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Abstract

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Keywords (separated by '-') *Haminoea hydatis* - Axonal tracing - Cephalic sensory organs - Homology - Innervation patterns

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ORIGINAL PAPER

Innervation patterns of the cerebral nerves in *Haminoea hydatis* (Gastropoda: Opisthobranchia): a test for intraspecific variability

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Abstract This study describes the innervation patterns for the cerebral nerves which project to the cephalic sensory organs (CSOs) in the opisthobranch *Haminoea hydatis* (Linnaeus 1758) and uses axonal tracing techniques (backfilling) to reveal the central cellular origins for these cerebral nerves. Cell clusters projecting into the cerebral nerves can be defined by their positions in the ganglion relative to other clusters, nerve roots and lobes. The number of cell clusters and the relative sizes of somata are constant in a given cluster, whereas the absolute number of somata and absolute sizes of single somata in a given cluster increase with the size of the animal. Additionally, the invariable morphological characteristics of the cell clusters are used to define criteria for the assessment of possible homology for the clusters innervating the CSOs in Opisthobranchia. The data suggest two different strategies to accommodate the increasing body size; first, the additions of nerve cells and second, the growth of nerve cells.

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Keywords *Haminoea hydatis* · Axonal tracing · Cephalic sensory organs · Homology · Innervation patterns

Introduction

Gastropoda are guided by a variety of cephalic sensory organs (CSOs) which are believed to have primarily chemosensory and mechanosensory functions (Davis and Matera 1982; Emery 1992; Audesirk 1979; Bicker et al. 1982; Chase 2002; Croll et al. 2003). In Opisthobranchia, these CSOs present an assortment of forms and include the rhinophores, labial tentacles, oral veils, Hancock′s organs and cephalic shields (Croll 1983; Boudko et al. 1999; Dayrat and Tillier 2002; Croll et al. 2003). Homology of the different types of CSOs in the various opisthobranch subgroups has not yet been investigated in detail, although the Hancock's organ of Cephalaspidea has been suggested to be homologous with the rhinophores of other Opisthobranchia (Hoffmann 1939; Edlinger 1980; Huber 1993;, Gosliner 1994) based primarily on their patterns of innervation by cerebral nerves. Homology of the nerves has in turn been assessed on the locations of their ganglionic origin and their peripheral terminations. The high variability of innervation patterns found in Crustacea (Hayman-Paul 1991) and other invertebrates (Goodman et al. 1979; Arbas 1991; Kutsch and Breidbach 1994), however, suggests a need to refine this criterion for assessment of homology.

In the present paper, we extend the use of innervation patterns to study homology of the CSOs by using a "backfilling" or *axonal filling/tracing technique*, which allows the visualisation of complex details in the morphologies for individual somata projecting into each nerve (Altman and Tyrer 1980; Fredman 1987; Kerkhoven et al. 1991). The

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terms homology and homologisation are used here to describe innervation patterns only to connote an assessment of hypothesised homology, since homology itself is hypothetical and cannot be conclusively be proven.

Homology at the cellular level has already been discussed by Croll (1987) in Gastropoda and by Kutsch and Breidbach (1994) in Crustacea and some criteria for cellular homology have been established. The current study serves to test whether patterns of individual neurons can be used as a morphological complex for the homologisation of nerves in order to evaluate whether these patterns provide a better method than the ganglionic origin of the nerves as a criterion for homology, as proposed by Hoffmann (1939) and Huber (1993).

Here we examine the innervation patterns and cellular origins of the four cerebral nerves which innervate the CSOs in *H. hydatis* (Opisthobranchia, Cephalaspidea), a herbivorous species of the cephalaspid Opisthobranchia that lives in the European North Atlantic Ocean and the Mediterranean Sea. The investigation focuses on the definition of criteria for the assessment of homology of cellular innervation patterns in Opisthobranchia. We survey whether constant cell clusters in the central nervous system (CNS) can be identified to innervate certain CSOs and whether these cell clusters differ with the size and thus maturity of individual animals of the same species. In particular, the intraspecific variability for one nerve, the nervus labialis (N2), is tested. A bifurcation of the N2 was described as an apomorphy of the Opisthobranchia (Salvini-Plawen and Steiner 1996) and we found a high variability of the CSOs innervated by the N2 in different opisthobranch taxa, e.g. labial tentacles (Anaspidea), oral veils (Pleurobranchoidea) or rhinophores (Sacoglossa). We discuss constant properties of cell clusters which may serve as potential criteria for homologisation of innervation patterns. These criteria will be used in a subsequent comparative investigation in order to homologise cellular innervation patterns of various types of CSOs in different Opisthobranchia.

Materials and methods

Animals

Haminoea hydatis (Linnaeus 1758) (Cephalaspida, Opisthobranchia) were collected from the wild at Plèneuf (Brittany, France) and from a colony at the Aquazoo Düsseldorf (Germany), and were used to establish a stable laboratory population, maintained in closed seawater aquaria at 17°C and under ambient light. They were fed pieces of *Ulva lactuca*, *Ulva rigida* and *Cladophora spec*.

Tracing studies

Animals were relaxed with an injection of 7% magnesium chloride and the CNS, consisting of the cerebral, pleural, parietal and pedal ganglia, was removed and placed in a small Petri dish containing filtered artificial seawater (ASW; Tropic Marin, REBIE, Bielefeld, Germany) as saline. We then followed the procedures from Croll and Baker (1990) for Ni^{2+}–lysine (Ni–Lys) tracing of axons. Briefly, the nerves of the right cerebral ganglion were dissected from the connective tissue. The nerves were cut and the distal tip was gently drawn into the end of a tightly fitting glass micropipette using suction provided by an attached 2.5 ml syringe. The saline in the micropipette was replaced by a Ni–Lys solution (1.9 g NiCl-6H_2O, 3.5 g L-lysine freebase in 20 ml double-distilled H_2O) and the preparation was incubated for 12–24 h at 8°C to allow transport of the tracer. The micropipette was then removed and the ganglia were washed in ASW three times. The Ni–Lys was precipitated by the addition of 5–10 drops of a saturated rubeanic acid solution in absolute dimethylsulfoxide (DMSO). After 45 min the ganglia were transferred to 4% paraformaldehyde (PFA) and fixed for 4–12 h at 4°C. Thereafter the ganglia were dehydrated by an increasing ethanol series (70/80/90/99/99% each 10 min), cleared in methylsalicylate and mounted dorsal side up in Entellan (VWR International) on a glass slide. Altogether we performed over 35 replicates for the N2 in specimens, ranging from 5 to 25 mm in length, but samples with only a partial staining of the nerve were not used because of possible incomplete innervation patterns. Our criterion for a well staining was a uniformly dark blue nerve as it joined the ganglion, an indication for intact axons (Fredman 1987). Thus, only 23 replicates were analysed for the right nervus labialis (N2), covering a wide range of specimens from juvenile to adult stages (Fig. 1). Additionally, we tested the variability of the innervation patterns for animals of nearly the same size (samples 16–18, Fig. 1).The other cerebral nerves of *H. hydatis* were studied in five replicates each, and only large individuals of approximately equal sizes (above 12 mm in shell length) were used and only the right nerve was filled. For all cerebral nerves we performed controls for the nerves of the left cerebral ganglion (n = 1–4).The Ni–Lys tracings were analysed by light microscopy (Leica TCS 4D). Camera lucida drawings were digitalised following the method of Coleman (2003)??? adapted for CorelDRAW 11.

Correlation analyses

For the correlation analyses, we used three different morphological sizes (Fig. 1; Table 1):

(1) the product of the maximum length and breadth of the shell, (2) the length of the cerebral commissure and (3)

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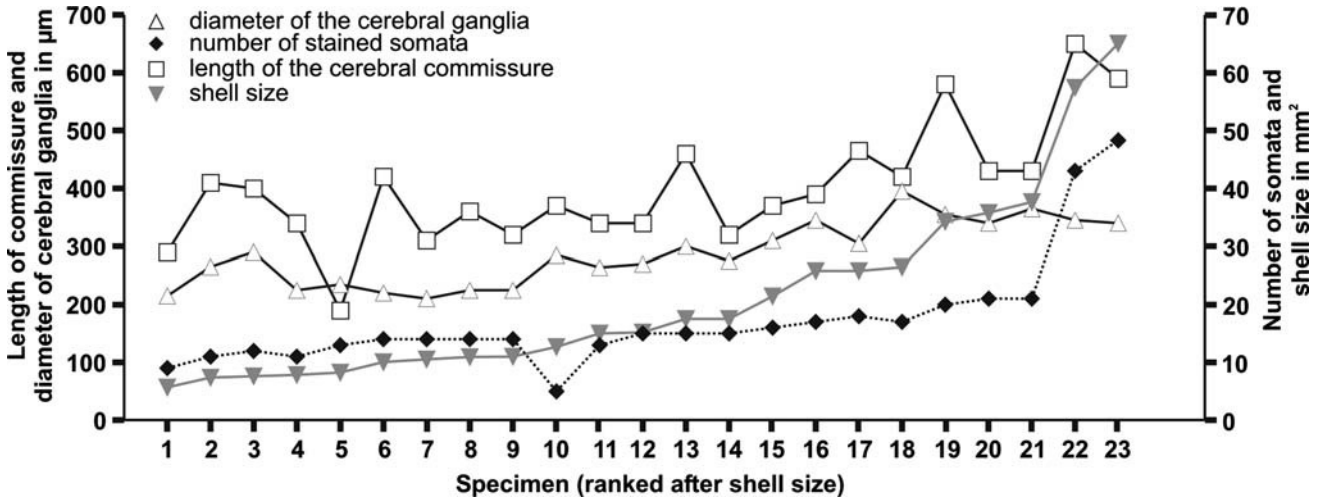

Fig. 1 Graph showing sizes and number of cells in the cerebral cell clusters. The *x*-axis represents the investigated animals ($n = 23$). The left *y*-axis shows the length of the commissure in µm and the mean diameter of the CG (cerebral ganglion) in µm, the right *y*-axis represents the shell size (length × breadth) in mm^2 and the number of somata in the clusters

the average of the maximal diameter of both cerebral ganglia. All measurements were performed on digital images, using Leica IM50 Software. Neither the length of the whole slug nor the size of peripheral structures such as the lip organ was used, because preliminary experiments indicated that these measures were found to depend greatly on the degree of relaxation of the animal.

Correlation analyses were performed using the statistical software PRISM4 (GraphPad Software Inc.). We tested for a Pearson correlation (Pearson *r*) assuming a Gaussian distribution for the data set, and also for a nonparametric correlation (Spearman *r*) with no assumption of distribution. For both the correlation analyses we used two-tailed correlation analyses with a 95% significance level.

Results

Organisation and innervation of the cephalic sensory organs in *Haminoea hydatis*

The CSOs are innervated by four, bilateral pairs of cerebral nerves in *H. hydatis* (Figs. 2, 3, 4), as indicated by the abbreviations modified from Edlinger (1980). The nervus oralis (N1) innervates the lip and the anterior cephalic shield (CS). The bifurcated nervus labialis (N2) innervates the lip organ (LO) and the anterior part of the Hancock`s organ (HO). The very short nerve nervus rhinophoralis (N3) terminates in the rhinophoral ganglion (RhG) which innervates the posterior part of the HO via four similarly short nerves. And last, the nervus clypei capitis (Nclc) innervates the posterior cephalic shield. We observed no variability of these nerves in all investigated specimens (over 40 preparations) with regard to regions of terminal innervation or even of major branch points.

Ni–Lys tracing

Five replicate backfills were performed for the N1, N3, Nclc and N2, using only the nerves of the right cerebral ganglion. The controls for the left cerebral ganglion ($n = 1$–4) shows no significant variation from the tracings for the right cerebral ganglion (data not shown). The characteristic patterns of labelled somata for all nerves are shown in Fig. 5a–d, including the approximate pathways of the stained axons. The identified clusters were named with abbreviations signifying the ganglion in which they are located, the nerve filled and a number indicating the order of their description (for example, Cnlc3, cerebral nervus labialis cluster 3; Pnoc1, parietal nervus oralis cluster 1).

In this study, we defined clusters of nerve cells, grouped on the basis of their close proximity within the ganglia and the tight fasciculation of their axons projecting into the filled nerve. While somata were often closely packed within individual clusters, they were occasionally more dispersed in other clusters. For example, the somata of clusters Pdncc3 and Pdncc4 (Fig. 5C) were distributed over a relatively large portion of the surface of the pedal ganglion but their axonal pathways were clearly distinguishable as two separate courses. We therefore designated the populations as separate clusters.

For the N1 ($n = 5$), we identified six cerebral clusters (Cnoc1-6), one pleural cluster (Plnoc1), one cluster in the right parietal ganglion (Prnoc1) and two pedal cluster (Pdnoc1-2) in each sample (Fig. 5a). These clusters were found in all preparations and the variation between the

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Table 1 Table of the number of specimen, shell size calculated by the product of length and breadth in mm^2 and maximum diameter of somata (in μm) within the cerebral clusters projecting into the N2

Specimen (+shell size in mm^2)	Size of somata (μm) within Cnlc1	Size of somata (μm) within Cnlc2	Size of somata (μm) within Cnlc3	Size of somata (μm) within Cnlc4	Size of somata (μm) within Cnlc5
1	7	4	6	12	5
5.67	8	14	11		
	9				
2	9	8	9	11	19
7.4	12	11	14	25	21
	14				
3	17	12	14	14	8
7.6	21	13	21	16	21
				17	29
4	18	13	19	17	6
7.82	19	14		18	24
		31			27
5	18	17	9	19	12
8.25	19	18	24	21	31
	21	29			36
6	21	16	8	19	21
10.08	22	19	17	22	31
		31	26	23	34
7	18	26	15	16	21
10.53	22	32	21	19	24
	24		28	24	34
8	13	10	21	20	19
10.92	15	28	22		21
		33	24		23
					24
					36
9	12	17	13	19	17
11.02	13		15	20	18
	14			21	20
	19				
	20				
10	23	17	21	23	21
12.71					
11	19	19	25	19	12
15.04	24	21		19	29
		23		21	33
					34
12	4	8	4	9	3
15.18	8	32	7	16	14
	12		16	29	26
	17				
13	13	15	11	4	17
17.5	17	23	21	12	19
	18	23		26	23
		26			

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Table 1 continued

Specimen (+shell size in mm^2)	Size of somata (μm) within Cnlc1	Size of somata (μm) within Cnlc2	Size of somata (μm) within Cnlc3	Size of somata (μm) within Cnlc4	Size of somata (μm) within Cnlc5
14	18	5	8	17	9
25.8		7	9	19	10
		8	9		18
		27	12		
		29	15		
		10	11		
15	6	8	14	24	12
17.5	11	12	17	28	18
	13	26	19		27
	24				
16	15	8	19	25	21
21.45	15	23	20		23
	16	30			26
	17	33			28
					33
17	14	12	6	6	12
25.8	15	15	12	16	14
	21	29	15	17	28
	36			24	
18	22	17	20	24	41
26.46	23	24	23	41	
	24	27	31		
	26	28	33		
		41	38		
19	9	17	9	13	22
34.3	12	21	11	18	24
	16	23	12	19	28
	18		14	22	
			16		
			25		
20	7	26	12	20	9
35.77	12	27	16	21	22
	13	28	18		27
	15		18		28
	16				29
	17				
	18				
21	17	29	15	16	21
37.63	22	30	16	18	22
	24	31	17	22	40
	29		22	24	
			24		
			26		
			33		

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Table 1 continued

Specimen (+shell size in mm^2)	Size of somata (µm) within Cnlc1	Size of somata (µm) within Cnlc2	Size of somata (µm) within Cnlc3	Size of somata (µm) within Cnlc4	Size of somata (µm) within Cnlc5
22	12	18	12	21	28
57.42	17	19	15	22	29
	19	23	27	27	31
	19	24	28	28	32
	20	28	30	30	32
	21	29			33
	22	31			34
	23	32			35
	23	36			
	23	54			
	24				
	24				
	27				
	29				
	31				
23	26	17	23	24	26
65.1	26	18	24	26	27
	27	18	27	27	28
	28	22	31	31	29
	28	23	36	41	32
	29	24	37	42	35
	29	27	38	44	41
	34	34	39	49	44
	35	36			
	35	48			
	36	59			
	37				
	38				

samples was restricted to only small differences in the number of somata (1–2 additional or missing somata) in some clusters. The single pleural cluster Plnoc1 is characterised by one large soma at the medial margin, and 8–10 small ones in the centre of the ganglion. The only parietal cluster, Prnoc1, is located on the medial margin of the right parietal ganglion. In the pedal ganglion, we identified two clusters: Pdnoc1, lying on the medial side near the pleural connective and above the pedal commissure, and Pdnoc2, at the posterior margin of the ganglion.

The innervation pattern for the N3 ($n = 5$) consisted of six cerebral (Cnrc1-6) and three pedal clusters (Pdnrc1-3) were identified (Fig. 5b). The position and the patterns of the first cerebral cluster Cnrc1 were very similar to an additional single cluster (Cclnrc1) in the left cerebral ganglion, indicating that these symmetric clusters may have bilateral projections in the N3. Additionally, two single somata occurred in both cerebral ganglia in nearly the same position at the root of the cerebral commissure (Fig. 5b, black arrows), again possibly indicating bilateral projections. The intraspecific variability between the five samples amounted to only very small differences in the number of somata in some clusters.

The innervation pattern ($n = 5$) for the Nclc consisted of five cerebral (Cncc1-5) and four pedal clusters (Pdncc1-4) (Fig. 5c). In comparison to the other cerebral nerves we found a higher absolute number of somata in the pedal clusters of this nerve. Additionally, cerebral and pedal clusters showed comparable number of somata. This was not found for the other nerves where cerebral somata always outnumbered pedal ones.

The innervation patterns for the last cerebral nerve, the N2 of large individuals (shell size >30 mm^2, Table 1) consisted of five cerebral clusters (Cnlc1-5), three pedal clusters (Pdnlc1-3) and a single soma in both pedal ganglia at nearly the same relative position, possibly indicating

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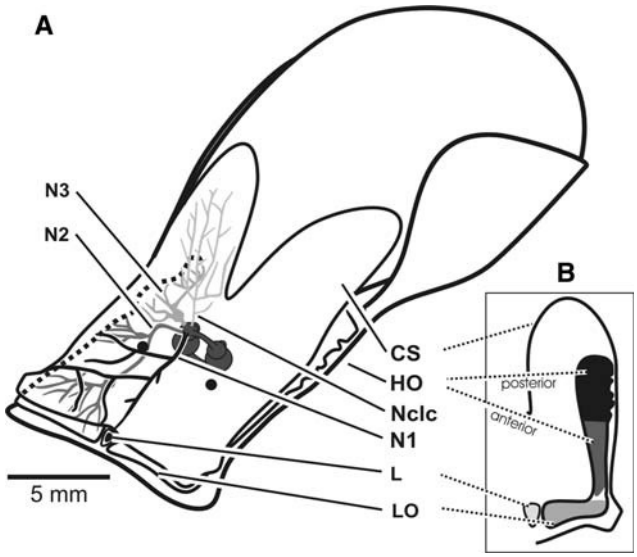

Fig. 2 **a** The four cerebral nerves (excluding the optical nerve) and the cephalic sensory organs of *H. hydatis.* Only the right cerebral nerves are shown. **b** Organisation of the lip organ and the Hancock′s organ in a dorsal view of the left side (N1, nervus oralis; N2, nervus labialis; N3, nervus rhinophoralis; Nclc, nervus clypei capitis, L, lip, LO, lip organ; HO, Hancock′s organ; CS, cephalic shield)

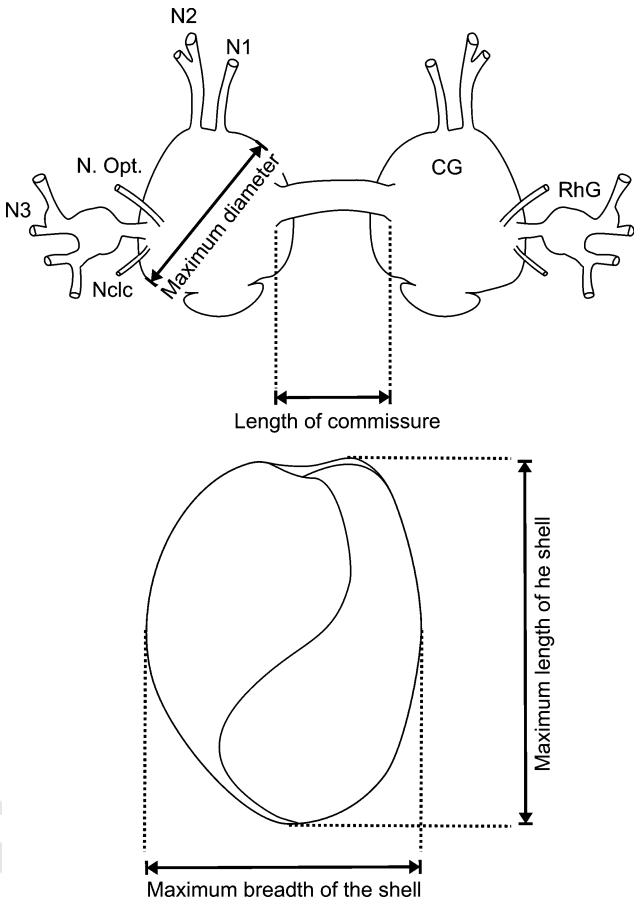

Fig. 3 Part of the CNS and of the shell in *H. hydatis*. The sizes, measured for the correlation analysis are shown: the maximum diameter of the cerebral ganglia, the length of the commissure and the length and breadth of the shell (N1, nervus oralis; N2, nervus labialis; N3, nervus rhinophoralis; Nclc, nervus clypei capitis; N.Opt, nervus opticus; RhG Rhinophoral ganglia; CG, cerebral ganglia)

bilateral projections (Fig. 5d). The three pedal clusters (Pdnlc1-3) in the innervation pattern for the N2 only occur in the labelling of larger individuals, (samples 19–23) and not in smaller individuals (samples 1–18). In comparison to the innervation patterns of other nerves, the clusters were easier to identify based on their positions as we found clear spatial separations. We found no significant differences in number of cell somata between specimens with shells of roughly similar sizes (Fig. 1, e.g. samples 16–18).

Variability of nervus labialis (N2) labelling

A specific aim of this study was to test the variability of axonal projections from identified clusters into specific nerves innervating the CSOs. For this purpose, we used the largest cerebral nerve, the nervus labialis (N2). First, we found no significant variability between innervation patterns for the left or the right N2. The staining patterns were nearly mirror images with all identified clusters containing cells of comparable sizes and numbers (data not shown).

To test developmental variability we compared labelling in animals of varying body sizes. All measured body sizes, the number of cerebral clusters and the total number of stained somata in the cerebral clusters are shown in Fig. 1. The data indicate a constant number of cerebral clusters in all individuals, but with increasing body size we found increasing numbers of cells (from 8–65) in the several clusters. Analyses showed a high correlation between the absolute number of somata projecting into the N2 and the size of the animals (Figs. 1, 6). For both the Pearson and the non-parametric correlation analyses, we obtained similar significant correlations between the measures of body size and the number of labelled somata. We found significant correlations for the number of cells with the animal's shell size (Pearson $r = 0.92470$, $P < 0.0001$; Spearman $r = 0.9312$, $P < 0.0001$), with the length of the commissure (Pearson $r = 0.74070$, $P < 0.0001$; Spearman $r = 0.6895$, $P = 0.0003$) and the average diameter of the cerebral ganglia (Pearson $r = 0.4988$, $P = 0.0154$; Spearman $r = 0.\ 7505$, $P < 0.0001$). In both the analyses, we found the highest correlation between the shell size and the number of innervating somata in the cerebral ganglia.

Additionally we measured the maximum diameter of each soma in each of the cerebral clusters Cnlc1–5 (Fig. 7). All clusters showed an increase of soma size with increasing shell size.

In summary, the results of this study demonstrate that all four stained cerebral nerves can be traced to specific clusters distributed across the cerebral, pleural and pedal

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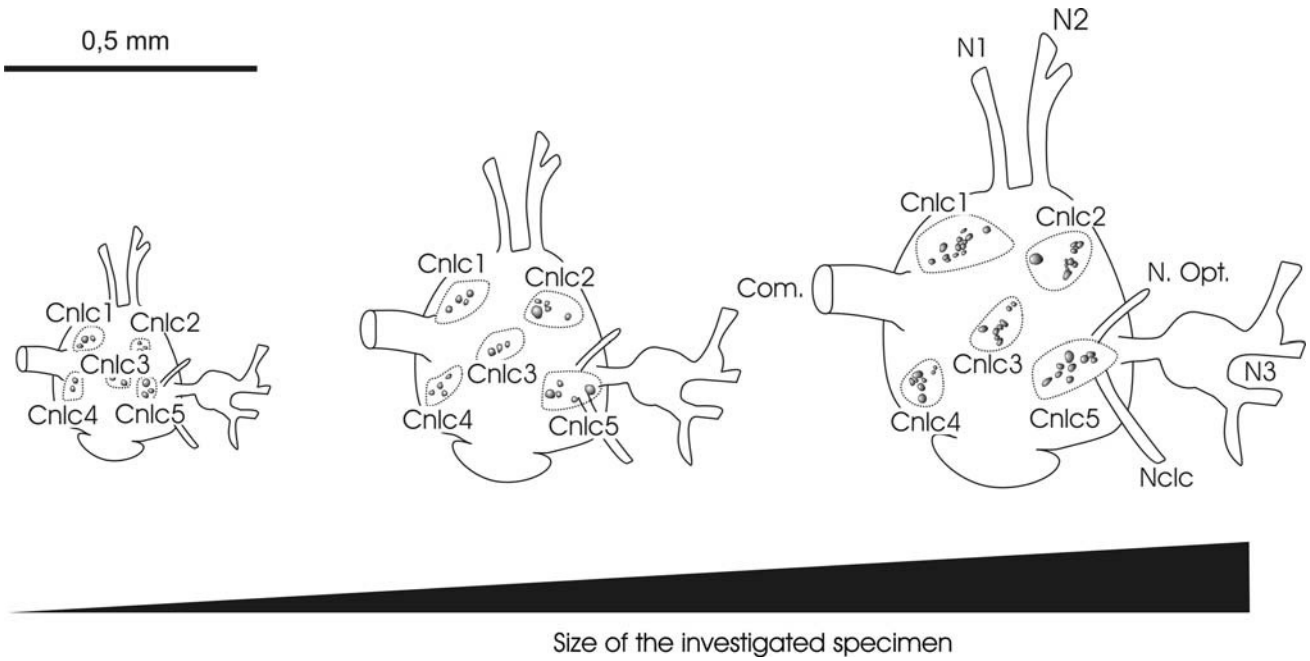

Fig. 4 Outline of cell clusters provided by the N2 in several right cerebral ganglia of individuals of different sizes. The size and position of the cells were digitalized from camera lucida drawings (N1, nervus oralis; N2, nervus labialis; N3, nervus rhinophoralis; Nclc, nervus clypei capitis; N. opt, nervus opticus; Com, cerebral commissure)

ganglia. The identities of cerebral clusters are specific for each nerve and independent of the size of the individual. Most somata projecting into the different cerebral nerves are located in the cerebral clusters. However, we also found relatively high numbers of somata in the pedal ganglia directly projecting into the CSOs via the cerebral nerves. While the identities of the various clusters are specific to the nerves and independent of the sizes of the specimens, the diameters and the absolute numbers of somata within the clusters depend on the size of the animal.

Discussion

The aim of this study was to provide a description of the innervation patterns for cerebral nerves of *H. hydatis* in order to define a morphological character complex for the assessment of probable homologies of the nerves and hence the homologisation of the CSO that they innervate. A specific goal of the current investigation, therefore, was to characterise several detailed features of the innervation patterns, including the size, position and number of neuronal somata within the central ganglia projecting into each of the specific nerves. Additionally, we tested the intraspecific variability of the patterns of these somata in order to provide a basis for identification of specific innervation patterns for each cerebral nerve. Previous studies have reported high variability of certain innervation patterns in Crustacea (Hayman-Paul 1991) and other invertebrates (Goodman et al. 1979; Arbas 1991; Kutsch and Breidbach 1994). We therefore systematically examined cellular characteristics of innervation patterns for different nerves [nervus oralis (N1), nervus labialis (N2), nervus rhinophoralis (N3), nervus clypei capitis (Nclc)], as well as differences in laterality and correlations between the size of animals and innervation patterns of one specific nerve (N2).

Innervation patterns of the four cerebral nerves

Recapitulating, our results clearly indicate that cellular innervation patterns of all four cerebral nerves could be described by specific cerebral, pleural, parietal and pedal cell clusters, which are cardinally characterised by their relative positions and axonal pathways in the respective ganglion (Fig. 5a–d). In addition, we found that these clusters were less characterised by similar relative sizes of somata within clusters. The lack of the pedal clusters for the N2 of small individuals may correspond to ontological changes in behaviour (e.g., onset of sexual behaviour). The abrupt appearance of the clusters only in larger specimens is less consistent with methodological problems (e.g., fragility of smaller nerves) which should be continuously variable over size.

With the purely anatomical nature of this study, it is, of course, impossible to assign specific functions to the various cell clusters, but projection patterns from the different ganglia might broadly correlate with general functions. For example, neurons mediating consummatory feeding behav-

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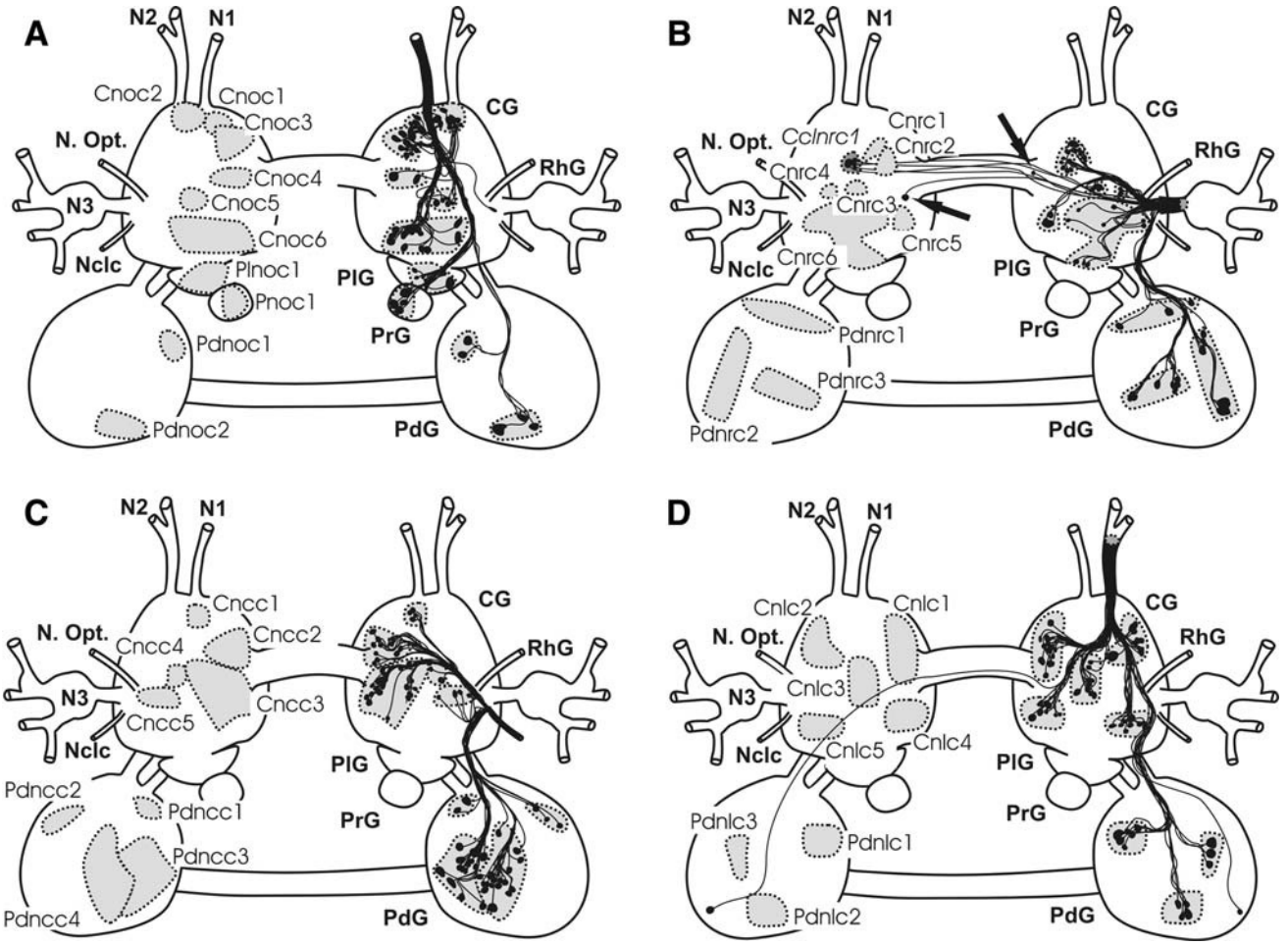

Fig. 5 **a** Outline of cell clusters providing the N1. **b** Outline of cell clusters providing the N3. **c** Outline of cell clusters providing the Nclc. **d** Outline of cell clusters providing the N2. The size and position of the somata were digitalized from a camera lucida drawing, the distribution of the axons are averaged from all replicates (N1, nervus oralis; N2, nervus labialis; N3, nervus rhinophoralis; Nclc, nervus clypei capitis; N. opt., nervus opticus CG, cerebral ganglia; RhG, rhinophoral ganglia; PlG, pleural ganglia; PdG, pedal ganglia; PrG, right parietal ganglia)

iours have been widely described in the cerebral ganglia (and also buccal ganglia not examined here) in other gastropods (Elliott and Susswein 2002). One might therefore expect neurons innervating organs mediating contact chemoreception and mechanoreception to similarly be located in the cerebral ganglia. Conversely the pedal ganglia are especially known to coordinate locomotion and might be expected to be more closely related to a distance chemoreceptive organ. These considerations are supported by the fact, that backfilling N1, which innervates the lip, a contact chemoreceptor, revealed the lowest amount of pedal somata, whereas backfilling N3, which innervates the posterior Hancock′s organ, a putative distance chemoreceptor, revealed a higher number of pedal somata. The highest number of pedal somata, however, was found backfilling the Nclc. This nerve innervates the posterior cephalic shield. The cephalic shield plays an important role for locomotion of *H. hydatis*, since it is used as a plough. During a long part of the daylight phase, *H. hydatis* is entrenched in the sand, probably as a protection against predators and the cephalic shield appears to aid in burrowing in the substrate (unpublished observations; Hoffmann 1939). Therefore, a higher number of pedal neurons providing this locomotory organ seems reasonable. Nevertheless, the exact function of the pedal somata is not clarified yet. More comparable data about other cephalaspid taxa with other strategies against predation or living in rocky habitats are needed.

Intraspecific variability of innervation patterns

While the backfilling of each nerve yielded a consistent pattern of clusters, which, in turn, each contained somata of similar relative sizes in larger specimens, we also found three sources of intraspecific variability. In smaller specimens, we observed: (1) lesser numbers of cells in clusters, (2) smaller cells within the clusters and (3) fewer clusters.

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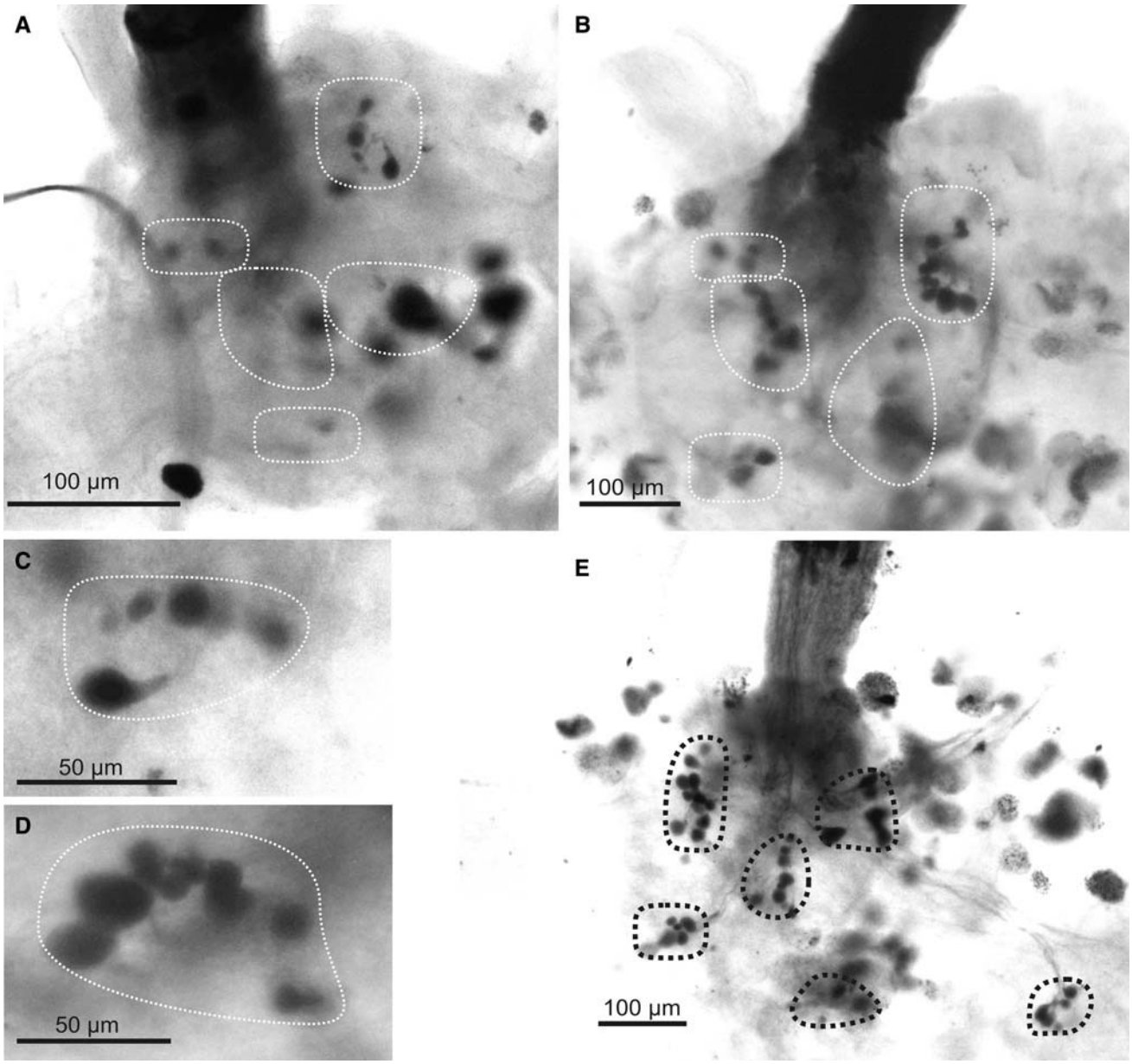

Fig. 6 Histological slides showing the difference of innervation patterns for the N2 between a small (**a**) and a large individual (**b**) and for the cluster Cnlc2 in the pictures **c** (small individual) and **d** (large individuals). Caused by the plasticity and the pigmentation of the ganglia, camera lucida drawings are more adequate to show the staining than photos. **e** Staining with all cerebral and one pedal cluster visible

These changes correlated with all of the different measurements of animal size used in this study. With the axonal tracing technique it is possible to identify characteristic cell clusters, but an identification of single cells is not given, as more characters are needed for a secure identification. Therefore, it is not possible to perform equivalent correlation analyses between shell size and size of single somata. Additionally for a correlation analysis of shell size and single soma size the data set (*n*) is too small.

The measurement which provided the highest correlation was the shell size, which has been used as a standard method (Hubendick 1951) of describing the size of soft-bodied shelled animals, like bivalves and gastropods. Measurements of ganglionic structures, which are directly influenced by the addition or the growth of neuronal somata, might be expected to provide higher correlations, but are also subject to shrinkage during histological processing. In fact, preliminary experiments attempting to measure the size of CSOs were also confounded by variable degrees of contraction during dissection in addition to subsequent histological distortions. Moreover, we did not use the age, because we observed an extremely high variation in body size from individuals of the same clutch (unpublished observations). Thus, we believe that body size provides a

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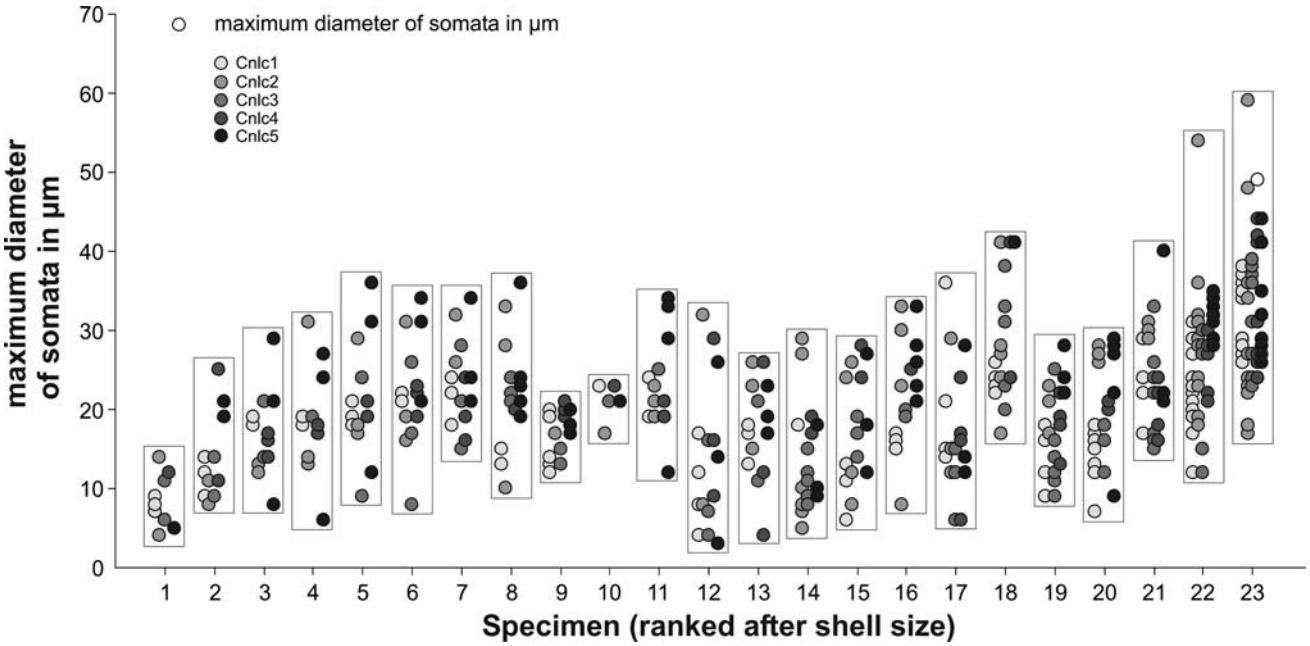

Fig. 7 Graph showing the distribution of somata sizes in the cerebral cell clusters (Cnlc1-5). The *x*-axis represents the investigated animals ($n = 23$), in the same order like in Table 1. Each *rectangle* represents one individual. Every cluster is represented by a different *greyscale*, and the clusters are ordered on the *x*-axis from left to right. The *y*-axis shows the maximal length of the somata in µm

poor indication for age, as has also been postulated for other gastropods such as *Lymnaea stagnalis* (Croll and Chiasson 1989).

We propose several explanations for increasing numbers and sizes of neuronal somata as well as the addition of pedal clusters in relation to the size of the animal. First, the N2 innervates the lip organ and the anterior Hancock′s organ, both of which are sensory organs (Edlinger 1980; Huber 1993). With growth of the animal, the sensory epithelia and associated glandular cells and muscles enlarge. Larger numbers of cells are therefore needed to innervate these structures. Second, the sizes of the somata also increase with increasing size of the animals. This could be explained with larger somata supporting larger axonal arborisations in either the periphery or in the central ganglia. Third, the addition of new pedal clusters may correlate with developmental changes in behaviour and physiology, which comprise predation, habitat and of course sexual maturity. Specifically, new clusters of cells may be added to the nervous system to mediate the appearance of new behaviours.

Our study employed a neuroanatomical technique to investigate innervation patterns in an opisthobranch gastropod. However, our results are consistent with the previous work in molluscs and other taxa using immunocytochemical or additional histological techniques. Other studies also found size-dependent or developmental changes like additional somata, cell clusters or growth of somata in the whole CNS, as it was observed in our study (Ogawa 1939; Stewart et al. 1986; Hauser and Koopowitz 1987; Cash and Carew 1989). For example, investigations on serotonin-like immunoreactive neurons of nudibranchs (Newcomb et al. 2006) showed that the size of somata in the CNS is correlated to brain size. Moreover, Newcomb et al. (2006) found a weak correlation of number of neurons in the CNS to brain size and also reported a higher intraspecific variation for neurons in pedal than cerebral clusters. Additionally Croll and Chiasson (1989) reported an increase in the numbers of neurons, mainly in identifiable clusters of neurons, and an increase in the size of somata for serotonergic neurons during the postembryonic development in the CNS of the basommatophoran snail *Lymnaea stagnalis*. They also noted the addition of clusters of neurons in various central ganglia including the pedal ganglia. This is congruent to our own investigation of additional pedal clusters labelled by N2 backfilling in large individuals which may be caused by developmental changes. The pedal ganglia are especially known for the coordination of locomotion which may undergo developmental changes in its chemosensory control. The preferred food resources of *H. hydatis,* green macroalgaes like *Ulva lactuca* or *Enteromorpha spec*., occurs in patches and in smaller individuals the mobility of the specimen is restricted. Small individuals with less mobility may therefore rely more heavily upon contact chemoreceptors to find food, whereas larger individuals with a higher mobility have the possibility to find new patches of adequate food sources with their distance chemoreceptor (Chester 1993).

While our findings are consistent with the previous literature indicating changes in the number of cells and clusters

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with increasing body size, we cannot discount possible contributions of systematic biases due to technical difficulties. Specifically, smaller specimens may have greater numbers of incomplete nerve fills despite our rigorous adoption of criteria for completeness. Further studies might employ double-labelling techniques combining backfills with immunocytochemical labels for transmitter contents to surmount such problems. However, regardless of the source of variability, our results clearly demonstrate that the number of cells within cerebral clusters and the numbers of pedal clusters are not likely to be adequate characters for homologising cerebral nerves across the Opisthobranchia.

Criteria for assessment of primary homology

To date, the homology of cerebral nerves in heterobranch gastropods has only been assessed using their ganglionic origins (Huber 1993). Whereas the N3 can be easily identified by the ganglionic origin, we believe that this criterion is insufficient for other nerves like the Nclc (Edlinger 1980) or the differentiation between the inner and the outer branch of the bifurcated N2. Such issues can also be entrapped by circular arguments for the homologisation of sensory organs, whereby nerves are named according to the structure which they innervate, but, in turn, the structures were homologised by the nerves which project to them. This paper therefore defines the following criteria for an assessment of homology of cell clusters projecting into the cerebral nerves of *H. hydatis*, because these innervation patterns provide more complex characteristics than ganglionic origins of nerves:

(1) the number of cerebral cell clusters. Presumably each cluster represents cells or regions with particular projections and different functions. This constancy in presence of neuronal structures in the cerebral ganglion has recently been postulated as a criterion for homology by Newcomb et al. (2006).
(2) the distribution of the axonal pathways. While the final arborisation of the axons can be variable (Croll 1987; Chiasson et al. 1994; Kutsch and Breidbach 1994), the major pathways of tracts projecting to the different nerves was found to be highly consistent in the present study.
(3) the position of the cell clusters in relation to each other and to ganglionic structures, like nerve roots, commissures and connectives. In fact, the position of clusters has been used widely as a means for their identification in gastropods, even through wide ranges of ontogeny (Croll and Chiasson 1989). Nevertheless a displacement of whole clusters during development, as described by Newcomb et al. (2006) for serotonin-like immunoreactive somata has been noted and further studies are needed to test this criterion between different taxa.
(4) the relative size of somata within each cluster in relation to other somata in the same cluster. This is the weakest criterion, as a high variability in size (Croll and Chiasson 1989) and a correlation between soma size and brain size was observed in serotonergic neurons of other gastropod taxa (Newcomb et al 2006).

Conclusions

As stated earlier, the goal of this paper was to establish the use of backfilling techniques to provide better means for evaluating probably homology of nerves than simply relying upon the positions of their origins from the central ganglia. However, we also acknowledge the eventual need for further criteria to assess homology, including the neurotransmitter content and other physiological features as well as patterns of developmental genes expressed by the specific populations of neurons.

In conclusion, we suggest that axonal tracing techniques provide a morphological character complex to describe and identify neuronal structures, and in consequence to postulate homology of cerebral clusters. The observations of variability and our definition of criteria can now be applied to evaluate hypothesised homologies of nerves and the organs which they innervate more broadly in Opisthobranchia .

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