 6A). Touching the posterior end of the leech also elicited movements at the anterior end of the leech, and, in this case, probing was more typical of control leeches, whereas pivoting was more common following ablation. The overall effect of surgery was significant (ANOVA,  $F_{2,38}=19.8$ ,  $P<0.001$ ,  $N=41$ ) due to significant differences between control and ablated and between nerve cut and ablated leeches (Fig. 6C).

#### Severity of ablation

In the previous analyses, leeches with ablations were lumped into a single group, but we also wanted to know whether ablating only part of the SubEG would produce either a qualitative or a quantitative difference. To ask this question, we focused on locomotory behaviours for three reasons. First, locomotion requires coordination of the whole body and thus might be affected by brain function. Second, unlike many elicited behaviours that were produced wherever the animal was touched, locomotion was common only in response to posterior touch, so we could focus our analysis on posterior touch. And third, although locomotion was produced in all three experimental groups following posterior touch, the form it took varied with experimental treatment (Figs 2A, 6A).

Ablating some or all of the SubEG affected both the level and the form of spontaneous locomotion, but surprisingly there were no statistically significant differences among leeches that were missing different numbers of neuromeres (Tukey's HSD,  $P>0.05$ ; Fig. 7A). All leeches with SubEG ablations were inactive much of the time. However, when they did move around spontaneously, they typically swam or executed only

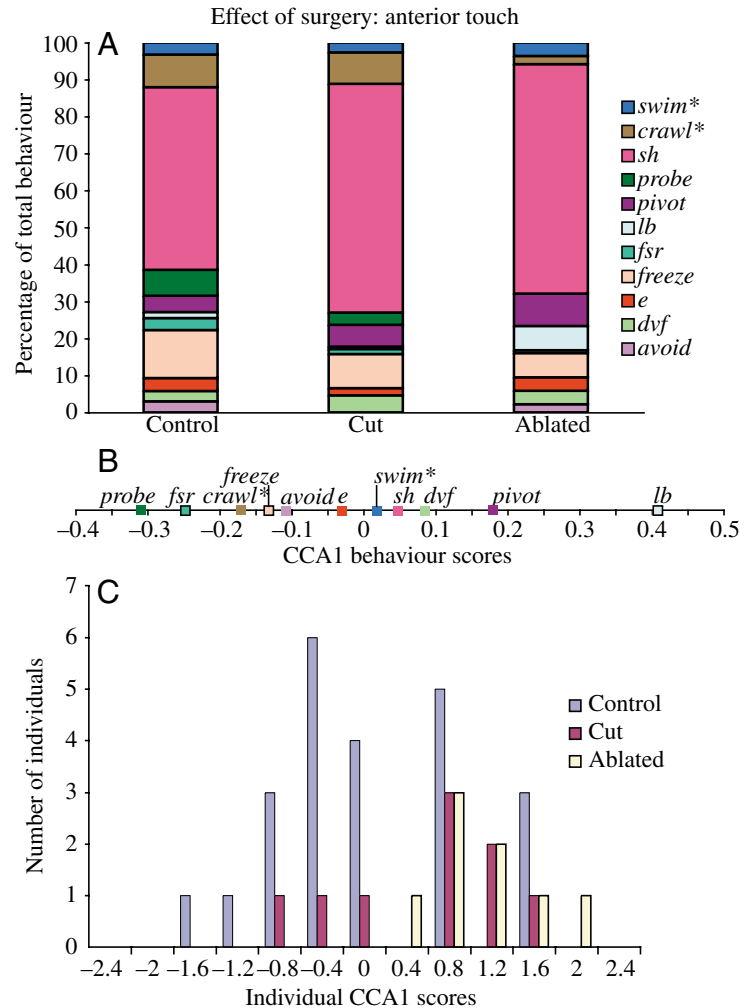


Fig. 4. Effects of surgery on behaviours elicited by touching the anterior of the body. (A) Behaviours elicited in the three experimental groups by anterior stimulation. Shortening was the most common response in all three groups. Control,  $N=22$ ; nerve cuts,  $N=9$ ; neuromeres ablated,  $N=10$ . (B) CCA1 behaviour scores. (C) Individual CCA1 scores of leeches in each experimental group. The distribution of scores for 'control' leeches and the distribution for 'ablated' leeches are farthest apart, and the distribution of scores for 'cut' leeches lies between the two. See Table 1 for a description of the behaviours.

part of the crawling cycle (*ic*), unlike controls and animals with nerve cuts, which crawled normally (Fig. 2A). In addition, once leeches with ablations began to swim, they typically continued for much longer than either control leeches or those with nerve cuts (data not shown), a pattern that was seen in all ablation groups regardless of severity. Similarly, we found no statistically significant differences among leeches missing different amounts of the SubEG when we considered elicited locomotion (Fig. 7B). These results suggest that many of the SubEG neurons affecting locomotion may be located in anterior neuromeres, so ablating posterior neuromeres in addition to the anterior ones produces relatively little additional effect.

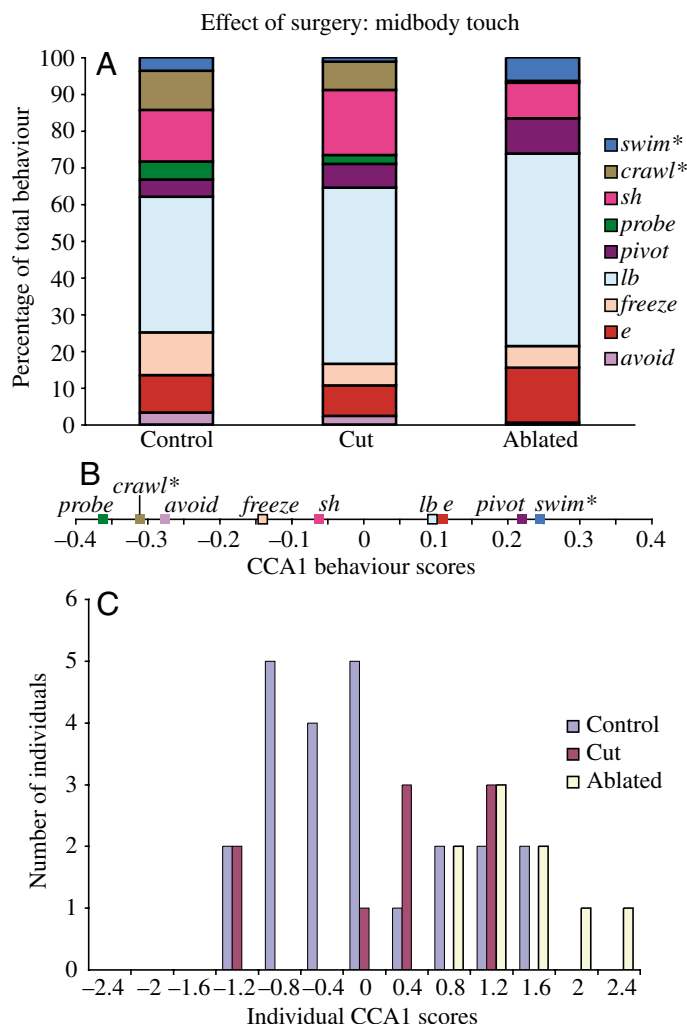


Fig. 5. Effects of surgery on behaviours elicited by touching the midbody. (A) Behaviours elicited in the three experimental groups by midbody stimulation. Local bending was the most common response in all three groups. Control,  $N=22$ ; nerve cuts,  $N=9$ ; neuromeres ablated,  $N=10$ . (B) CCA1 behaviour scores. (C) Individual CCA1 scores of leeches in each experimental group. The distributions of scores for 'control' leeches and for 'ablated' leeches are farthest apart; the distribution of scores for 'cut' leeches lies between the other two. See Table 1 for a description of the behaviours.

### Discussion

Surgically manipulated leeches exhibited different behavioural profiles to control leeches. Leeches with ablations of part or all of the SubEG and those with deafferented SubEGs were generally less active than controls, and when they moved about they were more likely to swim and less likely to crawl. This shift in behaviour suggests an important role for the SubEG in regulating whole-body behaviours.

#### General level of activity

The most general conclusion from our results is that the head brain plays a major role in setting the level of arousal, responsiveness and general activity in the whole animal.

Control leeches that crawled and actively probed provided a stark contrast to surgically manipulated leeches, which spent more than half of their time doing nothing at all or performing only slow probing behaviours. This result is consistent with the results reported by Bullock (Bullock and Horridge, 1965) and suggests that generalized excitation descends from the SubEG to the rest of the CNS. All neuromere ablations in this study severed the connection between the SupraEG and the ventral nerve cord, and future experiments will explore the effects of ablating just the SupraEG, while leaving all other connections of the SubEG intact.

#### Changes in whole-body behaviour

Crawling behaviour consists of a set of sequential actions that must be executed correctly and in the proper order if they are to produce forward motion (Baader and Kristan, 1995; Cacciatore et al., 2000; Stern-Tomlinson et al., 1986). Following ablations, leeches produced some parts of crawling behaviour, but they were typically uncoordinated, poorly executed and ineffective. The SubEG controls the use of the front sucker, which may explain part of this erosion of effective crawling following ablations. However, cutting the nerves projecting from the SubEG, which disables the front sucker as effectively as ablations, produced relatively little effect on crawling, whereas even partial ablation of the SubEG all but eliminated normal crawling (Fig. 7). We conclude that the SubEG, in addition to controlling the use of the front sucker, must contribute to organizing movements of the entire body during crawling; i.e. the elongation and contraction along the entire body, as well as placement of the rear sucker, which is likely also to depend heavily on neurons in the posterior brain.

The marked increase in both spontaneous and elicited swimming that we observed following ablations in the SubEG confirmed previous reports that the SubEG suppresses swimming (Broduehrer and Friesen, 1986). Leeches lacking a SubEG swam effectively but seemed unable to stop; they often swam for much longer periods of time than did control leeches. Similarly, adult leeches swim for much longer periods immediately after the connection between the head brain and the ventral nerve cord is severed (an effect well known among bait shop salespeople in the American Midwest). Following ablations, leeches produced swimming that appeared to be normal, which strongly suggests that the ablations left connections within the ventral nerve cord unchanged. We suggest that swimming is suppressed much of the time in intact leeches by neurons of the SubEG, such as Tr2 and SIN-1 (Broduehrer et al., 1995a,b; Taylor et al., 2003). Tonic suppression of swimming by the SubEG may then permit crawling in the absence of stimuli that lead specifically to swimming, but, when cephalic inhibition is lost, the default locomotory behaviour appears to be swimming.

We are beginning to understand the cellular basis for the choice between swimming and crawling. In the head brain, a neuron called R3b1 can cause either swimming or crawling depending on whether the leech is in deep water, in which case it swims, or in shallow water, in which case it crawls (Esch et



al., 2002). Interfering with the acquisition of sensory information from receptors in the head changes this behavioural choice. For example, in one set of experiments on adult leeches, the head brain was deafferented and the animals were tested in water whose depth was equal to the thickness of the body. Leeches with deafferented brains were highly likely to swim in this condition, whereas normal leeches were most likely to crawl (S. Copado, W. B. Kristan, III and W. B. Kristan, Jr, unpublished data). Thus, it appears that identifiable cells in the SubEG participate in processing sensory information coming from cephalic receptors and may play a central role in behavioural selection.

#### Locally controlled behaviours

Many studies have explored behaviours that are locally controlled by neurons in segmental ganglia of *Hirudo* (Kristan et al., 2005; Kristan, 1982). This pattern of local control remained intact following deafferentation or ablation of the SubEG. In our experiments, moderate touch to locations in the anterior, middle and posterior regions of the body elicited generally the same behaviours regardless of which surgical group the individual belonged to: shortening in response to anterior touch, local bending following midbody touch, and locomotion following posterior touch (Figs 3–6). If anything, these responses to touch were slightly enhanced following SubEG ablations, suggesting that modulatory signals descend from the SubEG. Thus, although our results indicate that the SubEG exerts some control over behaviour, the distributed nature of the leech nervous system allows many behaviours to be controlled on a local level, much as vertebrate stretch reflexes are confined to one or a few spinal segments with modulation descending from higher centres (Kandel et al., 2000).

#### Ablations vs nerve cuts

In each of our analyses, cutting the nerves connecting the SubEG to the periphery of the head produced a pattern of behaviour intermediate between the control group and the leeches that were missing at least some of the SubEG (Figs 2–7). In advance of the analyses, there was no compelling reason to predict this outcome, and, although the CCA took account of the experimental group to which each individual belonged, it imposed no particular ordering of the groups. This result suggests that one important role of the SubEG is to integrate signals from cephalic sensory neurons with signals from neurons in the rest of the body; when sensory input from the head is no longer available, there is erosion in the behaviour, but the animal neither falls into lassitude nor shifts to an entirely novel set of behaviours.

#### Comparison with other animal groups

The literature describing brain structure and function in other invertebrate groups is enormous and complex but, until

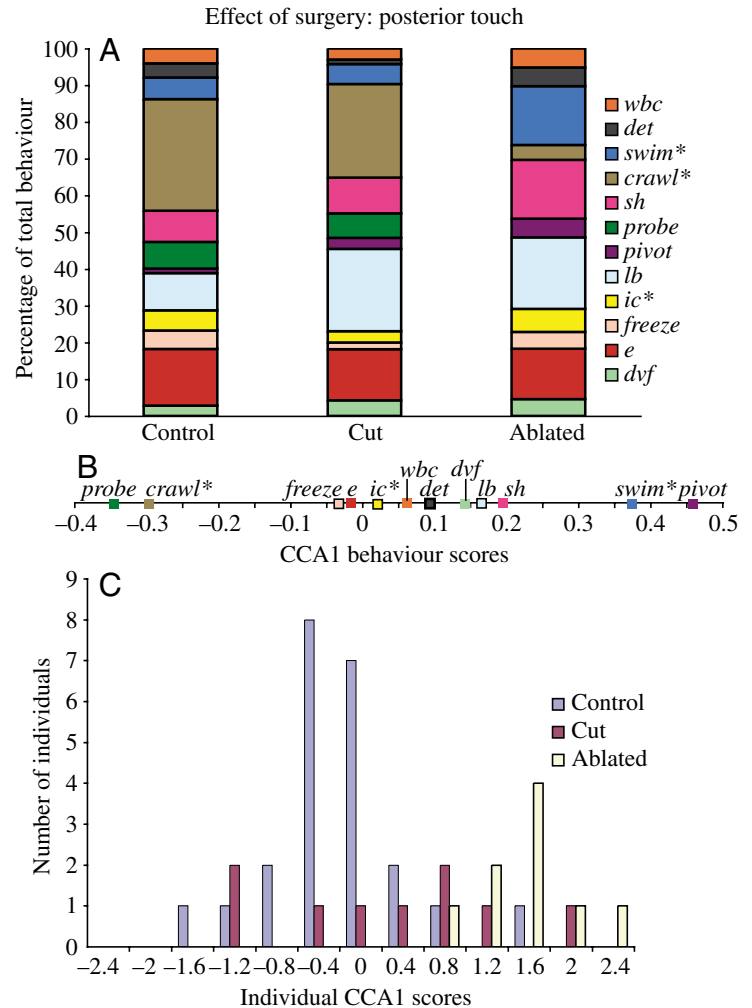


Fig. 6. Effects of surgery on behaviours elicited by touching the posterior. (A) Behaviours elicited in the three experimental groups by posterior stimulation. Locomotory behaviours (indicated by \*) were the most common behaviours elicited by posterior touch. Control,  $N=22$ ; nerve cuts,  $N=9$ ; neuromeres ablated,  $N=10$ . (B) CCA1 behaviour scores. (C) Individual CCA1 scores of leeches in each experimental group. The distribution of scores for 'control' leeches and 'ablated' leeches are farthest apart, and the distribution of scores for 'cut' leeches lies between the other two. See Table 1 for a description of the behaviours.

recently, tended to be biased toward exquisite descriptions of anatomy (Bullock and Horridge, 1965; Gupta, 1987). Much attention has been paid to the brains of arthropods, especially the complex brains of insects. Progress has been made in elucidating function in the brain regions devoted to sensory processing, e.g. the olfactory system (Christensen and Hildebrand, 2002; Galizia and Menzel, 2000) and the visual system in insects (Egelhaaf and Borst, 1993; Egelhaaf and Kern, 2002). The mushroom bodies in the insect brain seem to be association centres that play a variety of roles. They are thought to participate in the control of locomotion (Heisenberg, 1998; Zars, 2000), they are typically associated with olfaction (Strausfeld et al., 1998) and they contribute to learning and

memory (Menzel, 2001; Strausfeld et al., 1998). All of these structures are located in the supraesophageal brain, whose structure can be highly elaborate. The subesophageal ganglion

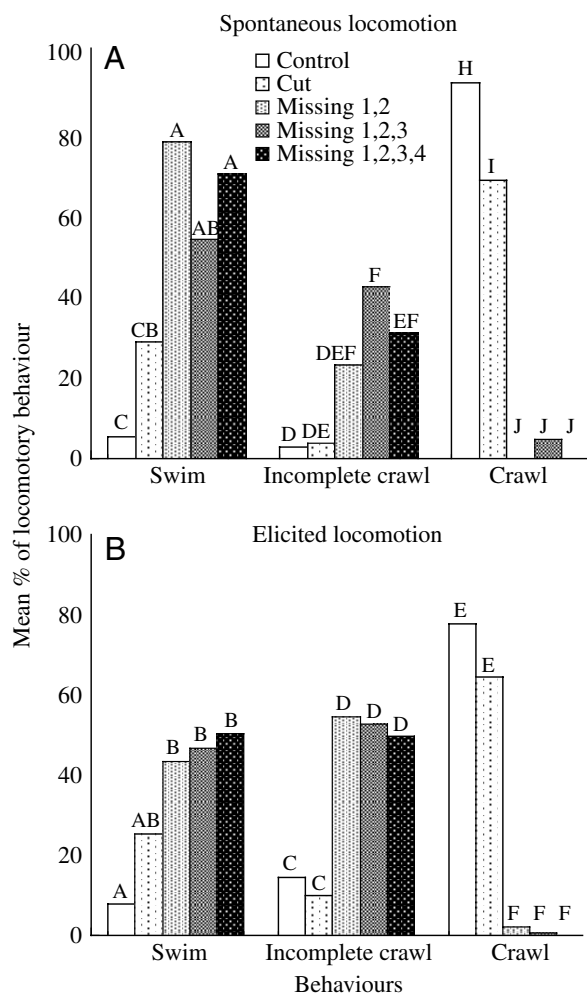


Fig. 7. Effect of surgical treatments on locomotory behaviours. (A) Percentage of the three locomotory behaviours produced spontaneously by leeches in the five experimental groups. Control,  $N=46$ ; cut,  $N=9$ ; missing 1, 2,  $N=10$ ; missing 1, 2, 3,  $N=10$ ; missing 1, 2, 3, 4,  $N=9$ . (B) Locomotion elicited by moderate touch to the posterior dorsal body. Control,  $N=46$ ; cut,  $N=9$ ; missing 1, 2,  $N=10$ ; missing 1, 2, 3,  $N=11$ ; missing 1, 2, 3, 4,  $N=10$ . Locomotion was much less common in animals with ablations than in controls or animals with nerve cuts (Fig. 6A). Locomotion included only swimming, crawling and incomplete crawling, so the sum of the bars for each experimental condition is 100% and the percentages for the three modes of locomotion are not independent. Within each set of bars (that is, for each behaviour within each panel), conditions that are not significantly different from one another are labelled by the same letter, A, B, C, etc., and bars that are labelled by different letters are significantly different from one another (differences among surgical treatment groups were tested with a Kruskal–Wallis test, and *post-hoc* comparisons among groups were done using a Tukey's HSD procedure on the ranked data). Bars that are labelled with two or more letters are not significantly different from bars labelled with any of the letters. This analysis includes a total of six extra controls, and eight leeches with ablations were used in only this analysis.

(SubEG) in arthropods has a simpler structure, and it exerts both excitatory and inhibitory control, providing coordination of several behaviours, including locomotion (Altman and Kien, 1987) and stridulation (Heinrich, 2002; Heinrich et al., 1998).

The brain in molluscs varies from relatively simple ganglion-like collections of neurons, as in snails (Chase, 2000), to the highly complex brains of the cephalopods (Williamson and Chrachri, 2004). Here, too, we know more about anatomy than about function, except in specific instances, such as olfactory function in the brain of the slug *Limax* (Cooke and Gelperin, 2001; Gelperin et al., 2000) and control of escape behaviour in the squid *Loligo* (Williamson and Chrachri, 2004). In addition, particular regions of the brain in *Octopus* are required for learning sensory discriminations.

Our results suggest that, in spite of the distributed nature of the annelid CNS and the relative morphological simplicity of the head brain, the SubEG plays many of the roles seen in a large variety of other animals, including the vertebrates: it provides general excitation that increases the activity of the animal; it is a source of inhibition that shapes the behaviour exhibited by the animal either subtly, as in the local responses to touch or, dramatically, as in the changes in locomotory pattern that accompany ablation of the SubEG; it integrates sensory information coming from receptors in the head with signals coming from other parts of the body; and it plays a crucial role in orchestrating whole-body behaviour such as crawling. We therefore suggest that these functions of the brain may well be primitive characters that have been preserved through evolution. Other abilities, such as learning and memory, have been added to the work of the brain in many taxa, but the relatively large size and small number of neurons in the leech SubEG may provide an excellent opportunity to understand many brain functions at a cellular and network level.

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