The Evolution of the Cephalic Sensory Organs within the Opisthobranchia

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Für meine Mutter

Rötlich dämmert es im Westen,
Und der laute Tag verklingt,
Nur daß auf den höchsten Ästen
Lieblich noch die Drossel singt.

Jetzt in dichtbelaubten Hecken,
Wo es still verborgen blieb,
Rüstet sich das Volk der Schnecken
Für den nächtlichen Betrieb.

Tastend streckt sich ihr Gehörne.
Schwach nur ist das Augenlicht.
Dennoch schon aus weiter Ferne
Wittern sie ihr Leibgericht.

Schleimig, säumig, aber stete,
Immer auf dem nächsten Pfad,
Finden sie die Gartenbeete
Mit dem schönsten Kopfsalat.

Hier vereint zu ernsten Dingen,
Bis zum Morgensonnenschein,
Nagen sie geheim und dringen
Tief ins grüne Herz hinein.

(Wilhelm Busch)

II. Zusammenfassung

Das Ziel der vorliegenden Doktorarbeit war es, die Evolution der Kopfsinnesorgane der Opisthobranchia zu rekonstruieren. Bei den Opisthobranchia handelt es sich um eine äußerst diverse Gruppe überwiegend mariner Gastropoden innerhalb der Euthyneura. Die Kopfsinnesorgane oder cephalic sensory organs (CSOs) weisen innerhalb der verschiedenen Großgruppen der Opisthobranchia eine sehr hohe morphologische Variabilität auf, und finden ihre Ausprägung in verschiedenen Formen von Labialtentakeln, Mundsegeln, Rhinophoren, Lippenorganen, Kopfschilden und dem so genannten Hancockschen Organ. Die Homologieverhältnisse der CSOs waren bislang ungeklärt.

Der Ansatz der vorliegenden Studie war es, neurobiologische Methoden zu verwenden um die CSOs zu charakterisieren und zu homologisieren, da sich bisherige Methoden wie Histologie und anatomische Studien als unzureichend herausgestellt haben, die Homologieverhältnisse zu klären. Die dabei verwendeten Methoden wurden bislang nur in funktionellen Fragestellungen verwendet, daher stellt dieser Ansatz eine Neuerung in der vergleichenden Morphologie dar.

Bei diesen Methoden handelt es sich primär um das so genannte Axonale Tracing oder Backfilling und um immunohistologische Untersuchungen der Verteilung der Neurotransmitter Serotonin (5HT), FMRFamide und Tyrosin Hydroxylase (TH). TH ist selbst kein Neurotransmitter, sondern ein Enzym, welches zum Nachweis von Catecholaminen verwendet wird. Der Nachweis der vorher erwähnten Neurotransmitter wurde angestrebt, da sie innerhalb der Gastropoden stark vertreten sind und zahlreiche Studien, die jedoch nicht vergleichend konzipiert waren, zur Verteilung der genannten Neurotransmitter bei verschiedenen Gastropoden vorliegen.

Die Methode des Axonalen Tracings, bei der alle Somata angefärbt werden, die ein Axon in einen spezifischen Nerven entsenden, wurde primär genutzt, um die cerebralen Nerven, welche die CSOs innervieren, zu homologisieren. Bei einem Axonalen Tracing erhält man ein so genanntes zelluläres Innervierungsmuster, welches eine hohe Komplexität besitzt. Bisherige Studien homologisierten die cerebralen Nerven anhand ihrer Termination, d.h. der von ihnen innervierten CSOs, zugleich wurden jedoch im Zirkelschluss die CSOs anhand ihrer nervösen Innervierung homologisiert. Das Axonale Tracing ermöglichte es, diesen Zirkelschluss zu vermeiden.

Bevor jedoch die zellulären Innervierungsmuster als morphologischer Merkmalskomplex verwendet werden konnten, um Homologiehypothesen zu postulieren, musste die intraspezifische Variabilität dieses Merkmalskomplexes untersucht werden, und es mussten Homologiekriterien für zelluläre Innervierungsmuster definiert werden. Dieses geschah mit Hilfe der Untersuchung der zellulären Innervierungsmuster der zerebralen Nerven von *Haminoea hydatis*, einem Opisthobranchia aus der Gruppe der Cephalaspidea. Hierbei wurde besonders auf Variabilität der Innervierungsmuster bezüglich der Körpergröße geachtet, da dies bereits in früheren Studien beobachtet werden konnte und sich variable Aspekte eines morphologischen Merkmalskomplexes nicht eignen, um Homologiehypothesen zu formulieren.

Als Ergebnis dieser Untersuchung stellte sich heraus, dass die Somata eines zellulären Innervierungsmusters Konglomerate in der Form von Clustern bilden. Die Anzahl der Somata und ihre Größe sind abhängig von der Größe der untersuchten Individuen.

Nicht variable Merkmale der Innervierungsmuster konnten gefunden werden. Hieraus ließen sich folgende Homologiekriterien ableiten:

- Anzahl und Position der Cluster
- Der Verlauf der Axone der in einem Cluster zusammengefassten Somata
- Relative Größe und Position einzelner Somata eines Clusters in Relation zueinander

Nach der erfolgten Definition der Homologiekriterien wurden die zellulären Innervierungsmuster der zerebralen Nerven verschiedener Großgruppen der Opisthobranchia verglichen. Die untersuchten Taxa umfassten die folgenden Großgruppen, Aplysiomorpha (*Aplysia punctata, Aplysia californica, Petalifera petalifera*), Pleurobranchomorpha (*Pleurobranchaea meckeli, Berthella plumula*), Nudibranchia (*Archidoris pseudoargus*), Cephalaspidea (*Haminoea hydatis, Scaphander lignarius*) und Acteonoidea (*Acteon tornatilis*).

Um eine Rekonstruktion der Evolution zu ermöglichen wurden weiterhin Außengruppen wie die Caenograstropoda (*Littorina littorea*) und die Pulmonata (*Achatina fulica*) untersucht. Als Ergebnis stellte sich heraus, dass die zellulären Innervierungsmuster überraschend stark konservierte Strukturen darstellen, welche sich eignen, um die zerebralen Nerven innerhalb der untersuchten Taxa zu homologisieren. Hierbei ist anzumerken, dass die Opisthobranchia und die Pulmonata zwei Paar Kopfsinnesorgane und in der Regel vier zerebrale Nerven besitzen, die Caenogastropoda jedoch nur ein Paar Tentakel und drei zerebrale Nerven. Die zellulären Innervierungsmuster des Tentakelnerven, welcher das einzige Tentakelpaar der Caenogastropoden innerviert, entspricht dem kombinierten zellulären Innervierungsmuster der zwei zerebralen Nerven, welche in den anderen untersuchten Taxa, die beiden Paare Kopfsinnesorgane innervieren.

Daher ist an dieser Stelle anzunehmen, dass der Tentakelnerv (Nervus tentacularis) der Caenogastropoda dem Nervus labialis und dem Nervus rhinophoralis der Opisthobranchia und der Pulmonata entspricht. Nur anhand der zellulären Innervierungsmuster lässt sich jedoch nicht klären, ob die drei cerebralen Nerven der Caenogastropoda ursprünglich sind und sich in den Euthyneura, denen die Opisthobranchia und die Pulmonata angehören, in zwei Nerven aufgespalten haben, oder ob der Tentakelnerv der Caenogastropoda, das Ergebnis einer Fusion zweier Nerven ist.

Nachdem nachgewiesen wurde, dass zelluläre Innervierungsmuster als morphologischer Merkmalskomplex verwendet werden konnten, um Homologiehypothesen für die zerebralen Nerven und in Folge, unter Ausschluss des vorher erwähnten Zirkelschlusses, für die CSOs selbst zu postulieren, wurden weitere Datensätze

verwendet, um diese Hypothesen zu bestätigen oder zu verwerfen. Bei diesen weiteren Datensätzen handelt es sich überwiegend um die Neurotransmitterverteilung innerhalb der CSOs. Als Ergebnis lässt sich feststellen, dass die zwei Paar Kopfsinnestentakel innerhalb der Euthyneura spezialisierte Sinnesorgane darstellen. So ist die hohe Dichte von TH enthaltenden Somata in den vorderen Kopfsinnesorganen (anterior sensory organs – ASO) ein Indiz für eine Spezialisierung als Mechano- und Kontaktchemorezeptor, während die hinteren Kopfsinnesorgane (posterior sensory organs – PSO), eine charakteristische Verteilung von FMRFamidhaltigen Strukturen (Glomeruli) aufweisen, die charakteristisch für ein olfaktorisches Sinnesorgan sind.

Eine solche Unterteilung und Spezialisierung konnte in dem untersuchten Caenogastropoden (*Littorina littorea*), der nur ein paar Tentakel hat, nicht gefunden werden. TH kommt in geringeren Zelldichten in der gesamten Kopfregion von *Littorina littorea* vor, und die Glomeruli fehlen. Dies erscheint plausibel, da die meisten Opisthobranchia Nahrungsspezialisten sind, während viele Caenogastropoda Generalisten sind. Es ist daher davon auszugehen, dass sich im Lauf der Evolution ein einzelnes unspezifisches Tentakelpaar zu zwei Paar spezialisierten Kopfsinnesorganen mit unterschiedlichen Funktionen entwickelt hat.

Im Zusammenhang mit der phylogenetischen Position der Caenogastropoda in Bezug auf die Euthyneura, den vorher beschriebenen zellulären Innervierungsmustern der Caenogastropoda und der unspezifischen Funktion der Tentakel der Caenogastropoda, ist daher anzunehmen, dass drei zerebrale Nerven ein plesiomorphes Merkmal sind, welche sich innerhalb der Euthyneura in zwei zerebrale Nerven aufgespalten haben.

Zusammenfassend lässt sich erklären, dass es anhand der verwendeten neurobiologischen Methoden möglich war, plausibel gestützte Homologiehypothesen für die CSOs der Opisthobranchia zu formulieren. Anstelle früher verwendeter, zum Teil widersprüchlicher Begriffe wie Labialtentakel oder Rhinophoren wurden Kategorien von CSOs postuliert. Diese Kategorien sind Lip (Lippe), ASOa und ASOb (der zerebrale Nerv der innerhalb der Euthyneura die ASOs innerviert ist gegabelt und innerviert Strukturen mit wahrscheinlich unterschiedlichen Funktionen) und die PSOs.

Nach der erfolgten Homologisierung der CSOs wurde ihre Evolution unter Berücksichtigung der sparsamsten Erklärung, auf der Grundlage einer molekularen Phylogeniehypothese a posteriori rekonstruiert.

Es wurde postuliert, dass das Grundmuster der Euthyneura, zwei paar Kopfsinnesstrukturen besitzt. Die ASOs sind hierbei noch relativ unspezialisiert und wurden als lobenartige Strukturen postuliert, die PSOs hingegegen als eine Art basale Tentakel (Rhinophoren), welche innerhalb der Opisthobranchia unterschiedliche Ausprägung erfuhren und homolog zu den Ommatophoren der Pulmonaten sind. Damit widerlegte die vorliegende Studie die bislang gängige Annahme eines Kopfschildes und des Hancockschen Organs im Grundmuster der Opisthobranchia. Es wird davon ausgegangen, dass diese Organe eine Anpassung an eine grabende Lebensweise sind, bei der Tentakel, als mechanischer Belastung ausgesetzte Strukturen, eher hinderlich sind.

III. Abstract

The term cephalic sensory organ (CSO) is used for specialised structures in the head region of adult Opisthobranchia. These sensory organs show a high diversity in form and function, and the gross morphology of these organs differs considerably among taxa. They can be identified as cephalic shields, oral veils, Hancocks organs, lip organs, rhinophores or oral tentacles. Because of this extremely high diversity, the homology and the evolution of these organs have not been clarified yet. My intention was to use neuroanatomical data sets in order to find putative homologous CSOs. In this study, I will show data about immunohistochemical neurotransmitter content and cellular innervation patterns and their applicability as morphological characters for the homologisation of structures. I support earlier investigations that neurotransmitter content is often related to function. In contrast, axonal tracing patterns can be used to homologise nerves. Overall the aim of this study was to reconstruct the evolution of the CSOs of the Opisthobranchia, by projecting our neuroanatomical data sets onto a molecular phylogeny.

1. Introduction

„The behaviour of every animal depends on its perception of the external world. In the case of gastropods, their world has no sounds and, in most cases, no sights. Gastropods do have eyes, but in only a few species are they used for object recognition. Thus, the distance perception of gastropods usually depends on olfaction, and their perception of near objects is dependent on a combination of chemoreception and mechanoreception."

Ronald Chase, 2002

Gastropoda are guided by a variety of cephalic sensory organs (CSOs), believed to possess chemo- and mechanosensory functions (Audesirk 1979, Bell and Tobin 1982, Bicker et al. 1982, Davis and Matera 1982, Croll 1983, Emery 1992, Boudko et al. 1999, Künz and Haszprunar 2001, Dayrat and Tillier 2002, Chase 2002, Croll et al. 2003). According to Jahan-Pawar (1972), Audesirk (1975), Bicker et al. (1982) and Croll (1983) the CSOs are primarily involved in chemoreception. Chemoreception is generally the most important modality for gastropods (Audesirk 1975, Chase 2002, Wertz et al. 2006, Wertz et al. 2007). Chemical senses are used in finding food, avoiding predators, homing and interacting with conspecifics (Emery 1992). However, the CSOs are also sensitive to mechanical stimuli (Jahan-Pawar 1972, Bicker et al. 1982), water currents (Wolter 1967, Storch and Welsch 1969) and light (Chase 1979, Jacklet 1980).

The Opisthobranchia comprise a species rich and diverse group of rather specialized, highly evolved, mostly marine slugs and snails within the Heterobranchia with up to 6000 extant species confined in nine taxa. The cephalic sensory organs exhibit a very prominent but also very variable character complex in these taxa, with each subgroup possessing a more or less characteristic set of CSOs. Within the Acteonoidea but also the Cephalaspidea and the interstitial Acochlidiacea, the CSOs manifest as lip organ,

Hancocks organ and cephalic shield. The taxon Nudipleura is divided into the Nudibranchia, which show a variety of labial tentacles, oral veils, massive rhinophores but also a Hancocks organ (i.e. *Tritonia diomeda*) and the Pleurobranchomorpha with very prominent oral veils and curled rhinophores. The Umbraculida, like the Aplysiomorpha, present a set of labial tentacles and rhinophores. In some aplysiomorph species oral lobes and a Hancocks organ are also present. The two pelagic taxa, the Gymnosomata and the Thecosomata (both taxa are combined in the taxon Pteropoda) possess labial tentacles and rhinophores. The ninth subgroup within the Opisthobranchia, the Sacoglossa, only possess one pair of tentacles, which is very uncommon in these gastropods, as most opisthobranch taxa exhibit two pairs of CSOs. Although the gross morphology of the CSOs is relatively well described, extensive detailed comparative studies of this extremely diverse character complex, allowing for the assessment of homology of the organs, are lacking to date.

1.1 Current homology hypotheses

Although homology of the different types of CSOs has never been assessed based on detailed comparative data, several hypotheses of homology of the different sensory organs in Opisthobranchia have been postulated in the past. Based on the neuroanatomy, Huber (1993) postulated that the labial tentacles of the Aplysiomorpha and the Pleurobranchomorpha are homologous structures to the anterior Hancocks organ, which is located below the cephalic shield within the taxon Cephalaspidea. Briefly, the Hancocks organ is presently divided into an anterior and posterior section (Edlinger 1980), which are innervated by different cerebral nerves. In the past, the Cephalaspidea (including the Acteonoidea) were considered to take a basal position within Opisthobranchia (Boettger 1954, Ghiselin 1966, Schmekel 1985). This view even nowadays sometimes holds up (Myers et al. 2008). This taxonomic placement implicated that the cephalic shield, lip organ and Hancocks organ are plesiomorphic structures within Opisthobranchia and subsequently, that the tentacles and rhinophores of other opisthobranch taxa are derived structures.

However, recent molecular investigations (Vonnemann et al. 2005, Klussmann-Kolb et al. 2008) suggest two major clades within the Opisthobranchia which neither support a monophyletic taxon for the Cephalaspidea containing the Acteonoidea, nor the basal position of the Cephalaspidea. Therefore the possibility of the existence of tentacles or rhinophores in the ground pattern of the Opisthobranchia can no longer be excluded and merits particular attention. Gosliner (1994) postulated an independent development of the rhinophores within the different opisthobranch taxa. Later, Wägele and Willan (2000) postulated homology for the rhinophores of the Nudipleura comprising the taxa Nudibranchia, which exhibit massive rhinophores as well as the Pleurobranchomorpha, which display a curled version of the rhinophores. Nevertheless, the authors could not support this hypothesis with detailed data about the histology and morphology of these structures. Different hypotheses exist about the origin of the opisthobranch rhinophores. One hypothesis suggested that the rhinophores evolved from the Hancocks organ (Hoffmann 1939, Bullock and Horridge 1965, Schmekel 1985, Huber 1993, Mikkelsen 1996). In a second hypothesis, Gosliner (1994) postulated that the Hancocks organ and the rhinophores are analogous structures. In addition a homology of the opisthobranch

rhinophores or the Hancocks organ with the caenogastropod tentacles has been excluded (Hoffmann 1939, Schmekel 1985, Huber 1993).

The homology hypotheses for the CSOs of the Opisthobranchia in earlier investigations are often based on the innervation by presumably homologous nerves (Hanström 1929, Hoffmann 1939, Huber 1993). Hereby, the nerves were primarily homologised in respect to their ganglionic origin and their peripheral terminations. This approach implicates failure since the CSOs are homologised based on the homology of the cerebral nerves, while the cerebral nerves in turn are homologised according to homology hypotheses of the CSOs. The high variability of nervous innervation patterns found in Crustacea (Hayman-Paul 1991) and other invertebrates (Goodmann et al. 1979, Arbas 1991, Kutsch and Breidbach 1994), however, suggests a need to refine this criterion for assessment of homology for the CSOs.

1.2 Concept

The present study provides the first comparative investigation of the anatomy, immunohistochemistry and cellular innervation of the CSOs in Opisthobranchia. These data will be used to assess for homology of these organs in this group of Gastropoda. In a second step, a posteriori, the hypotheses on homology will be mapped onto an independent phylogenetic hypothesis in order to trace the evolution of this character complex. In order to homologise the CSOs several approaches will be used, regarding the assumption that a high complexity and similarities of all kinds (Bock 1989) are fundamental criteria for an explanation of homology.

The cellular and not the nervous innervation patterns of the cerebral nerves, providing the respective sensory organs, will be compared by a method called axonal tracing or backfilling, which allows the visualisation of complex details in the morphologies of individual somata projecting into a respective nerve (Altman and Tyrer 1980, Fredman 1987, Kerkhoven et al. 1991). Homology at the cellular level has already been discussed by Croll (1987) in Gastropoda and by Kutsch and Breidbach (1994) for Crustacea and some criteria for cellular homology have hereby been established. Using the axonal tracing technique, a morphological character complex, which is more complex than the ganglionic origin as used in earlier investigations (Huber 1993), will be reconstructed. Nevertheless, the innervation by homologous nerves does not always result in a homology of organs (Dayrat and Tillier 2002). Due to the extreme diversity of the CSOs within the Opisthobranchia, homoiology or parallelism revealing the several types of CSOs can not be excluded. In this context, additional aspects of the CSOs will be investigated in this study. These additional aspects include neuroanatomy and immunohistochemistry, which will be used to confirm the primary homology hypotheses based on the homology of the cerebral nerves. Earlier investigations using immunohistochemistry have shown that the neurotransmitter content of nervous cells can be used for evolutionary questions, as the neurotransmitter content is often conserved within the molluscan nervous system (Newcomb et al. 2006). The distribution of neurotransmitters in the CSOs has been studied in different gastropod taxa (Ono and McCaman 1984, Croll and Lo 1986, Salimova et al. 1987, Longley and Longley 1986, Sudlow et al. 1998, Hernadi and Elekes 1999, Moroz et al. 1997, Croll

2001, Croll et al. 2003, Croll and Dickinson 2004, Wertz et al. 2006, Newcomb et al. 2006, Wertz et al. 2007). These studies primarily focused on the distribution of serotonin. Serotonin (5-HT) is a biogenic monoamine which is synthesized in the nervous system from the amino acid tryptophan (S.-Rozsa 1984) and supposed to have a neuromodular function. Investigations of other neurotransmitters or neuropeptides were less extensive. Only few comparative studies exist until now (Croll et al. 2003, Newcomb et al. 2006, Faller et al. *in revision*).

In the present study the distribution of serotonin, tyrosine hydroxylase (TH) and FMRFamides in the cephalic sensory organs of several different opisthobranchs are compared. This will enable the comparison of these distributions among different opisthobranch taxa, in order to reveal insights into the function of the different types of CSOs. TH is an enzyme which catalyses the initial step in the conversion of tyrosine to the catecholamines (S.-Rozsa 1984), and therefore is an indication for catecholamines. Catecholamines have been detected in the central nervous systems of the gastropod *Helix* (Hernadi et al. 1993, Bernocchi et al. 1989, Hernadi and Elekes 1999), in the central and peripheral nervous systems of *Aplysia* (Salimova et al. 1987, Croll 2001) and in the CSOs of *Phestilla sibogae* (Croll et al. 2003). FMRFamide (Phe-Met-Arg-Phe-NH2)-related peptides comprise a family of neuropeptides which were isolated first from the ganglia of the clam *Macrocallista nimbosa* (Price and Greenberg 1977), but are also ubiquitous in other molluscs (Price et al. 1987) and across most of the invertebrates (Predel et al. 2004, Berg et al. 2007). According to Cottrell (1989) FMRFamides are chemical messengers in both the central and peripheral nervous systems. They have been detected in the central nervous systems of the gastropods *Helix pomatia* (Elekes and Nässel 1990) and *Limax marginatus* (Suzuki et al. 1997) and in the CSOs of *Phestilla sibogae* (Croll et al. 2003). With these comparative data on immunohistochemistry of the CSOs, the homology hypotheses based on the cellular innervation patterns will be evaluated.

1.3 Objective

The aim of this study is to describe the structure, cellular innervation and function of the CSOs in Opisthobranchia. This will be done using neurobiological methods. These methods, until now, were mainly used in the context of functional questions. Furthermore, comparative studies of these kinds of data regarding evolutionary aspects like the evolution of morphological structures are lacking. I will test if axonal tracing patterns can be used to homologise the cerebral nerves. Moreover, I will evaluate, if homology hypotheses regarding cerebral nerves as well as data on immunohistochemistry of sensory epithelia allow for assessment of homology of the respective organs. Based on a current phylogenetic hypothesis I will trace the evolution of the CSOs. Furthermore, I will reconstruct ground patterns for specific clades and postulate the evolution of the ground patterns in different lineages forwards the CSOs present in the investigated extant taxa.

This study represents a new approach within the comparative morphology and anatomy of Gastropoda to verify homology hypotheses for complex morphological structures. In future, this approach could be the base of studies dealing with questions about homology hypotheses and the evolution of structures.

2. Material and Methods

2.1 Material

The neuroanatomy of representatives of five of the main opisthobranch suborders was investigated: the Acteonoidea with *Acteon tornatilis*, the Cephalaspidea with *Haminoea hydatis* and *Scaphander lignarius,* the Aplysiomorpha (= Anaspidea) with *Aplysia californica, Aplysia punctata* and *Petalifera petalifera*, the Nudibranchia with *Archidoris pseudoargus* and the Pleurobranchomorpha with *Pleurobranchaea meckeli* and *Berthella plumula*. Additionally, I investigated the Caenogastropoda with *Littorina littorea* and the Stylommatophora with *Achatina fulica*. All investigated species, their origin, and the used methods are shown in table 1.

Table 1: List of all investigated species, their locations, collectors, and used methods: NA/Neuroanatomy, AT/Axonal tracing, IH/Immunohistochemistry.

Investigated species	Location	Collector	NA	AT	IH
Acteon tornatilis	St. Michel-en-Grève, Brittany, France	Sid Staubach	X	X	X
Pleurobranchaea meckeli	Blanes, Spain	Sid Staubach, Yvonne Gryzimbowski	X	X	X
Berthella plumula	Roscoff, Brittany, France	Sid Staubach	X	-	X
Archidoris pseudoargus	Roscoff, Brittany, France	Sid Staubach	X	X	X
Aplysia punctata	Roscoff, Brittany, France	Sid Staubach	X	X	X
Aplysia californica	National Resource for Aplysia Facility at the Rosenstiel of Marine and Atmospheric Sciences	-	X	X	X
Petalifera petalifera	Banyuls, France Cubagua, Venezuela	Sid Staubach, Sylvia Grune	X	-	X
Haminoea hydatis	Plèneuf, Brittany, France	Ulrike Schulte-Oehlmann	X	X	X
Scaphander lignarius	Blanes, Spain	Sid Staubach, Yvonne Gryzimbowski	X	-	X
Achatina fulica	Terrarium population	Carmen Zinßmeister	X	X	-
Littorina littorea	North Sea, Vollerwiek	Eberhard Kolb	X	X	X

2.2 Species

The classification of species followes Bouchet et al. (2005). Here the Acteonoidea belong to the informal group of the lower Heterobranchia.

2.2.1 *Acteon tornatilis* LINNAEUS, 1758

Taxonomic position:

Heterobranchia

Lower Heterobranchia

Acteonoidea

Acteon tornatilis occurs at the North Atlantic coast of Europe from Iceland to Norway, the British Isles, France, Southern Portugal and Gibraltar. This heterobranch species shows a heavily calcified spiral shell and a thin horny operculum. The shell is pinkish brown with two white bands on the shell. The white soft body with the prominent bifurcated cephalic shield can fully retract into the shell. The relaxed animal reaches about 3 cm in length and is found burrowed in sandy sediments from the low intertidal to over 200 meters of depth. It is reported to feed on polychaete worms such as *Owenia fusiformis* and *Lanice conchilega* (Yonow 1989). *Acteon tornatilis* (Fig. 1) was collected in the wild at St. Michel-en-Grève (Brittany, France) and stored alive at our lab in Frankfurt in closed seawater aquaria at 17º C and under ambient light conditions (12h light / 12h dark rhythm) until further investigations were conducted.

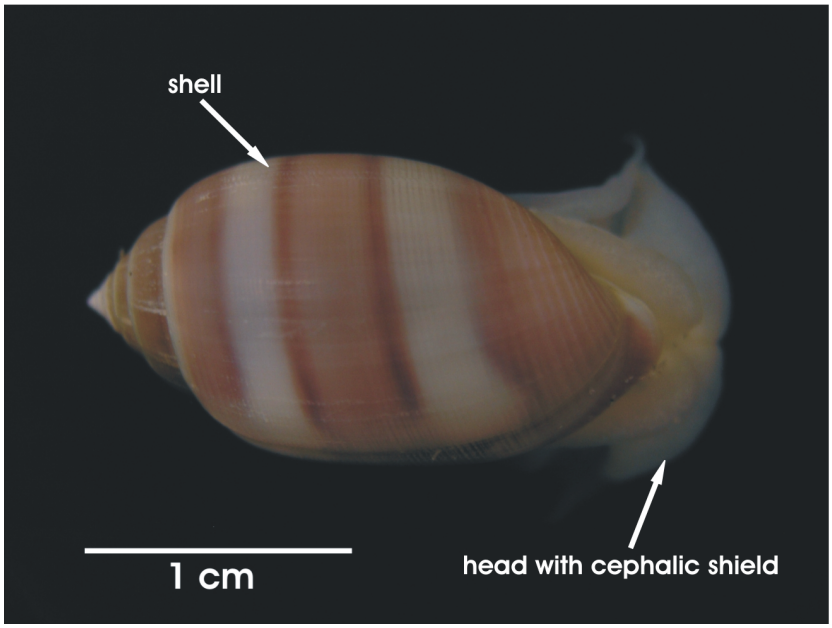


Figure 1: Dorsal view of *Acteon tornatilis*

2.2.2 *Pleurobranchaea meckeli* LEUE, 1813

Taxonomic position:

Heterobranchia

Opisthobranchia

Pleurobranchomorpha

Pleurobranchaea meckeli (Fig. 2) occurs at the Atlantic coast from Greenland to Gibraltar and in the Mediterranean Sea. The species grows up to 20 cm. The body colour is very variable. Several species of the genus Pleurobranchaea have been described in the Mediterranean and the Eastern Atlantic. Externally all of them look extreme similar. Therefore I used the anatomy traits defined by Marcus and Gosliner (1984) to identify the species. Characteristically for the CSOs are a prominent oral veil and rhinophores. *Pleurobranchaea meckeli* was collected in Blanes (Spain) by fishermen via dredging at depths of 60 to 80 meters. Specimens were investigated immediately upon collection.

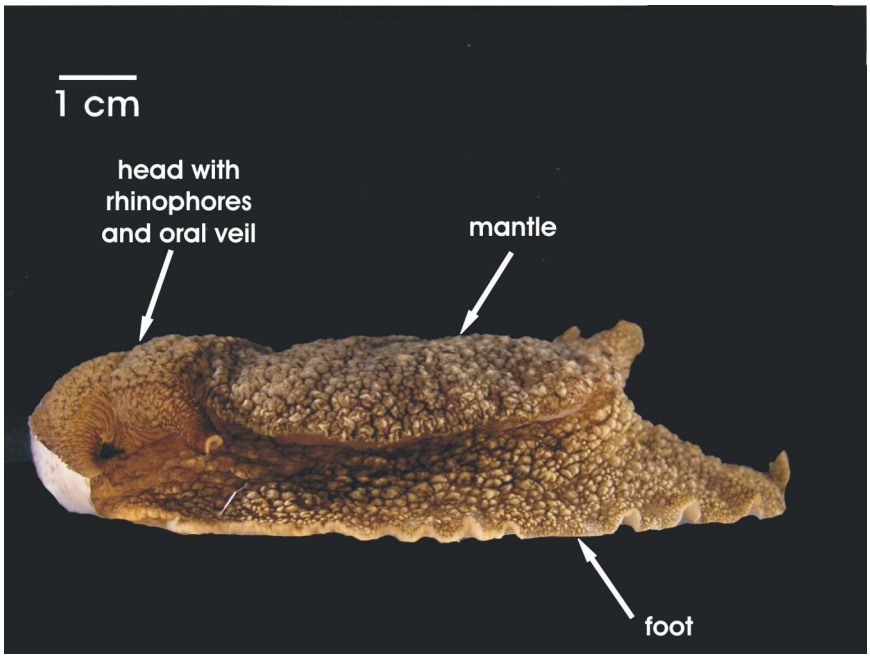


Figure 2: Lateral view of *Pleurobranchea meckeli*

2.2.3 *Berthella plumula* (MONTAGU, 1803)

Taxonomic position:

Heterobranchia

Opisthobranchia

Pleurobranchomorpha

Berthella plumula (Fig. 3) occurs at the Northeastern Atlantic from Norway to Gibraltar and in the Mediterranean Sea. *Berthella plumula* has a thin transparent internal shell. The shell is about half the body length, which may reach about 60 mm with 30 to 40 mm being more common. The skin has stellate calcareous spicules over the whole body including the oral veil and the rhinophores. In colour *Berthella plumula* is pale lemon-yellow to orange. If attacked the skin can secrete defensive sulphuric acid. Thompson (1976) suggested that *Berthella plumula* may feed on tunicates but further published observations to support this are missing. *Berthella plumula* was collected in the wild at Roscoff (Brittany, France). They were then stored alive at our lab in Frankfurt under the same conditions like *Acteon tornatilis*.

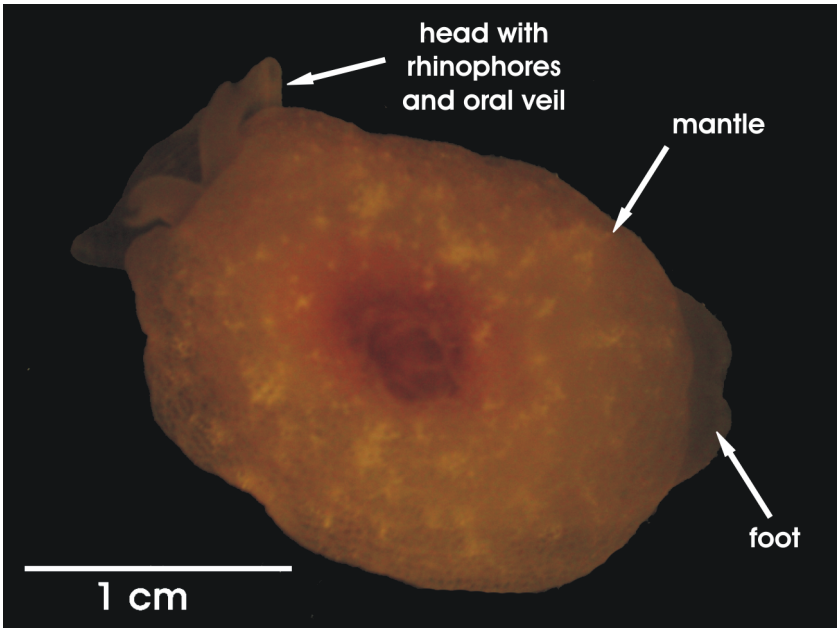


Figure 3: Dorsal view of *Berthella plumula*

2.2.4 *Archidoris pseudoargus* (RAPP, 1827)

Taxonomic position:

Heterobranchia

Opisthobranchia

Nudibranchia

Archidoris pseudoargus (Fig. 4) occurs at the European coast from Norway to Spain, and in the Mediterranean Sea, from the intertidal to 300 m of depth. It is common on the British Isles where it is known as the Sea Lemon. *Archidoris pseudoargus* is a large dorid reaching over 120 mm in length. The mantle is covered with tubercules, and has a mottled colour pattern of brown, pink, green, yellow and white blotches. It feeds on siliceous sponges including *Halichondria panicea* and *Hymeniacidon perleve* (Swennen 1961). In many parts of Europe it is often identified as *Archidoris tuberculata* (MULLER, 1778). *Archidoris pseudoargus* was collected in the wild at Roscoff (Brittany, France). The specimens were then stored alive at our lab in Frankfurt under the same conditions like *Acteon tornatilis* and *Berthella plumula.*

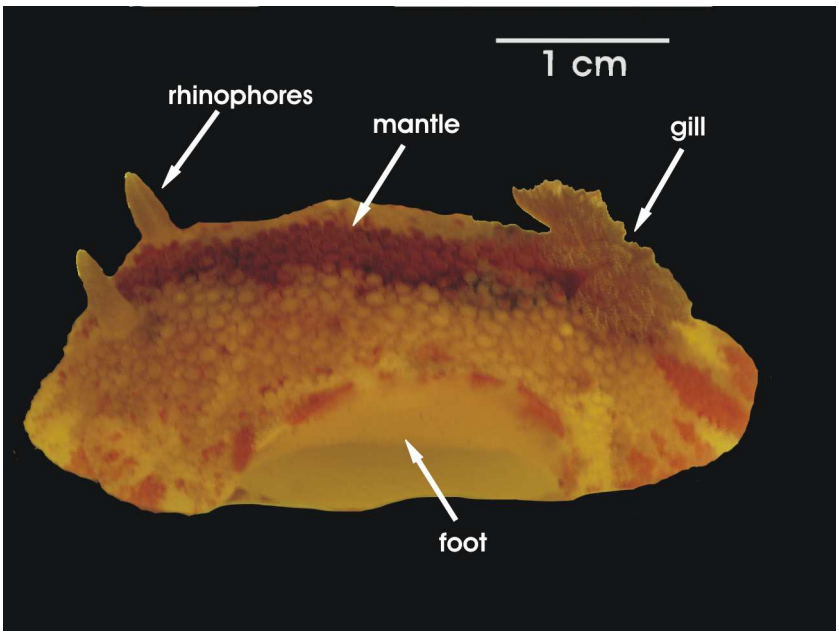


Figure 4: Lateral view of *Archidoris pseudoargus*

2.2.5 *Aplysia punctata* CUVIER, 1803

Taxonomic position:

Heterobranchia

Opisthobranchia

Aplysiomorpha

Aplysia punctata (Fig. 5) occurs in the Northeast Atlantic from Greenland to the Mediterranean Sea. This species grows up to 20 cm. The body colour is very variable, ranging from olive-green, brown, red, purplish-black, with blotches of grey, white, often with black or dark-brown spots and veining. The body is long and narrow and the parapodia join rather high posteriorly. The CSOs form very prominent labial tentacles and rhinophores. When disturbed it produces both purple and white secretions. *Aplysia punctata* was collected in the wild at Roscoff (Brittany, France). They were then stored alive at our lab in Frankfurt, maintained in closed seawater aquaria at 17º C, under ambient light and fed with frozen pieces of the green algae *Ulva lactuca* and *Polysiphonia spec.*

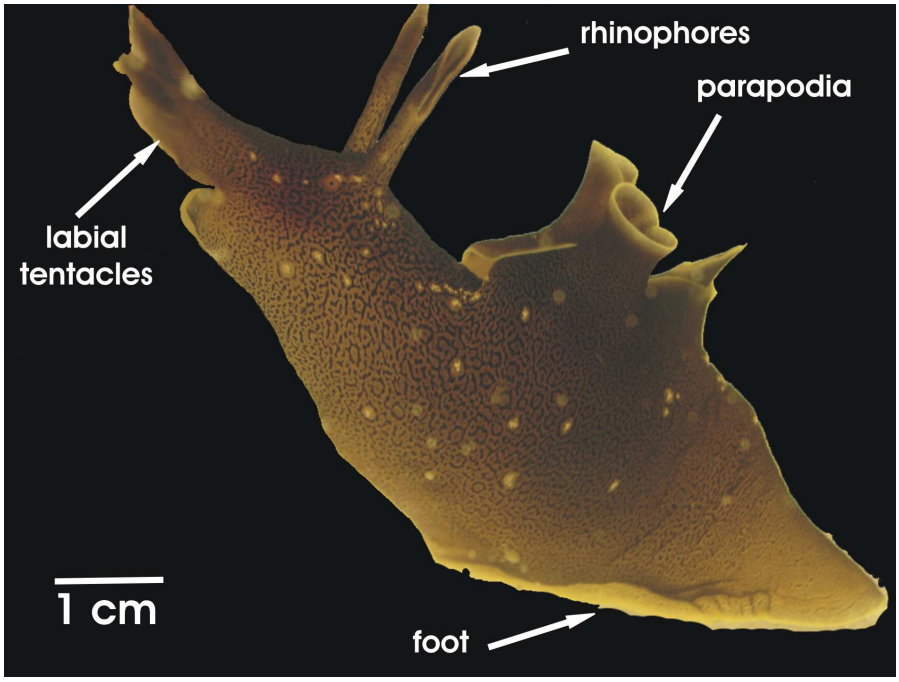


Figure 5: Lateral view of *Aplysia punctata*

2.2.6 *Aplysia californica* COOPER, 1863

Taxonomic position:

Heterobranchia

Opisthobranchia

Aplysiomorpha

Aplysia californica occurs along most of the Californian coast and in the Gulf of California. It is a very large sea hare, with a length up to 75 cm but more often around 40 cm (Cooper 1863, Rivero et al. 2003). *Aplysia californica* has become a very valuable laboratory animal for research on the nervous systems and behaviour. Therefore it is used as a model organism in invertebrate neurobiology. The animals were purchased from the Aplysia Resource Facility of the Rosenstiel School of Marine and Atmospheric Sciences alive, and stored at our lab in Frankfurt under earlier mentioned conditions. They were also fed with frozen green algae (*Ulva lactuca* and *Polysiphonia spec.),* which were collected in Roscoff (Brittany, France).

2.2.7 *Petalifera petalifera* (RANG, 1828)

Taxonomic position:

Heterobranchia

Opisthobranchia

Aplysiomorpha

Petalifera petalifera is reported from the Mediterranean Sea and eastern Atlantic but was also found in Venezuela (own investigations) and the East coast of Australia (own investigations, unpublished data). It is possibly more widely spread, but a better understanding of the distribution of the genus worldwide is required. *Petalifera petalifera* resembles a very flattened sea hare, in which the parapodia are reduced and fused, except for a small postero-dorsal opening in the mantle cavity, which is covered by a pair of small rounded flaps. The body is translucent with a dense scattering of green to brown specks. The translucent body enables this species to be well-camouflaged on the sea grass leaves and algae on which it lives. It grows up to 40 mm in length. Specimens were collected in Banyuls sur mer (France) by dredging sea gras at depths of 20 to 40 meters and at the Isla Margaritha (Venezuela) subtidally by snorkeling. Specimens were investigated immediately upon collection.

2.2.8 *Haminoea hydatis* (LINNAEUS, 1758)

Taxonomic position:

Heterobranchia

Opisthobranchia

Cephalaspidea

Haminoea hydatis (Fig. 6) occurs along the South and West coast of the British Isles. But it is more common from the Atlantic coast of France to the Mediterranean Sea (Thompson 1976). It has a fragile transluminecent inflated shell which grows to about 15 mm in length. The body reaches up to about 30 mm in length. The parapodial lobes are relatively small, leaving most of the shell exposed. *Haminoea hydatis* was collected from the wild at Plèneuf (Brittany, France). Characteristical CSOs for the Cephalaspidea are a cephalic shield, a lip organ and a Hancocks organ. The animals were used to establish a stable laboratory population, maintained in closed seawater aquaria at 17º C and under ambient light. They were fed pieces of the green algae *Ulva lactuca*, *Ulva rigida* and *Cladophora spec*.

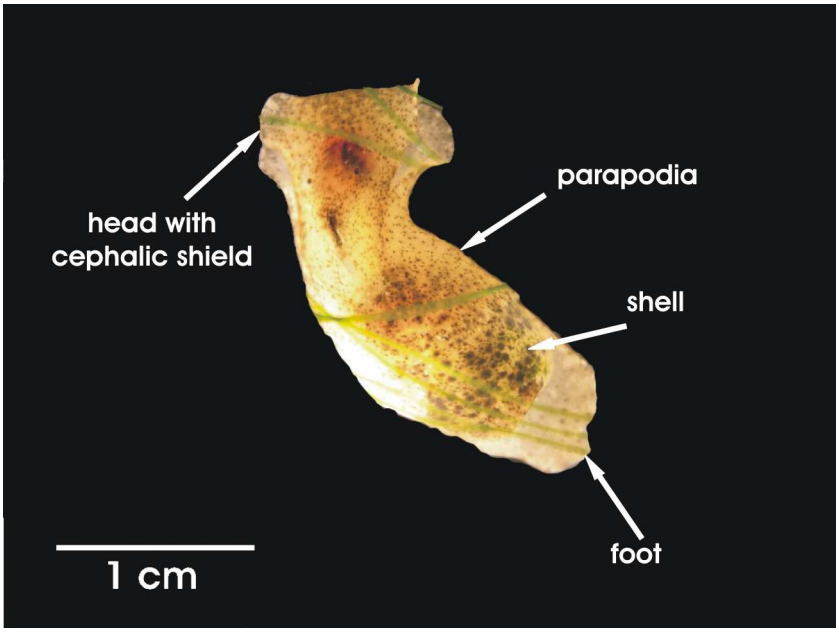


Figure 6: Dorsal view of *Haminoea hydatis*

2.2.9 *Scaphander lignarius* (LINNAEUS, 1758)

Taxonomic position:

Heterobranchia

Opisthobranchia

Cephalaspidea

Scaphander lignarius (Fig. 7) occurs at the North-eastern Atlantic coast and in the Mediterranean Sea. The nutbrown shell with small white stripes is relatively massive and up to 70 mm in length. Characteristically for the soft body is a dominat white cephalic shield, which is also described as a cephalic disc (Thompson 1976). The cephalic disc cannot be retracted into the shell. *Scaphander lignarius* was collected in Blanes (Spain) by fishermen at depths up to 80 meters. Specimens were investigated immediately upon collection.

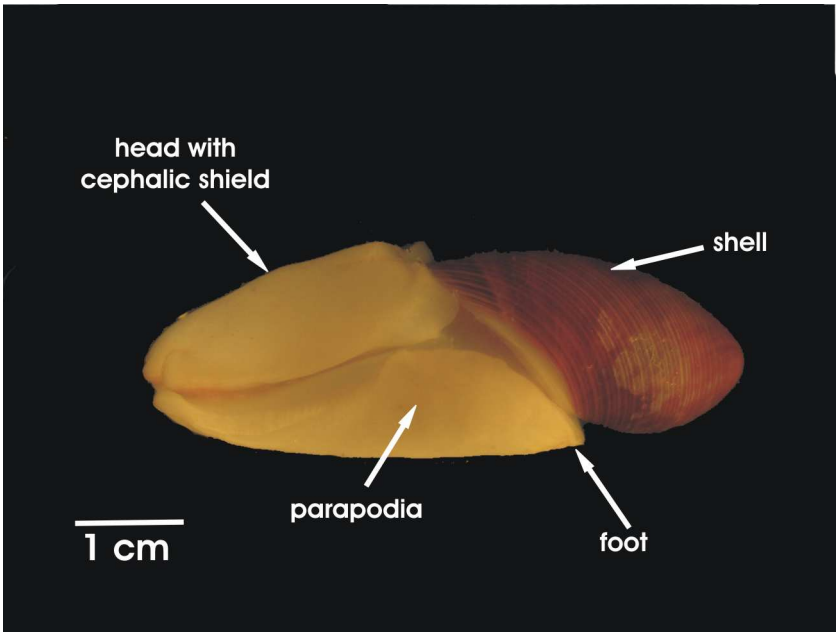


Figure 7: Lateral view of *Scaphander lignarius*

2.2.10 *Achatina fulica* BOWDICH, 1822

Taxonomic position:

Heterobranchia

Eupulmonata

Stylommatophora

Achatina fulica is a land snail which originally occured in eastern Africa (Kenia and Tansania) but it was imported to Asia and India where it has been established as a neozoan species. *Achatina fulica* has a narrow, conical shell, which is twice as long as it is wide and contains 7 to 9 whorls when fully grown. *Achatina fulica* shows two pairs of tentacles, the ommatophores and the rhinophores (Fig. 8). The shell is generally reddish-brown in colour with weak yellowish vertical markings but colouration varies with environmental conditions and diet. Adults of the species may exceed 20 cm in shell length but generally average about 5 to 10 cm. The average weight of the snail is approximately 32 grams (Cooling 2005). A lab population was established at our lab in Frankfurt.

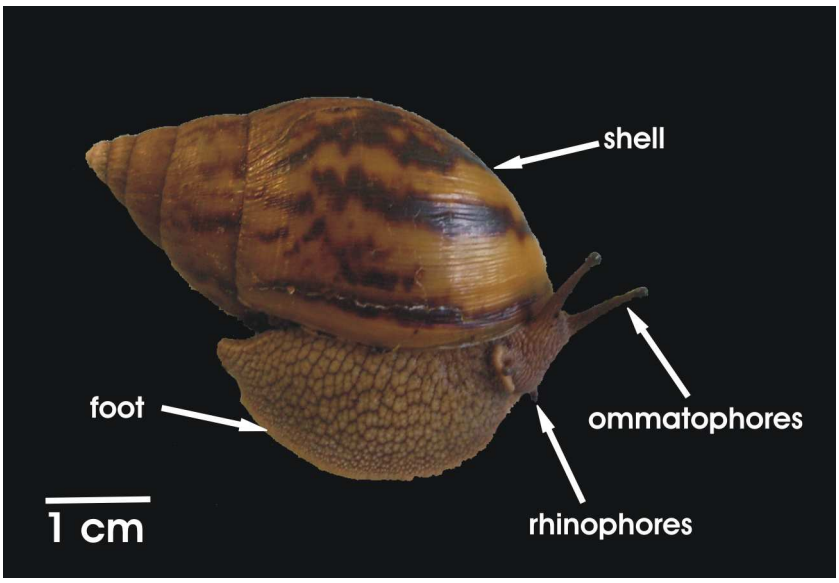


Figure 8: Lateral view of *Achatina fulica*

2.2.11 *Littorina littorea* (LINNAEUS, 1758)

Taxonomic position:

Caenogastropoda

Hypsogastropoda

Littorinimorpha

Littorina littorea (Fig. 9) is widely distributed among rocky shores from Northern Spain to the White Sea of Northern Russia. It occurs from the upper shore down to the sublittoral. It has a very massive black to brown shell, only one pair of tentacles and also a massive operculum. In sheltered conditions the specimens can also be found in sandy or muddy habitats such as estuaries and mud-flats. They are common grazers of microalgae. The species is fairly tolerant of brackish water. Animals were collected at the German North Sea (Vollerwiek, Eiderstedt) and stored alive under earlier mentioned conditions in our Lab at Frankfurt.

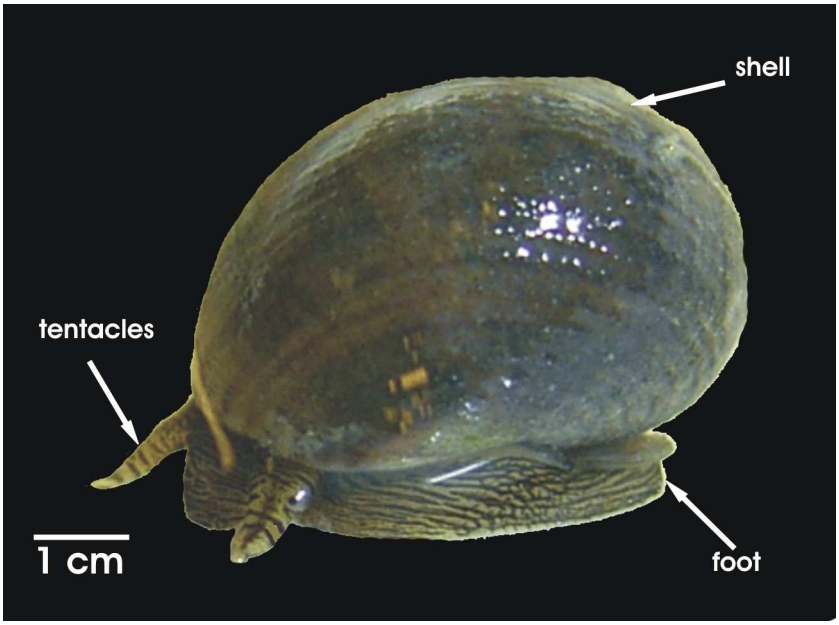


Figure 9: Lateral view of *Littorina littorea*

2.3 Methods

2.3.1 Axonal tracing

Animals were relaxed with an injection of 7% $MgCl_2$ (% in Volume) and the central nervous system, 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. I followed the procedures of Croll and Baker (1990) for Ni^{2+}-lysine (Ni-Lys) tracing of axons. The major cerebral nerves of eight species (table 1) were traced comparatively with at least ten replicates for each nerve per species. Therefore the nerves of the right cerebral ganglion were dissected free from the connective tissue. In addition control replicates for the cerebral nerves of the left hemisphere were performed. 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 8º 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 five to ten drops of a saturated rubeanic acid solution in absolute dimethylsulfoxide (DMSO). After 45 minutes the ganglia were transferred to 4% paraformaldehyde (PFA) and fixed for 4-12 h at 4º C. Thereafter the ganglia were dehydrated by an increasing ethanol series (70/80/90/99/99% each 10 minutes), cleared in methylsalicylate and mounted dorsal side up in Entellan (VWR International) on a glass slide. My criterion for a good staining was a uniformly dark blue nerve as it joins the ganglion. This is an indication for intact axons (Fredman 1987, Johnson et al. 1999). 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.

2.3.2 Intraspecific variability

I tested the intraspecific variability of innervation patterns for the Nervus labialis of *Haminoea hydatis*. Altogether I performed over 35 replicates in several specimens, ranging from 5 to 25 mm in length. Samples with only a partial staining of the nerve were not used because of possible incomplete innervation patterns. Thus, only 23 replicates were analysed for the right Nervus labialis (N2), covering a wide range of

specimens from juvenile to adult stages. Moreover, I tested the variability of the innervation patterns for animals of nearly the same size, and for the left Nervus labialis (n = 4).

For correlation analyses, in order to test if the innervation patterns are correlated to the size of the animal, I used three different morphological sizes:

1) the product of the maximum length and breadth of the shell, 2) the length of the cerebral commissure, and 3) the average of the maximum diameter of both cerebral ganglia. All measurements were performed on digital images, using the Leica IM50 Software. Neither the length of the whole slug nor the size of peripheral structures such as the lip organ were 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.). I 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 correlation analyses I used two-tailed correlation analyses with a 95% significance level.

2.3.3 Immunohistochemistry

I investigated the occurance and distribution of the three neurotransmitters, Serotonin (5HT), FMRFamide and Tyrosine hydroxylase (TH) which is a catalyst in catecholamine synthesis and thus indirectly labels catecholamines (Magoski and Bulloch 1997). Following the protocols shown in Table I (Supplement Data), I used as primary antibodies (PA) polyclonal 5HT (Acris Antibodies, Hiddenhausen, DP057) raised in rabbit, polyclonal FMRFamide (Diasorin, Stillwater via Immunostar Incooperated, Hudson, Wisconsin, 20091) raised in rabbit and monoclonal TH (Acris Antibodies, Hiddenhausen via Immunostar Incooperated, Hudson, Wisconsin, LNC1) raised in mouse. As secondary antibodies (SA) I used Rhodamine/TRITC (Dianova, Hamburg via Jackson ImmunoResearch laboratories, Inc., West Grove) and Fluorescein/FITC (Dianova, Hamburg via Jackson ImmunoResearch laboratories, Inc., West Grove). The SA for FMRFamide and 5HT were raised in goat, anti rabbit, meanwhile the SA for TH were raised in sheep, anti mouse. The specimens were relaxed by an injection of 7% $MgCl_2$ in the foot. Thereafter, the entire head region was dissected from the rest of the animal and immediately fixed. For 5HT and FMRFamide the fixation was done with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered

saline (PBS) at a pH of 7.3 overnight at 4º C. The tissue for TH was fixed in 99% methanol and 1% Acetic acid at – 18º C for 30 minutes, immediately followed by a decreasing methanol series (70%/50%/30%) for ten minutes at each concentration. After fixation the whole mounts were washed three times in PBS (five minutes the first two times and 60 minutes the third time). This washing procedure is equal between all steps. After the first washing the tissue was permeabilisated and blocked, using 4% Triton for the permeabilisation and 1% normal goat serum (NGS – for 5HT and FMRFamides) respectively normal sheep serum (NSS – for TH) for blocking overnight at 4º C. Before and after the whole mounts were exposed to the primary antibody (concentrations shown in supplement data, Tab. I) they were washed again. The exposition to the secondary antibody (concentrations see also supplement data Tab. I) was done avoiding light and followed again by washing. At last the whole mounts were mounted on glass slides in 3/1 glycerol in 0.5 M TRIS buffer with a pH of 8.0. Also 2% n-propyl gallate was added to the mounting medium, working as an anti fading agent. All complete mounts were analysed using a confocal laser scanning microscope (CLSM – Leica TCS SP5).

3. Results and Discussion

3.1 Terminology of cerebral nerves

In the past, the terminology for the nerves innervating various organs or structures in Gastropoda has been very inconsistent and confusing, thus hampering comparisons of innervation patterns across taxa. This has also been mentioned earlier by Hanström (1929). Huber (1993) described four cerebral nerves in the ground pattern of the Architectibranchia and most other Opisthobranchia. These four cerebral nerves innervate the cephalic sensory organs. My investigations of the neuroanatomy confirm this ground pattern in the investigated species. However, there is no common notation of these nerves in earlier investigation (Vayssière 1880, Hanström 1929, Hoffmann 1939, Huber 1993, Croll et al. 2003). Table 2 summarises terms used for the four cerebral nerves in Opisthobranchia, in representative studies on the neuroanatomy of these gastropods. The most common synonyms for the cerebral nerves and their innervation area are shown. In the present study, I use a modified notation from Edlinger (1980) who has focussed his investigations on the neuroanatomy of the CSOs of Acteonoidea and Cephalaspidea. Instead of using latin names, numbers were used as notations of the cerebral nerves, as it was also done by Vayssière (1880). The cerebral nerves were numbered from anterior to posterior. Therefore the Nervus oralis which innervates the lip is termed as the N1. The Nervus labialis, which is divided in two branches within the Opisthobranchia innervates the anterior CSOs like the labial tentacles, the lip organ and the oral veil and is named N2. The Nervus rhinophoralis which innervates the posterior CSOs like rhinophores or the Hancocks organ is termed N3. The fourth cerebral nerve, the Nervus clypei capitis innervates parts of the body wall or the cephalic shield. Sensory functions of these regions cannot be excluded. However, they seem to be more related to locomotion (Schmekel 1985). Therefore this nerve has not been termed with a number. Here I will term this nerve as Nclc.

As the investigations of Edlinger (1980) were published in German, these notations are less known in the international scientific community. However, for my investigations these terms fit best, as they are correlated to the position of the CSOs, unlike latin names, where sometimes the same notations have been used for different nerves. So the

N1 always projects most anteriorly on the median part of the cerebral ganglion and projects towards the most anterior CSO, the lip. The N2 originates commonly in the lateral anterior part of the cerebral ganglion and provides the anterior CSOs, meanwhile the N3 often arises in the posterior part of the cerebral ganglion and provides the posterior CSOs.

Table 2: Cerebral nerves in Opisthobranchia and their synonyms

Modified synonyms after Edlinger (1980) used in the present study	**Vayssière (1880)**	**Hanström (1929)**	**Huber (1993)**	**Croll et al. (2003)**	**Innervated CSO/Head region (see also Edlinger 1980, Huber 1993)**
N1	c1	Nervus labialis minor, Nervus oralis	Nervus labialis superior, Nervus oralis	Upper labial nerve	Lip
N2	c3	Nervus labialis superior, Nervus tentacularis	Nervus labialis, Nervus labiotentacularis, Nervus menti	anterior tentacle nerve	Anterior tentacle, Lip organ, oral veil, oral lobe, anterior Hancocks organ
N3	c4	Nervus tentacularis, Nervus rhinophoralis	Nervus rhinophoralis	posterior tentacle nerve	Rhinophore, posterior Hancocks organ, posterior tentacle
Nclc	c2	Nervus proboscidis	Nervus tentacularis, Nervus clypei-capitis	lower labial nerve	Anterior / lateral body wall, cephalic shield, cephalic disc

3.2 Cephalic sensory organs (CSOs)

In the following chapter I will describe the investigated CSOs, additionally I will compare them with earlier descriptions. The descriptions of the CSOs proceed from anterior to posterior of the head region.

Acteon tornatilis (Acteonoidea) possesses four types of CSOs: a lip, which is completely covered by the cephalic shield, a lip organ, Hancocks organ and a cephalic shield (Fig. 10A), as has been described earlier (Edlinger 1980). My investigations (chapter 3.3, 3.4, 3.7) lead to the conclusion that *Acteon tornatilis* has no Hancocks organ and it might be possibly reduced. This has been mentioned earlier by Schmekel (1985). Furthermore, the description of a separated lip organ at the anterior cephalic shield (Edlinger 1980) could not be confirmed by the present data (see also, Faller et al. *in revision*, Göbbeler and Klussmann-Kolb 2007). Therefore I define following CSOs for *Acteon tornatilis*: a lip which is completely covered by the cephalic shield, and a lip organ (the same structure was described as the anterior Hancocks organ (Edlinger 1980) which lies as a kind of groove underneath the lateral edge of the anterior cephalic shield. The lip organ comprises the ventral part of the cephalic shield and not a separated structure. The massive cephalic shield is completely divided into a left and a right hemisphere, but also into an anterior and posterior part or lobe.

Pleurobranchaea meckeli (Pleurobranchomorpha) has a lip, furthermore a massive dorsal oral veil with lateral tips which terminate in some kind of rolled labial tentacles. The rhinophores at the posterior end of the head are also rolled or curled (Figs. 10B, 10C, 12A).

Berthella plumula (Pleurobranchomorpha) also possesses a lip, an oral veil and rhinophores. Here the oral veil is positioned more ventrally and partly covered by the mantle. Moreover, it is a structure clearly separated from the head region unlike the oral veil of *Pleurobranchaea meckeli* (see also Faller et al. *in revision*, Göbbeler and Klussmann-Kolb 2007). The lateral sides of the oral veil do not terminate in rolled labial tentacles. Instead, I found long grooves along the lateral side of the trapeze like oral veil. The rhinophores are rolled structures which are positioned above the oral veil (Figs. 10D, 12B).

In *Archidoris pseudoargus* (Nudibranchia) I found the following CSOs: the lip, the oral tentacles and rhinophores (see also Faller et al. *in revision*). All CSOs in the

investigated species except for the lip are paired. The oral tentacles are only lobe like structures underneath the head and not visible from a dorsal view. They form a groove at the lateral edge. The rhinophores are retractable and are positioned on the head. The rhinophores are massive with up to 15 discs along the top (Figs. 10E, 13).

Aplysia punctata (Aplysiomorpha) and *Aplysia californica* (Aplysiomorpha) show the same set of CSOs (see also Faller et al. *in revision*, Göbbeler and Klussmann-Kolb 2007), a lip, very prominent labial tentacles with a thick base and a folded groove at the tip of the tentacles and the rhinophores. The latter are also prominent structures on the posterior end of the head. The base of the rhinophores is massive, on the top they form a spoon like groove (Figs. 10F, 14A) which was also described by Hoffmann (1939).

Petalifera petalifera (Aplysiomorpha) also has a lip, labial tentacles and rhinophores. Whereas the rhinophores are like the rhinophores of the other investigated Aplysiomorpha, the thick base of the folded labial tentacles is missing. Instead, *Petalifera petalifera* has separated oral lobes at the right and left lateral side of the lip (Figs. 10G, 14B).

Haminoea hydatis (Cephalaspidea) possesses a lip, a lip organ, a Hancocks organ and a cephalic shield. The lip organ is a separated structure underneath the cephalic shield near the lip. The Hancocks organ is also a separated structure underneath the cephalic shield. It is folded and positioned on the lateral side of the head region in some kind of channel formed by the cephalic shield and the foot (Figs. 10H, 10I, 15A). In earlier investigations the Hancocks organ has been divided into an anterior and a posterior part (Edlinger 1980). However, I consider the anterior Hancocks organ to be part of the lip organ (chapter 3.1) and therefore describe the latter as an anterior and a posterior lip organ (see also, Faller et al. *in revision*, Göbbeler and Klussmann-Kolb 2007). The cephalic shield of *Haminoea hydatis* is divided in the posterior part.

Scaphander lignarius (Cephalaspidea) possesses the same sets of CSOs like *Haminoea hydatis*. However, in *Scaphander* the lip organ seems to be part of the cephalic shield which is very prominent and also in common termed as cephalic disc (Figs. 10J, 15B). The same is true for the Hancocks organ. The cephalic shield is extremely massive and undivided with the shape of a disc. *Achatina fulica* (Stylommatophora) also has a lip, small anterior tentacles (“rhinophores”) and large posterior tentacles (ommatophores) (Fig. 16). The eyes are positioned at the top of the posterior tentacles. In contrast *Littorina littorea* (Caenogastropoda) (Fig. 17) shows a lip, but only one pair of tentacles. The eyes are located at the bottom of these tentacles.

In summary I found the lip as a very invariable, conserved structure in all investigated species. Within the Opisthobranchia the anterior CSOs like the oral veil or the labial tentacles show the highest variability. From my point of view they should be divided in two types of anterior sensory organs (ASO), an anterior (ASOa) and a posterior (ASOb) type. The posterior sensory organs (PSO) also show a high variability and include several types of rhinophores and the Hancocks organ. The term ASO was also used by Boudko et al. (1999) for the labial tentacles of *Phestilla sibogae* (Opisthobranchia, Nudibranchia). Here I restrict the term Hancocks organ only to the posterior part of the Hancocks organ described in earlier investigations (Edlinger 1980).

ASOs: the investigated species possess two types of oral veils, a dorsal veil which ends in labial tentacles like in *Pleurobranchaea meckeli* and a ventral veil with a lateral groove like in *Berthella plumula*. I found three kinds of lip organs: first a separated structure underneath the cephalic shield (*Haminoea hydatis*), secondly the lip organ as part of the cephalic shield (*Scaphander lignarius)* and thirdly a groove along the ventral side of the cephalic shield (*Acteon tornatilis*). The labial tentacles are shaped differently, too: massive labial tentacles with a folded top and a thick base (*Aplysia* species), folded with a separated oral lobe (*Petalifera petalifera)* or lobe like with a groove (*Archidoris pseudoargus),* very small tentacles above the mouth opening (*Achatina fulica*). These anterior tentacles are also called “rhinophores”.
PSOs: I also found three types of rhinophores, massive with a spoon like groove in the Aplysiomorpha, rolled in the Pleurobranchomorpha and massive with discs in *Archidoris pseudoargus*. The prominent posterior tentacles of *Achatina fulica* are also called ommatophores (omma = eye) as the eye is positioned at the top of these tentacles. Moreover, the Hancocks organs are differently shaped in the investigated species: in *Haminoea hydatis* the Hancocks organ is separated from the cephalic shield, whereas it is fused with the cephalic shield in *Scaphander lignarius*. The cephalic shields can be described as completely divided from anterior to posterior in *Acteon tornatilis*, only divided in the posterior part in *Haminoea hydatis* and completely undivided in *Scaphander lignarius*. *Littorina littorea* has only one pair of tentacles, and at this point I can not state if these tentacles are ASOs or PSOs.

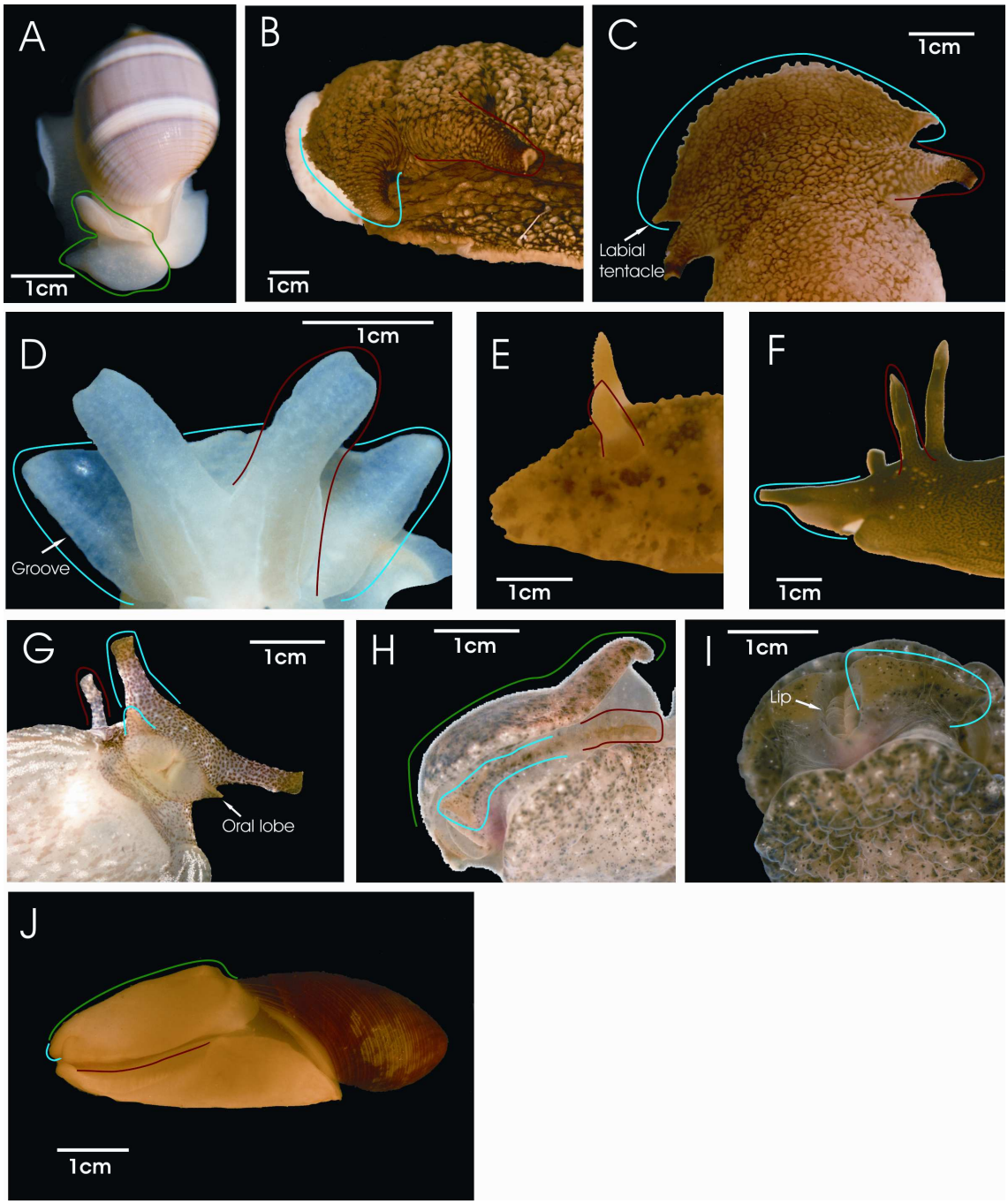


Figure 10: Various CSOs of the investigated species, anterior sensory organs (ASOs) outlined in blue, posterior sensory organs (PSO) outlined in red, cephalic shields outlined in green. **A:** *Acteon tornatilis*, cephalic shield (green); **B:** *Pleurobranchaea meckeli*, oral veil and labial tentacles (blue), rhinophores (red); **C:** *Pleurobranchaea meckeli*, oral veil and labial tentacles (blue), rhinophores (red); **D:** *Berthella plumula*, oral veil with groove (blue) and rhinophores (red); **E:** *Archidoris pseudoargus*, rhinophores (red); **F:** *Aplysia punctata*, labial tentacles (blue), rhinophores (red); **G:** *Petalifera petalifera*, labial tentacles and oral lobes (blue), rhinophores (red); **H:** *Haminoea hydatis*, anterior and posterior lip organ (blue), Hancocks organ (red) and cephalic shield (green); **I:** *Haminoea hydatis*, anterior lip organ (blue); **J:** *Scaphander lignarius*, lip organ (blue), Hancocks organ (red) and cephalic shield (green).

3.3 Neuroanatomy

In this part, I am going to describe the neuroanatomy of the head region of the investigated species. The description will be restricted to the cerebral nerves which innervate structures with a presumably sensory function. Commissures and connectives and the general structure of the central nervous system will only be mentioned with minor priority.

Within the investigated Opisthobranchia and in the stylommatophoran *Achatina fulica* I found a ground pattern of four cerebral nerves, which has also been described for the Opisthobranchia by Huber (1993). The first cerebral nerve (N1) cardinally innervates the lip and sometimes parts of the anterior head region. The second nerve (N2) is divided into an inner and an outer branch. A bifurcation of the N2 has been described as an apomorphy of the Opisthobranchia (Salvini-Plawen and Steiner 1996). This nerve innervates the anterior sensory organs (ASOa and ASOb) which are lip organs, labial tentacles, oral veils and lobes (see chapter 3.2). The third nerve (N3) often forms an additional ganglion, commonly termed as the rhinophoral ganglion. The nerve innervates the posterior sensory organs (PSO), here rhinophores and Hancocks organ. The last remaining cerebral nerve (Nclc), which is not related to a primary sensory organ (chapter 3.2), innervates either parts of the anterior to lateral body wall of the head region or the cephalic shield. The investigated caenogastropod *Littorina littorea* possesses only three cerebral nerves.

3.3.1 *Acteon tornatilis*

In *Acteon tornatilis* (Fig. 11) the N1 innervates the lip and small parts of the anterior part of the bipartite cephalic shield. The bifurcated N2 innervates the groove underneath the anterior cephalic shield, which I call the lip organ. In earlier investigations this pigmented groove was described as the anterior and posterior Hancocks organ (Edlinger, 1980). Edlinger (1980) described the N2 to be restricted to a structure around the mouth, which he termed as lip organ. However, the existence of such a structure cannot be confirmed by my investigations (see also chapter 3.2, 3.7). The N3 innervates a small part of the posterior cephalic shield, which has no significant sensory function (Faller et al. in revision, Göbbeler and Klussmann-Kolb 2007), and not the groove as mentioned by Edlinger (1980). The Nclc innervates the largest part of the posterior cephalic shield.

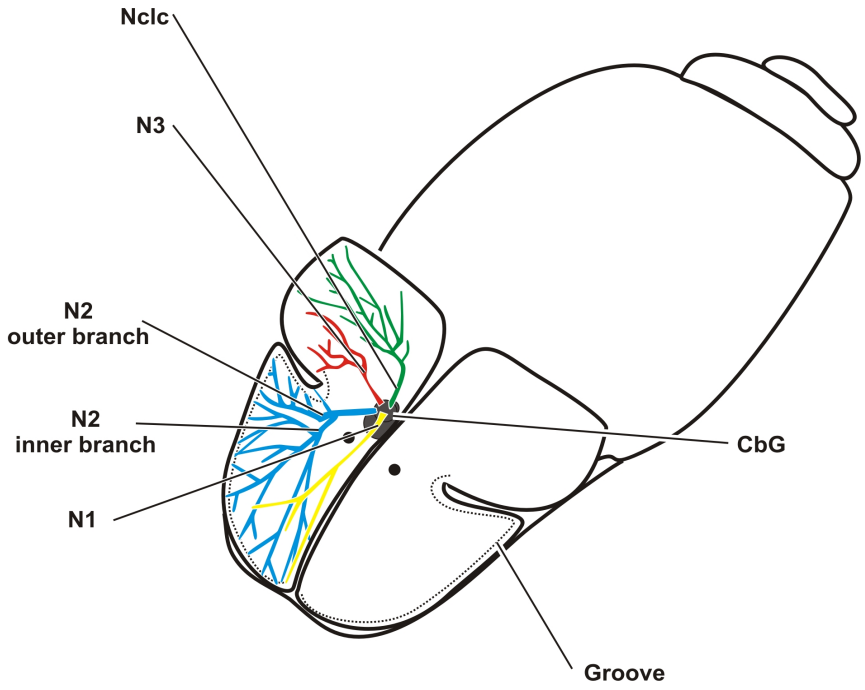


Figure 11: Neuroanatomical scheme of the four cerebral nerves of *Acteon tornatilis* (only right hemisphere shown). N1 in yellow, N2 in blue, N3 in red and the Nclc in green. The groove represents the lip organ; CbG – cerebral ganglion.

3.3.2 *Pleurobranchaea meckeli* and *Berthella plumula*

The investigated Pleurobranchomorpha (*Pleurobranchaea meckeli* and *Berthella plumula*) show a very similar neuroanatomy to each other (Figs. 12A, B). The N1 innervates the lip, the bifurcated N2 the ASO (here oral veil, labial tentacle and groove), the N3 the rolled rhinophores and the Nclc parts of the anterior and posterior body wall. In both investigated species the inner branch of the N2 innervates the median part of the oral veil. Whereas in *Pleurobranchaea meckeli* the outer branch of the N2 innervates the labial tentacles, in *Berthella plumula* it innervates the lateral groove of the oral veil. The N3 forms a small rhinophoral ganglion on the bottom of the nerve directly above the CNS in both species.

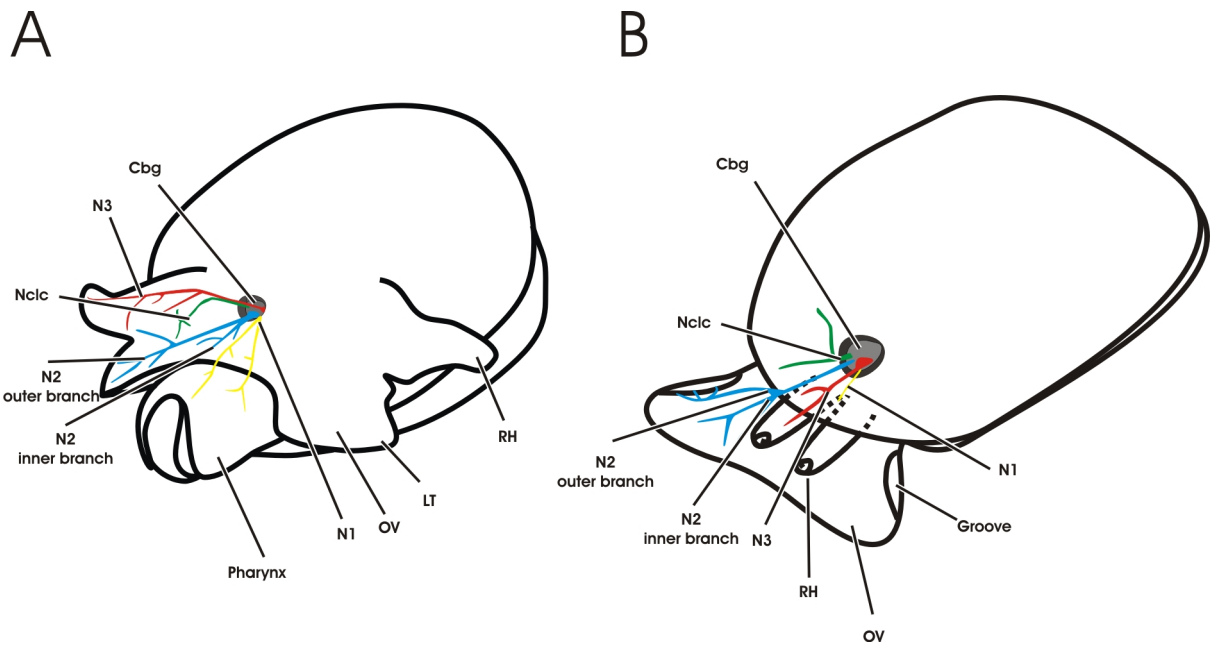


Figure 12: Neuroanatomical scheme of the four cerebral nerves of the investigated Pleurobranchomorpha (only right hemisphere shown). **A:** *Pleurobranchaea meckeli*; **B:** *Berthella plumula*; N1 in yellow, N2 in blue, N3 in red and the Nclc in green; CbG – cerebral ganglia, RH – rhinophore, LT – labial tentacle, OV – oral veil.

3.3.3 *Archidoris pseudoargus*

The investigated Nudibranchia *Archidoris pseudoargus* (Fig. 13) also shows a very similar distribution of the cerebral nerves as the Pleurobranchomorpha described before. The N1 innervates the lip, the bifurcated N2 the labial tentacles (ASO), the N3 innervates the massive disced rhinophores (PSO), with also a rhinophoral ganglion at the base of the nerve and the Nclc innervates the lateral and anterior head region.

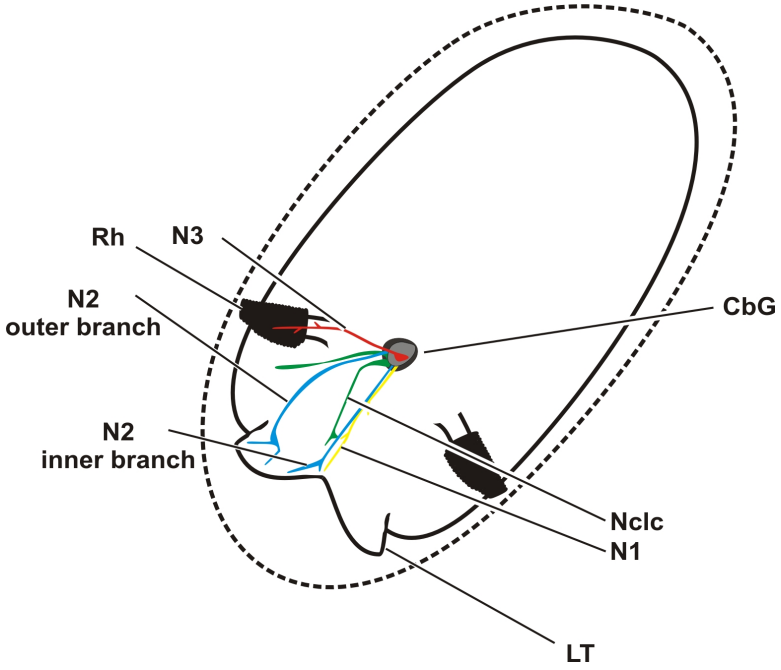


Figure 13: Neuroanatomical scheme of the four cerebral nerves of *Archidoris pseudoargus* (only right hemisphere shown). N1 in yellow, N2 in blue, N3 in red and the Nclc in green; CbG – cerebral ganglion, RH – rhinophore, LT – labial tentacle.

3.3.4 *Aplysia* spp. and *Petalifera petalifera*

I investigated three Aplysiomorpha, two species of the genus *Aplysia* and *Petalifera petalifera*. The organisation of the cerebral nerves has also been investigated in detail by Hoffmann (1939), Chase (2002), Croll (2001), Huber (1993) and Wollesen (2007a,b). I found no discrepancies with these earlier descriptions in my investigations. In all Aplysiomorpha I found a very similar neuroanatomy of the cerebral nerves (Figs. 14A, B). The N1 innervates the lip and anterior head region, the N2 the labial tentacles and also the oral lobes in *Petalifera petalifera* (ASO), the N3 the rhinophores and the Nclc the anterior and posterior body wall, also partly the lower part of the labial tentacles. In both species of the genus *Aplysia*, the inner branch of the N2 innervates the thick base of the labial tentacles, whereas in *Petalifera petalifera* it innervates the oral lobes. The oral lobes are clearly separated from the labial tentacles.

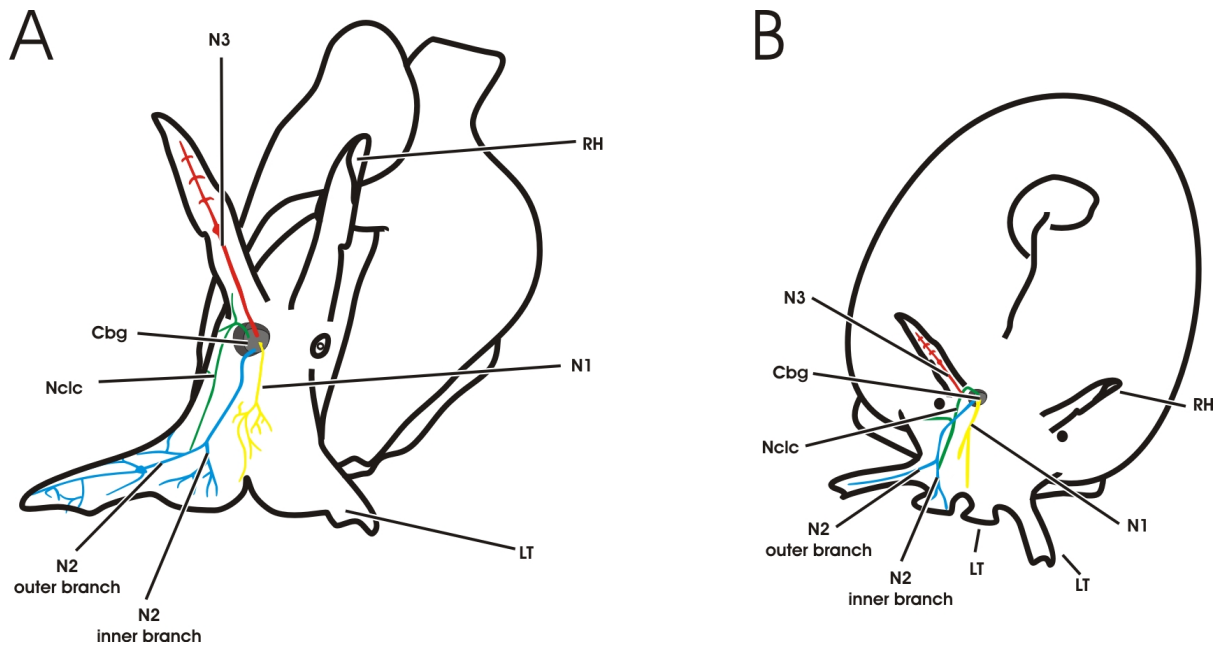


Figure 14: Neuroanatomical scheme of the four cerebral nerves of the investigated Aplysiomorpha, the two investigated *Aplysia* show no significant differences (only right hemisphere shown). **A:** *Aplysia punctata/californica*; **B:** *Petalifera petalifera*; N1 in yellow, N2 in blue, N3 in red and the Nclc in green; CbG – cerebral ganglion, RH – rhinophore, LT – labial tentacle.

3.3.5 *Haminoea hydatis* and *Scaphander lignarius*

I studied two species of the taxon Cephalaspidea, *Haminoea hydatis* and *Scaphander lignarius*. Here I found a higher variability in the neuroanatomy of the cerebral nerves than in other opisthobranch taxa like the investigated Pleurobranchomorpha or Aplysiomorpha. In *Haminoea hydatis* (Fig. 15A) the N1 innervates the lip and the anterior part of the cephalic shield. This differs to earlier descriptions of Vayssière (1880), Hoffmann (1939) and Edlinger (1980) about the nervous system of *Haminoea hydatis*. Hoffmann (1939) defined the N1 found in the current study as the Nervus clypei capitis internus (c1) and the Nervus clypei capitis externus (c2) which only provides the cephalic shield of *Haminoea hydatis*. However, Edlinger (1980) described the same nerve to innervate the lip organ. My own investigations showed that the N1 provides the lip but also the cephalic shield. The largest branch directly innervates the lip not the lip organ, like mentioned by Edlinger (1980). The N2 innervates the anterior (ASOa) and posterior (ASOb) lip organ. The N2 is divided into two branches as found in the other opisthobranch species, which have been described as two single nerves by Edlinger (1980). The first, or inner branch of the N2 provides the lip organ, the second, outer branch is related to the anterior Hancocks organ (Edlinger 1980, Huber 1993). This is congruent to the assumption of Hoffmann (1939) that the c3 (after Vayssière 1880) of *Haminoea hydatis* represents the Nervus labialis which innervates the lip organ. I cannot support Edlingers (1980) description of independent nerves for the lip organ and the anterior Hancocks organ.

The CSO termed as posterior lip organ in the present study by myself, was described by Edlinger (1980) as the anterior Hancocks organ (see also chapter 3.2). The N3 innervates the Hancocks organ, this is congruent with earlier investigations by Vayssière (1880) and Huber (1993). I found the Nclc to innervate the posterior cephalic shield. My description of the Nclc is also congruent to the basic Bauplan of the Architectibranchia and Bullomorpha described by Huber (1993).

In *Scaphander lignarius* (Fig. 15B) I found a variation of the pattern described for *Haminoea hydatis*. The N1 provides the lip and anterior cephalic shield, whereas the N2 innervates the lip organ, similar to the lip organ which has been described by Edlinger (1980) for *Acteon tornatilis*. The N3 innervates the Hancocks organ, which looks extremely similar to the lip organ of *Acteon tornatilis*. Both of these very similar CSOs are innervated by different nerves. As the cephalic shield of *Scaphander lignarius* is very muscular and prominent and the Nclc is very small, thus difficult to dissect, I cannot exclude that the fourth nerve (Nclc) innvervates the whole cephalic shield. In my investigations it seems to be restricted to the anterior part of the cephalic shield, which is in absolute contrast to the neuroanatomy of the Nclc in *Haminoea hydatis*.

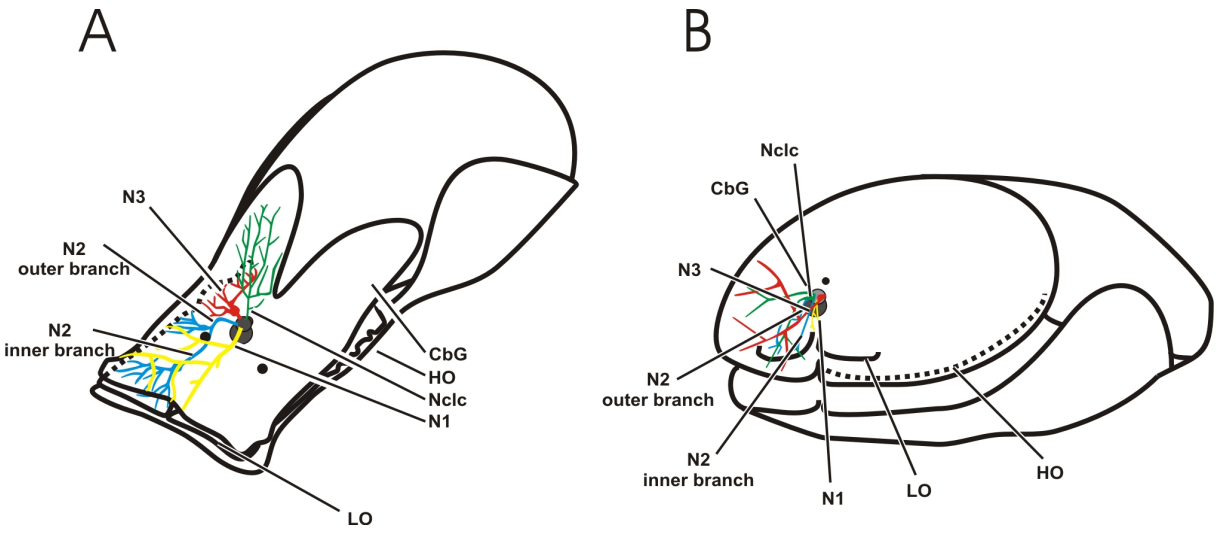


Figure 15: Neuroanatomical scheme of the four cerebral nerves of the investigated Cephalaspidea (only right hemisphere shown). **A:** *Haminoea hydatis*; **B:** *Scaphander lignarius*; N1 in yellow, N2 in blue, N3 in red and the Nclc in green; CbG – cerebral ganglion, HO – Hancocks organ, LO – lip organ.

3.3.6 *Achatina fulica*

In *Achatina fulica*, a pulmonate species, I found the same pattern of cerebral nerves like in the investigated opisthobranch taxa (Fig. 16). I can describe four cerebral nerves, the N1 innervates the lip, the also bifurcated N2 the anterior head region and the small anterior tentacles, which were also termed “rhinophores”. The N3 innervates the large posterior tentacles through a tentacle ganglion. These posterior tentacles are also called ommatophores (Ierusalimsky and Balaban 2007, Ierusalimsky and Balaban 2005). The Nclc provides the lateral and anterior head region. This organisation has been described to be common throughout the whole Stylommatophora (Ierusalimsky and Balaban 2007).

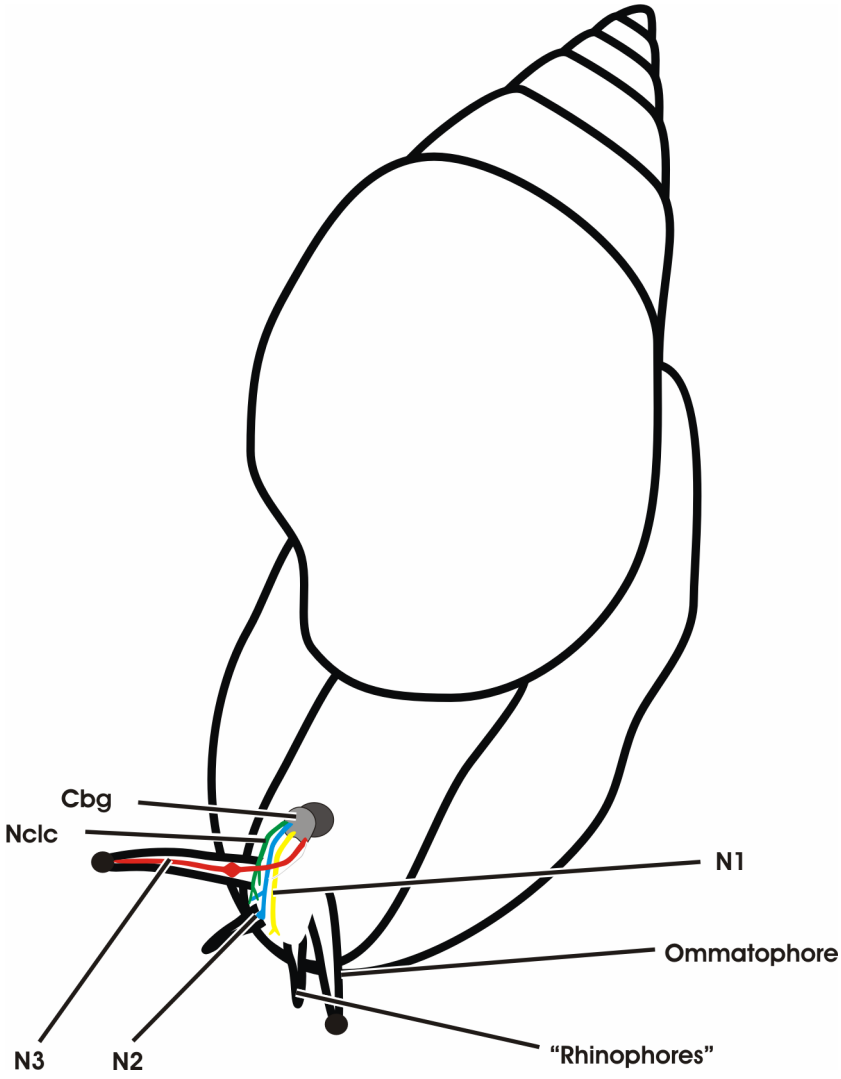


Figure 16: Neuroanatomical scheme of the four cerebral nerves of the investigated Stylommatophora *Achatina fulica* (only right hemisphere shown). N1 in yellow, N2 in blue, N3 in red and the Nclc in green.

3.3.7 *Littorina littorea*

In *Littorina littorea*, I found only three cerebral nerves (Fig. 17): an oral nerve, termed as N1 which innervates the lip and anterior head region, a tentacle nerve which provides the only pair of clearly identifiable CSOs (the tentacles), and a third nerve, termed as Nclc which also innervates the anterior head region. In the ground pattern for Caenogastropoda, Huber (1993) mentioned the N1 as Nervus labialis superior = Nervus oralis. Furthermore, Huber (1993) described the Nclc as Nervus labialis/ labiotentacularis/ menti, synonyms which are in general used for the N2, and the last cerebral nerve which provides the tentacles as the Nervus tentacularis. As it is not clear yet, if the Nervus tentacularis is homolougous to the N2 or the N3 of the Opisthobranchia, I mentioned this nerve here preliminary as tentacular nerve, without postulating homology hypotheses if this nerve is homologous to the opisthobranch N2 or N3

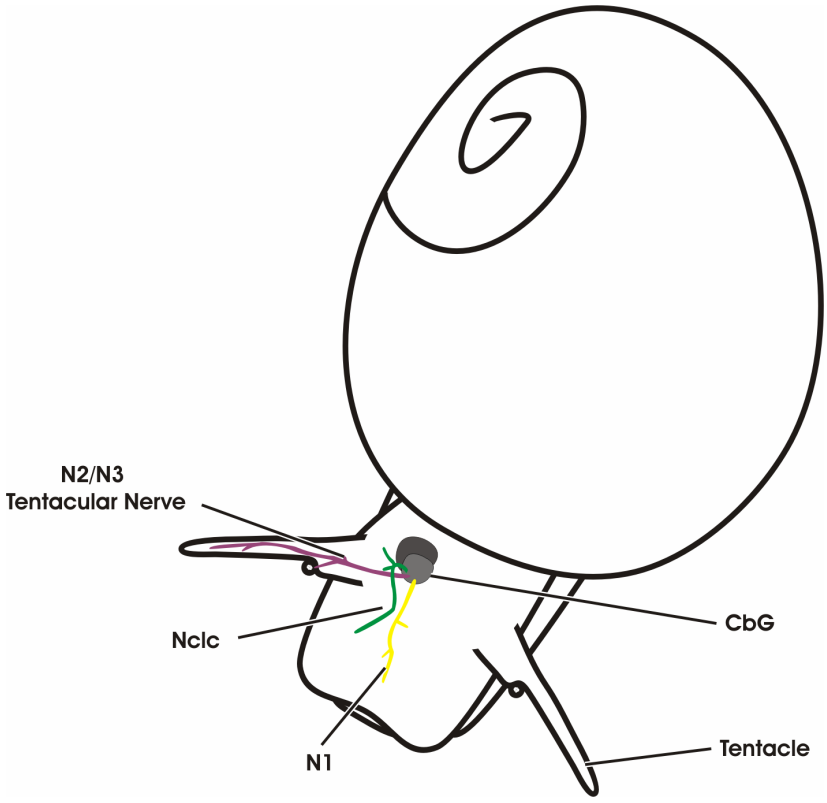


Figure 17: Neuroanatomical scheme of the three cerebral nerves of *Littorina littorea* (only right hemisphere shown). N1 in yellow, tentacular nerve in purple, and the Nclc in green; CbG – cerebral ganglion.

3.4 An evaluation of potential homology criteria for cellular innervation patterns and their intraspecific variation in *Haminoea hydatis*

This part of my study focuses on the definition of preliminary criteria for homologisation of cellular innervation patterns in Opisthobranchia. I survey, whether constant cell clusters in the central nervous system (CNS) can be identified to project into certain cerebral nerves innervating certain CSOs and whether these cell clusters differ with the size and the maturity of individual animals of the same species. In particular, the intraspecific variability of 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 I found a high variability of the CSOs innervated by the N2 in different opisthobranch taxa, e.g. labial tentacles (Anaspidea), oral veils (Pleurobranchomorpha) or rhinophores (Sacoglossa). I am going to discuss constant properties of cell clusters which serve as criteria for homologisation of innervation. These criteria for potential homology will be used in a subsequent comparative investigation in order to homologise cellular innervation patterns of various types of CSOs in different Heterobranchia with focus on the Opisthobranchia.

This part of my study has already been accepted for publication in a modified form by Zoomorphology (Staubach et al. *accepted 2008*). The manuscript is added in the supplement data.

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

The CSOs are innervated by four, bilateral pairs of cerebral nerves in *Haminoea hydatis* (Fig. 18), as indicated by the abbreviations modified from Edlinger (1980), see chapter 3.1. 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 Nervus rhinophoralis (N3) innervates the posterior part of the Hancock`s organ and the Nervus clypei capitis (Nclc) innervates the posterior cephalic shield. I observed no variability of these nerves in all investigated specimen (over 40 preparations) with regard to regions of terminal innervation or even of major branch points.

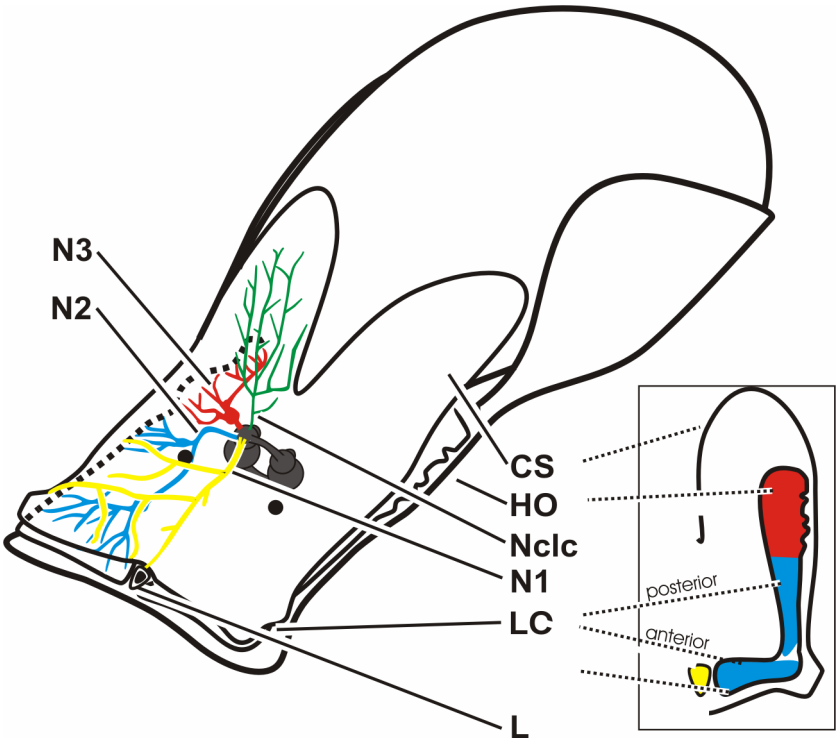


Figure 18: Schematic illustration of the central nervous system (CNS), the four cerebral nerves (excluding the optical nerve) and the cephalic sensory organs of *Haminoea hydatis.* The right partial illustration shows the organisation of the lip organ and the Hancocks organ (N1 - Nervus oralis, N2 - Nervus labialis, N3 - Nervus rhinophoralis, Nclc - Nervus clypei capitis, L – Lip, LO - lip organ, HO - Hancocks organ, CS - Cephalic shield.) Only the right cerebral nerves are shown.

3.4.2 Ni-Lys tracing

Five replicate backfills were performed for the N1, N3, Nclc and N2, using only the nerves of the right cerebral ganglion and the characteristic patterns of labelled somata for all nerves are shown in Figure 19 A-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: **C**erebral **N**ervus **l**abialis **c**luster **3**; Pnoc1: **P**arietal **N**ervus **o**ralis **c**luster **1**).

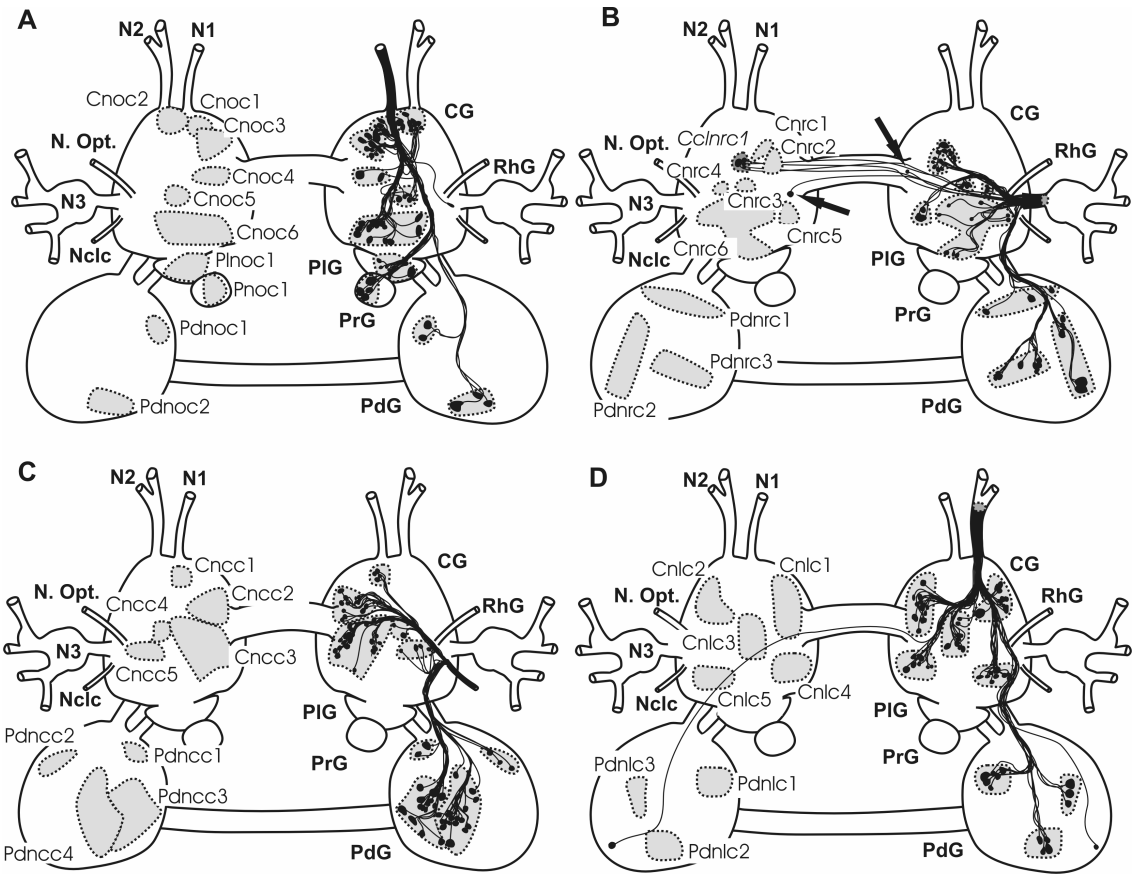


Figure 19: **A:** Schematic outline of cell clusters providing the N1. **B:** Schematic outline of cell clusters providing the N3. **C:** Schematic outline of cell clusters providing the Nclc. **D:** Schematic outline of cell clusters providing the N2. The size and position of the somata were digitalized from a camera lucida drawing, the distribution of the axons are averaged from all replicates (N1 - Nervus oralis, N3 - Nervus rhinophoralis, Nclc - Nervus clypei capitis, N. Opt. - Nervus opticus, CG – cerebral ganglia, RhG – rhinophoral ganglia, PlG – pleural ganglia, PdG – pedal ganglia and PrG – right parietal ganglia).

In this study, I defined clusters of nerve cells, grouped on the basis of their close positioning in the ganglia and the tight fasciculation of their axons projecting into the filled nerve. This is shown in Figure 20, which shows the pedal clusters for an axonal tracing of the N2. The close relationship of the somata within one cluster is clearly visible, but also that the axonal pathway group these somata to one cluster.

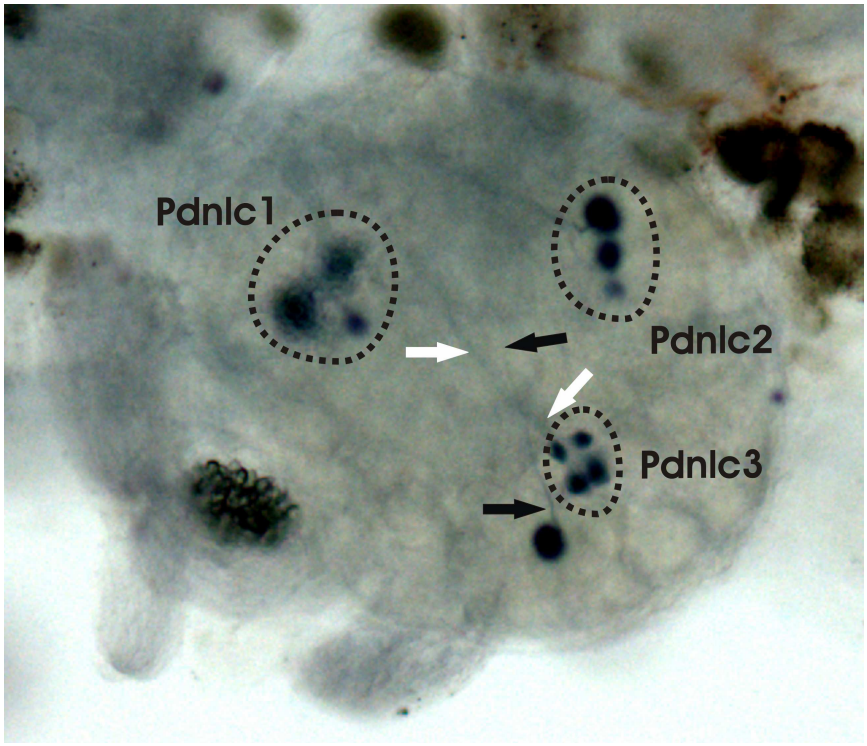


Figure 20: **P**e**d**al **N**ervus **l**abialis **c**lusters (Pdnlc) 1 to 3, dorsal photography of the pedal ganglia for an axonal tracing of the N2 in *Haminoea hydatis*. The white arrows mark the axonal pathways of the cluster Pdnlc 3 and the black arrows mark the axonal pathway of a single soma. This picture also shows the problems using photography to show the results of an axonal tracing, as it is quite difficult to focus all clusters (see Pdnlc 1 – out of focus) and nearly impossible to focus the axonal pathways.

As mentioned earlier, 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. 19C) were distributed over a relatively large portion of the surface of the pedal ganglion but their axonal pathways were clearly distinguishable into two separate courses. I therefore designated the populations as separate clusters.
For the N1 (n = 5) I 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. 19A). These clusters were found in all preparations and the variation between the samples was restricted to small differences (1-2) in the number of somata in some clusters only. The first cerebral cluster Cnoc1 is located right to the origin of the N1 directly under the root of the N2, it includes up to 12 somata, characteristically are one or two large somata and a bunch of nine to ten smaller somata right to the larger ones. The second cerebral cluster Cnoc2 lies at the same height, but is located on the left side of the N1. It consists of three large somata with two to three small ones behind. The next cluster Cnoc3 is located on the left hand of Cnoc2, under the Cnoc2 and above the root of the cerebral commissure. Characteristical patterns for Cnoc3 are a large soma and up to nine smaller ones. Cluster Cnoc4 lies directly underneath the clusters Cnoc1, Cnoc2, Cnoc5 and the root of the cerebral commissure on the right hand to Cnoc5. It consists of five to six small to medium sized somata. The next cluster Cnoc5 lies on the left side above Cnoc4, directly on the root of the commissure and simply consists of two large somata. The last cerebral cluster is located at the posterior margin of the cerebral ganglion near the pleural connective. It is the largest cluster with up to 15 medium sized somata. Characteristics are two larger somata on the right hand and a bunch of eleven to 13 somata on the left hand. The single pleural cluster Plnoc1 is characterised by one large soma at the right margin, and eight to ten small ones in the centre of the ganglion. The only parietal cluster, Prnoc1, is located on the left side of the right parietal ganglion, and its characteristics are three large somata and sometimes one medium sized soma. In the pedal ganglion I identified two clusters, Pdnoc1, a small cluster with only two medium sized somata, lying on the left side near the pleural connective and above the pedal commissure and Pdnoc2 at the posterior margin of the ganglion, consisting of one very large soma, and two medium sized ones.

The second traced cerebral nerve (n = 5) was the N3. This very short nerve terminates in the rhinophoral ganglion (RhG) which innervates the posterior part of the Hancock`s organ via four similar short nerves. Six cerebral (Cnrc1-6) and three pedal clusters (Pdnrc1-3) were identified (Fig. 19B). 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. 20B, 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 first cerebral cluster Cnrc1 is located little above the heigth of the cerebral commissure near the centre of the cerebral ganglia. It is characterised by one large and three medium sized somata, additionally a small soma occurs in two of the five samples. The position and the patterns of the cluster are very similar to the single cluster (Clnrc1) in the left cerebral ganglion, indicating that this cluster is a contralateral adequate to Cnrc1. Additionally, two single somata occur in both cerebral ganglia at nearly the same position, at the root of the cerebral commissure, maybe also contralateral. The second cerebral cluster Cnrc2 lies in direct neighbourhood of the first cluster and consists of seven to eight medium sized somata. The five medium sized somata of the third cerebral cluster (Cnrc3) are very close together. This cluster is located in the centre of the ganglion, in one line between the root of the cerebral commissure and the N3. The fourth cluster (Cnrc4) lies next to Cnrc3 on the right side, near the root of the N3. Four small somata are arranged nearly in a line from anterior to posterior. Cluster Cnrc5 is located at the inner posterior margin of the cerebral ganglion and consists of one very large soma and two medium sized somata. The last cerebral cluster Cnrc6 is very widespread and located right to the fifth cluster underneath the other cerebral clusters. Its characteristics are three smaller somata close together at the posterior margin of the ganglion and five to six more dispersed somata above. The first pedal cluster Pdnrc1 lies at the roots of the cerebral and pleural connectives and consists of two to three large somata in an extended row. The second cluster (Pdnrc2) is located at the outer margin of the ganglia and can be characterised by a patch of three large somata at the posterior end of the ganglion and a bunch of four to six medium sized somata above. The third pedal cluster (Pdnrc3) lies underneath Pdnrc1 on the left hand to Pdnrc2 and consists of a single larger soma between two bunches of medium sized somata with four to five somata each.

The smallest cerebral nerve, Nclc, innervates the posterior cephalic shield. The innervation pattern (n = 5) consists of five cerebral (Cncc1-5) and four pedal clusters (Pdncc1-4) (Fig. 19C). In comparison to the other cerebral nerves I 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.

Cncc1 lies at the root of the N1 and is a small patch of four medium sized somata. Cluster Cncc2 is more widespread and located at the anterior end of the cerebral commissure, characteristic is a patch of four to five small somata surrounded by seven of nine medium sized somata. The third and largest cluster (Cncc3) is also located at the root of the commissure but more posteriorly. It consists of two bunches of medium sized somata with a single soma lying between them, but more posteriorly. Cluster Cncc4 is located at the right hand of Cncc3 and has only two medium sized somata. The last cerebral cluster Cncc5 is located at the root of the Nclc and consists of three to four somata in a horizontal line.

The first pedal cluster (Pdncc1) is found at the root of the pleural ganglion and consists of one large and one medium sized soma. The second cluster (Pdncc2) lies on the right outer margin of the ganglion and is characterised by three medium sized somata in a row or semi circle. The last two clusters are more widespread and are located next to each other on the inner posterior part of the ganglion. Pdncc3 is located near the root of the pedal commissure on the left side with up to ten medium sized somata and five very large somata. The last cluster (Pdncc4) also has a high amount of somata (up to 17 medium and one very large somata) and is located at the right hand to cluster Pdncc3. The last cerebral nerve, the N2 innervates two cephalic sensory organs, the lip organ and the anterior Hancock´s organ (Edlinger 1980).

The innervation patterns of large individuals (shell size > 30 mm^2, Tab. II, Supplement data) consists of five cerebral clusters (Cnlc1-5), three pedal clusters (Pdnlc1-3) and a single soma in both pedal ganglia at nearly the same position possibly indicating bilateral projections (Fig. 19D). The first cluster (Cnlc1) is located above the anterior margin of the cerebral commissure, on the inner margin of the ganglia under the N1 root. In the ganglia of the largest animals it includes up to 15 somata in nearly a line from anterior to posterior, and a characteristically pattern were three larger somata lying more ventral in a row or a weak semi circle. My investigations indicate the addition of several smaller somata with an increasing size of the animal (see also Fig. 21). The second cerebral cluster (Cnlc2) is located at the same height like Cnlc1 but at the outer margin, right to the root of the N2 and is characterized by one large, six to eight middle sized and up to three very small somata in a row parallel to the large soma. The next cerebral cluster (Cnlc3) lies between Cnlc1 and Cnlc2 but more anteriorly, in one line with the root of the N1 and consists of three to nine medium sized somata. The cluster

Cnlc4 lies at the posterior inner margin of the cerebral ganglia, next to the cerebral commissure and anterior to Cnlc3. Cnlc4 is characterized by a mixture of maximal three large and five smaller somata. The last cerebral cluster (Cnlc5) is located at the posterior outer margin of the ganglia and next to Cnlc3 and is characterized by up to ten medium sized somata in a horizontal row. In larger individuals I found three additional cell clusters in the pedal ganglia (Pnlc1–3) and several contralateral somata in the left and right cerebral ganglia. The first pedal cluster of somata (Pnlc1) lies left to the root of the cerebral connective, at the inner margin of the ganglia and is characterized by six to eight large and medium sized somata. The second pedal cluster (Pnlc2) is located at the right outer margin under Pnlc1 and mostly consists of 3 somata of all sizes. The last pedal cluster (Pnlc3) lies between Pnlc1 and Pnlc3 at the anterior margin in one line with the base of the cerebral connective and includes one large soma and up to four medium sized somata. In comparison to the innervation patterns of other nerves, the clusters were easier to identify based on their positions as I found clearly spatial separations. I found no significant differences in number of cell somata between samples of roughly similar sizes (Fig. 21, e.g. samples 16-18). Similarly sized individuals were characterised by the shell size.

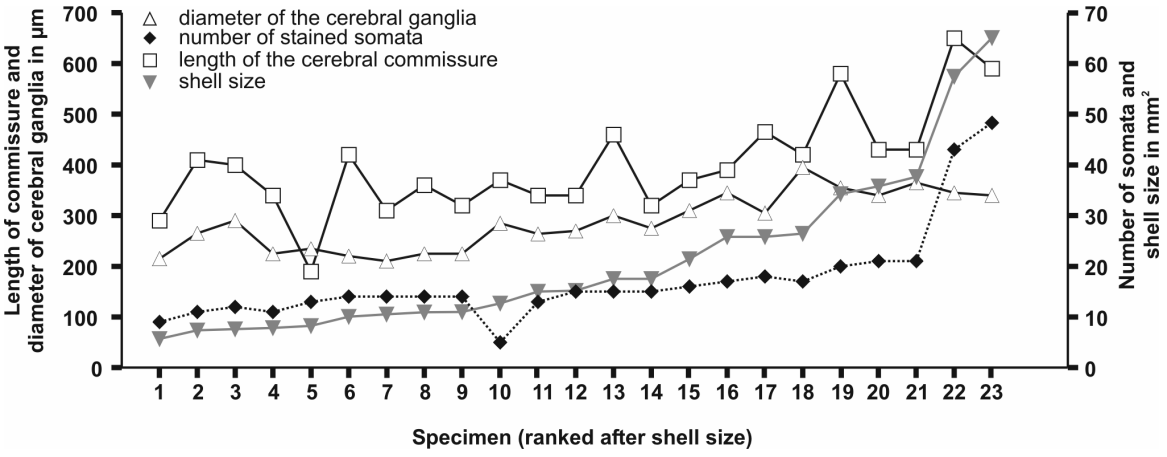


Figure 21: Graph showing sizes and number of cells in the cerebral cell clusters. The x-axis represents the investigated animals (n = 23). The left y-axis shows the length of the commissure in µm and the mean diameter of the cerebral ganglia in µm, the right y-axis represents the shell size (length*breadth) in mm^2 and the number of somata in the clusters.

3.4.3 Variability of N2 labelling

The specific aim of this part of my study was to test the variability of axonal projections from identified clusters into specific nerves innervating the CSOs, followed by a description of variable and invariable characters of cellular innervation patterns. The invariable characters should be used to define criteria for homology. For this purpose, I used the largest cerebral nerve, the Nervus labialis (N2). First, I found no significant variability between innervation patterns for the left or 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 I compared the backfilled labelling in animals of varying body sizes. All measured body sizes (Fig. 22), the number of cerebral clusters and the total number of stained somata in the cerebral clusters are shown in Figure 21.

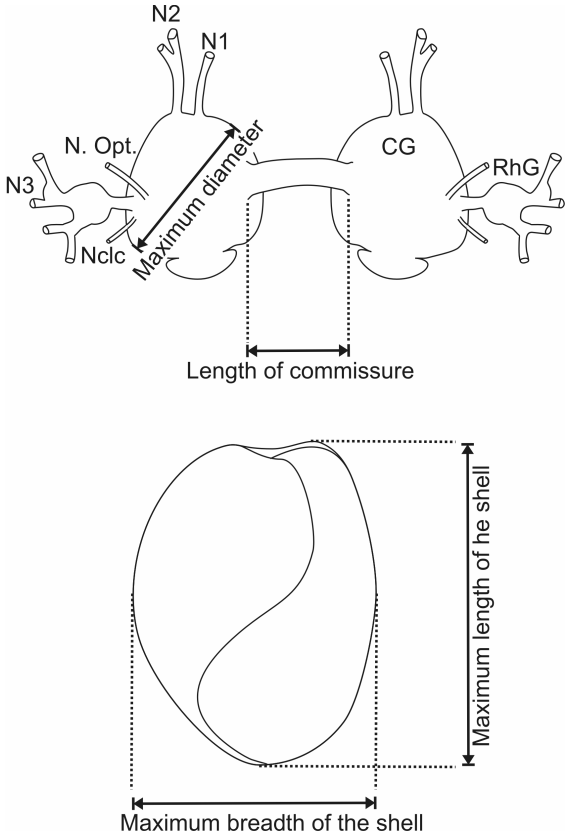


Figure 22: Schematic drawing of part of the CNS and of the shell in *Haminoea hydatis*. The sizes, measured for the correlation analysis are shown: The maximum diameter of the cerebral ganglia, the length of the commissure and the length and breadth of the shell. (N1 - Nervus oralis, N2 - Nervus labialis, N3 - Nervus rhinophoralis, Nclc - Nervus clypei capitis, N.Opt. - Nervus opticus, RhG - rhinophoral ganglia, CG - cerebral ganglia.)

The data indicate a constant number of cerebral clusters in all individuals, but with increasing body size I found increasing numbers of cells (from eight to 65 over all clusters) in several clusters. Correlation analyses showed a high correlation between the absolute number of somata projecting into the N2 and the size of the animals (Fig. 21, 23, 24).

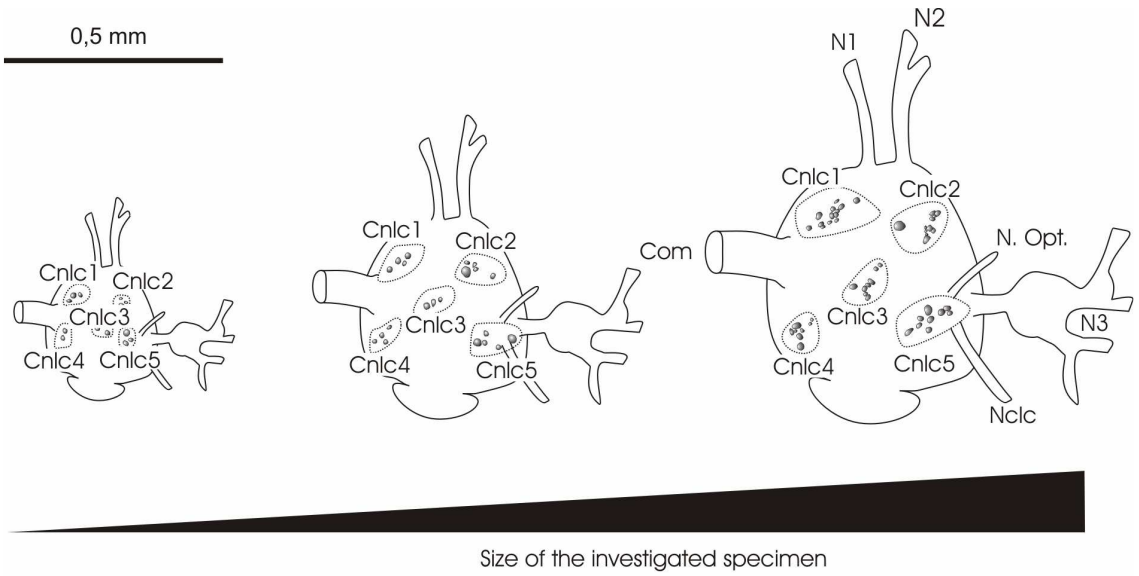


Figure 23: Schematic outline of cell clusters in several right cerebral ganglia of individuals of different sizes. The size and position of the cells were digitalized from camera lucida drawings (N1 - Nervus oralis, N3 - Nervus rhinophoralis, Nclc - Nervus clypei capitis, N. Opt. - Nervus opticus and Com - cerebral commissure).

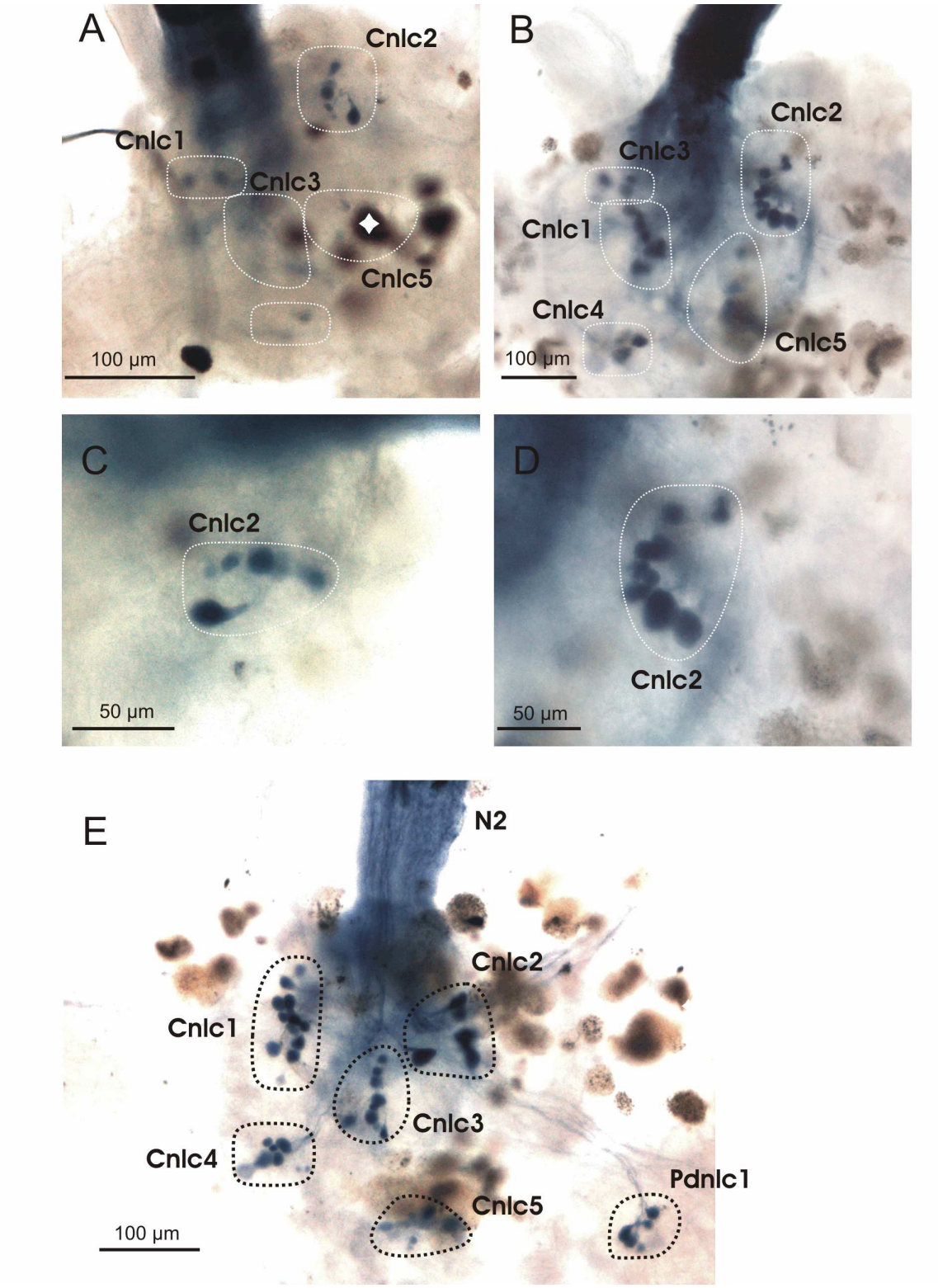


Figure 24: Showing the difference of innervation patterns for the N2 between a small (**A**) and a large individual (**B**), especially for the cluster Cnlc2 in the pictures **C** (small individual) and **D** (large individual). Caused by the plasticity and the pigmentation of the ganglia, camera lucida drawings are more adequate to show the staining than photos do. Picture **E** shows one of the less staining photography with all cerebral and one pedal cluster visible.

For both analyses, the Pearson and the nonparametric correlation analyses, I obtained similar significant correlations between the measured morphological sizes and the number of labelled somata. I 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 analyses I found the highest correlation between the shell size and the number of innervating somata in the cerebral ganglia. Additionally I measured the maximum diameter of each soma in each of the cerebral clusters Cnlc1–5 (Fig. 23).

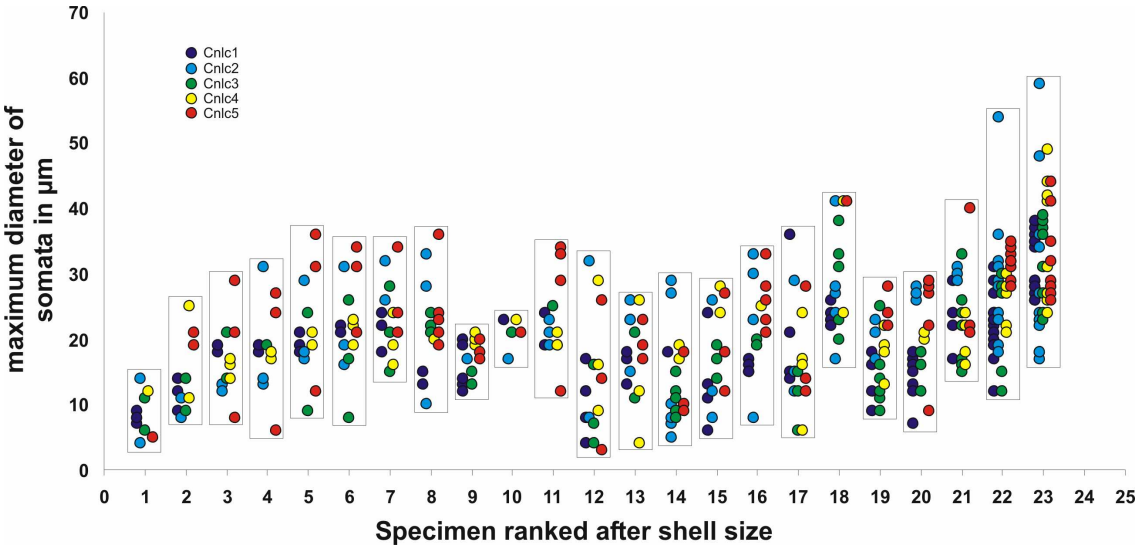


Figure 25: Graph showing the distribution of soma sizes in the cerebral cell clusters (Cnlc1-5). The x-axis represents the investigated animals (n = 23), in the same order like in Table II, (Supplement Data). Each rectangle represents one individual. Every cluster is represented by a different colour, 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.

All clusters showed an increase of soma size with increasing shell size. But the data set is too small for equivalent analyses of correlations between shell size and the size of single somata. Also with the axonal tracing technique it is possible to identify characteristic cell clusters, but an identification of single cells is not given. Therefore the increasing soma size can also be postulated as a trend. In contrast to the cerebral

clusters described above, the number of pedal clusters varied. Pedal clusters could not be found in smaller individuals (samples one to 18), whereas larger individuals possess three clusters in the right pedal ganglion (samples 19 to 23). All investigated specimen show no or three pedal clusters. This indicates that the three pedal clusters occur with increasing body size and are present in all large individuals.

To summarise, the results of this chapter demonstrate that all four stained cerebral nerves can be traced to specific cell clusters, which are distributed across the cerebral ganglia. The identities of cerebral clusters are specific for each nerve and independent of the size of the individual slug. Most somata projecting into the different cerebral nerves are located in the cerebral clusters. I also found relative high numbers of somata in the pedal ganglia projecting directly 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.

3.4.4 Discussion

The aim of this chapter was to provide a description of the innervation patterns for cerebral nerves of *Haminoea hydatis* in order to define a morphological character complex for the homologisation of the nerves and hence the homologisation of the cephalic sensory organs innervated by these nerves. Therefore, a specific goal of the current part of my investigations was to characterise several detailed features of the innervation patterns, including the size, position and number of neuronal clusters within the central ganglia projecting into each of the specific nerves. Additionally, I 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). Therefore, I systematically examined characteristics of cellular innervation patterns for different nerves (N1, N2, N3, Nclc), as well as differences in laterality and correlations between the size of animals and innervation patterns of one specific nerve (N2).

My results clearly indicate that efferent projections into all four cerebral nerves could be attributed to specific cerebral, pleural, parietal and pedal cell clusters, which are cardinally characterised by their relative positions and axonal pathways in the respective ganglion (Fig. 19 A-D). In addition, I found that these clusters were characterised by similar relative sizes of somata within clusters. I demonstrated remarkably little variability in these two characteristics when examining animals of comparable sizes, and in the case of the N2 also regardless of the laterality of the nerves. 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 behaviours have been widely described in the cerebral ganglia (and also buccal ganglia not examined here) in other gastropods (Elliot 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 for example 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 Hancocks organ, a 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 *Haminoea hydatis*, since it is used as a plough. During a long part of the daylight phase, *Haminoea 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 own observations, Hoffmann 1939). Therefore, a higher number of pedal neurons providing this locomotory organ seem reasonable. Nevertheless, the exact function of the pedal somata is not clarified yet. More comparable data about other cephalaspid taxa with diverse strategies against predation or living in rocky habitats are needed.

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, I also found three sources of intraspecific variability. In smaller specimen, I observed: 1) lesser numbers of cells in clusters, 2) smaller cells within the clusters, and 3) fewer clusters. These changes correlated with all of the different measurements of animal size used in this study. 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 sizes of CSOs were also confounded by variable degrees of contraction during dissection in addition to subsequent histological distortions. Moreover, I did not use the age, because I observed an extremely high variation in body size from individuals of the same clutch. Thus I believe that body size provides a poor indication for age, as has also been postulated for other gastropods such as *Lymnaea stagnalis* (Croll and Chiassion 1989).

I 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 Hancocks 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. Therefore larger numbers of cells are needed to innervate these structures. Second, the sizes of the somata also enhance 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 maturity. Specifically, new clusters of cells may be added to the nervous system to mediate the appearance of new behaviours.

My study employed a neuroanatomical technique to investigate innervation patterns in an opisthobranch gastropod. My results are consistent with previous work in molluscs and other taxa using immunocytochemical or additional histological techniques. Other studies also found size dependence or developmental changes like additional somata, cell clusters or growth of somata in the whole CNS as it was observed in my study (Ogawa 1939, Stewart et al. 1986, Hauser and Koopowitz 1987, Cash and Carew 1989). For example investigations on 5HT-lir (lir = like immuno reactivity) 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 the number of neurons in the CNS to brain size and also reported a higher intraspecific variation for neurons in pedal than in 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 with my own investigations of additional pedal clusters labelled by N2 backfilling in large individuals which might 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 *Haminoea hydatis,* green macroalgaes like *Ulva lactuca* or *Enteromorpha spec*., occur in patches and in smaller individuals the mobility of the animal is restricted. So it is possible that small individuals with less mobility are forced to find food with their contact chemoreceptor, whereas larger individuals with a higher mobility have the possibility to find new patches of adequate food sources with their distance chemoreceptor (Chester 1993) which involves locomotion.

While my findings are consistent with previous literature indicating changes in the number of cells and clusters with increasing body size, I cannot discount possible contributions of system biases due to technical difficulties. Specifically smaller specimen may have greater numbers of incomplete nerve fills despite my 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, my 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.

Knowledge of intraspecific variation in a character complex such as innervation patterns as discussed before is a prerequisite for comparing and homologising such character complexes in different taxa. In order to homologise structures it is important, that only constant or relatively invariable features should be considered for the homologisation. This is also true for homologising innervation patterns and in consequence organs or structures provided by these. Therefore I use characteristics of the innervation patterns of the cerebral nerves of *Haminoea hydatis* to define criteria for a subsequent homologisation of these nerves. Up to now, the homology of cerebral nerves in heterobranch gastropods has only been identified by the ganglionic origin of the nerves (Huber 1993). Whereas the N3 can easily be identified by the ganglionic origin, I 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. Therefore I hereby define the following criteria for homologisation of innervation patterns of the cerebral nerves of *Haminoea 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 highly variable (Croll 1987, Kutsch and Breidbach 1994), the major pathways of tracts projecting to the different nerves were 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 identifying them 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 5HT-lir 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).

As stated above, the goal of this study was to establish the use of backfilling techniques to provide better means for homologising nerves than simply relying upon the positions of their origins from the central ganglia. However, I 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 this way I will get a more detailed characterisation of the innervation patterns, a basic premise under the assumption that high complexity and similarities of all kinds (Bock 1989) are fundamental criterions for an explanation of homology.

In conclusion, I postulate the axonal tracing technique primary as a method for the homologisation of nerves. The axonal tracing technique gives us a morphological character complex to homologise cerebral clusters, and in consequence to describe and identify neuronal structures. This character complex has a higher complexity than ganglionic structures, therefore innervation patterns are more suitable to distinguish between homologous and analogous nerves. The observations about the variability and my definition of criteria for homologous innervation patterns of the cerebral nerves in Opisthobranchia can now be used to compare these patterns throughout different taxa of Opisthobranchia in order to homologise nerves and the organs which they innervate.

3.5 Potential homology of innervation patterns and cerebral nerves

In this chapter, I will compare the innervation patterns for the cerebral nerves of the investigated species. This chapter serves as an expanded test whether patterns of individual neurons can be used as a morphological complex for the homologisation of nerves. Here I will test if the homology criteria for the cellular innervation patterns, based on the intraspecific investigations in chapter 3.4 can be confirmed when comparing interspecific cellular innervation patterns. The primary aim of this chapter is to describe and compare tracing patterns between different taxa of Opisthobranchia, Stylommatophora and Caenogastropoda. Secondly I want to homologise the cerebral nerves and in follow to postulate primary hypotheses of homology for the CSOs. These primary homology hypotheses of the cerebral nerves innervating the CSOs of different Opisthobranchia orders should be part in homologising the CSOs. At this point I will not postulate final homologies for the CSOs themselves, this will be part of chapter 4.7. Nevertheless, the homology hypothesis of the cerebral nerves will give a first indication for the homologies of the CSOs. As basic pattern for the comparison of the cellular innervation patterns, I used the cellular innervation patterns described in chapter 3.4 for *Haminoea hydatis*.

3.5.1 Innervation patterns of *Acteon tornatilis*

By conducting the axonal tracing studies I am able to reconstruct cellular innervation patterns for the four cerebral nerves of *Acteon tornatilis*. The characteristic patterns of labelled somata for all nerves are shown in Figure 26A-D, including the approximate pathways of the stained axons. For the N1/Nervus oralis (n = 10) I can identify six cerebral clusters (Cnoc1-6) and one pedal cluster (Pdnoc1) in each sample (Fig. 26A). The variation between the samples is restricted to very few somata in some clusters. The cerebral clusters are distributed over the whole cerebral ganglion. The pedal cluster Pdnoc1 is located on the anterior margin of the pedal ganglion above the pedal commissure. The innervation pattern of the N2/Nervus labialis (n=10) consists of five cerebral clusters (Cnlc1-5) and three pedal clusters (Pdnlc1-3) (Fig. 26B). The cerebral clusters show distinct spatial separations and are easy to identify. The third traced cerebral nerve (n = 10) is the N3/Nervus rhinophoralis. Six cerebral (Cnrc1-6) and three pedal clusters (Pdnrc1-3) are identified (Fig. 26C). I found an additional single cluster (*Cclnrc1*) and a single soma in the left cerebral ganglion (see arrows in Fig. 26C). The contralateral cluster is located at the base of the N2 whereas the single soma is found at the root of the cerebral commissure. I observed slight intraspecific variability between the ten samples which amounted only to very few somata in some clusters. In the Nclc/Nervus clypei capitis, the innervation (n = 10) pattern consisted of five cerebral clusters (Cncc1-5) and a single soma at the lateral margin of the cerebral ganglion above the pedal connective (Fig. 26D). Additionally I found four pedal clusters (Pdncc1-4). The Nclc had the highest amount of pedal clusters in all investigated nerves. The number of pedal somata, however, is comparable to the number of pedal somata for the N2 innervation pattern (Fig. 26B).

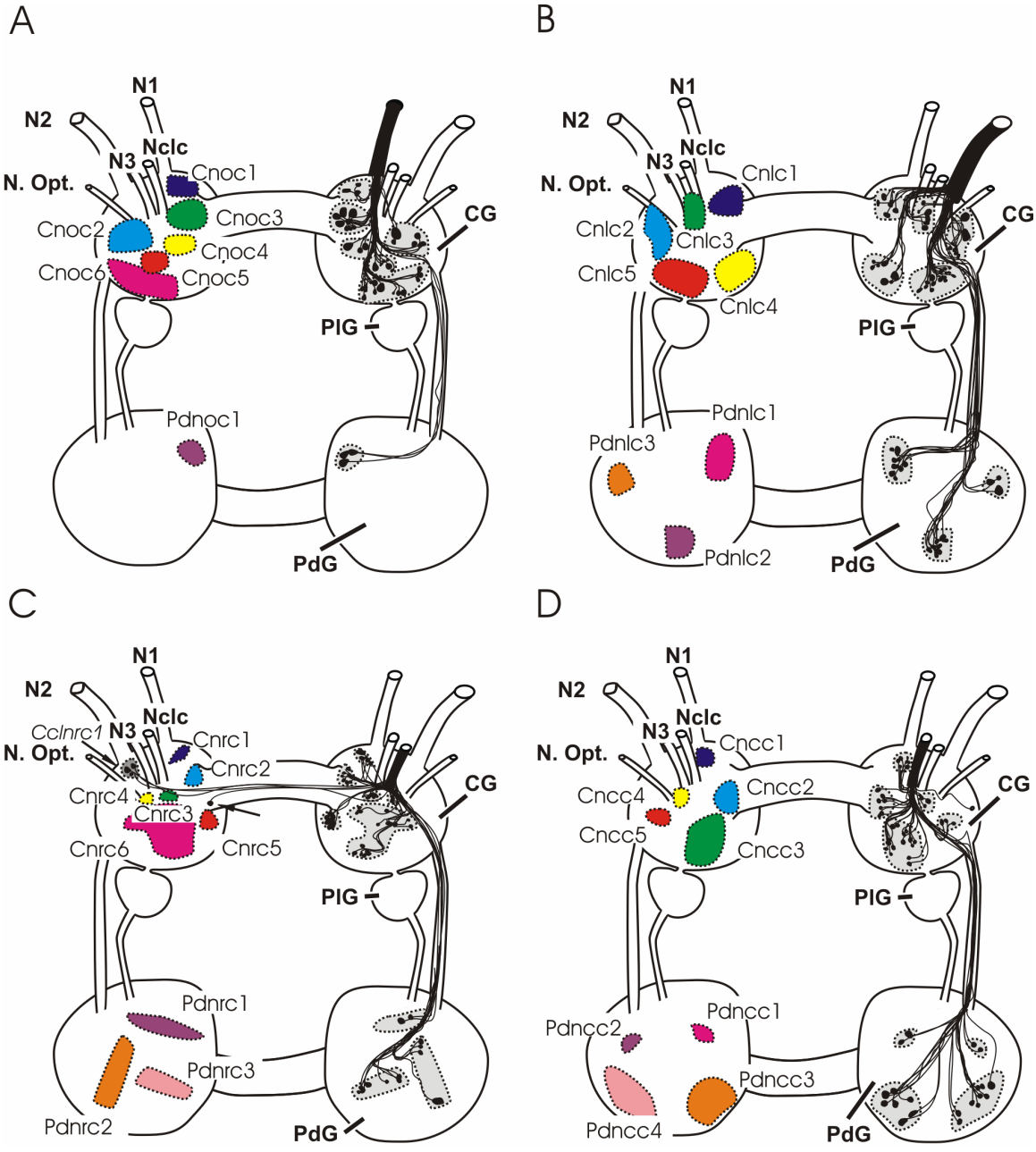


Figure 26: Schematic outline of somata and their axons projecting into the N1 (**A**), N2 **(B**), N3 (**C**) and Nclc (**D**) of *Acteon tornatilis***.** The size and position of the somata are digitalized from a camera lucida drawing, the distribution of the axons are averaged over all replicates. N1 - Nervus oralis, N2 - Nervus labialis, N3 - Nervus rhinophoralis, Nclc - Nervus clypei capitis, N. Opt. - Nervus opticus, CG - cerebral ganglia, PlG - pleural ganglia, PdG - pedal ganglia.

3.5.2 Innervation patterns of *Pleurobranchaea meckeli*

The characteristic patterns of labelled somata for all four cerebral nerves of *Pleurobranchaea meckeli* are shown in Figure 27A-D, including the approximate pathways of the stained axons. For the N1/Nervus oralis (n = 8) I also can identify six cerebral clusters (Cnoc1-6) in each sample (Fig. 27A). Again the variation between the samples is restricted to very few somata in some clusters. The cerebral clusters are primarily distributed in the median anterior region of the cerebral ganglion, except for Cnoc2 and Cnoc6. The innervation pattern of the N2/Nervus labialis (n = 9) consists of five cerebral clusters (Cnlc1-5) and three pedal clusters (Pdnlc1-3) (Fig. 27B). The cerebral clusters show distinct spatial separations and are easy to identify. The third traced cerebral nerve (n = 10) is the N3/Nervus rhinophoralis. Six cerebral (Cnrc1-6), one contralateral cerebral (Clnrc1) and two pedal clusters (Pdnrc1-2) are identified (Fig. 27C). The contralateral cluster is located in the anterior region of the cerebral ganglion near the base of the N2 like in *Acteon tornatilis*. I observed slight intraspecific variability between the samples which amounted only to very few somata in some clusters. In the Nclc/Nervus clypei capitis, the innervation (n = 10) pattern consisted of five cerebral clusters (Cncc1-5) (Fig. 27D). Unlike *Acteon tornatilis* I found no pedal clusters.

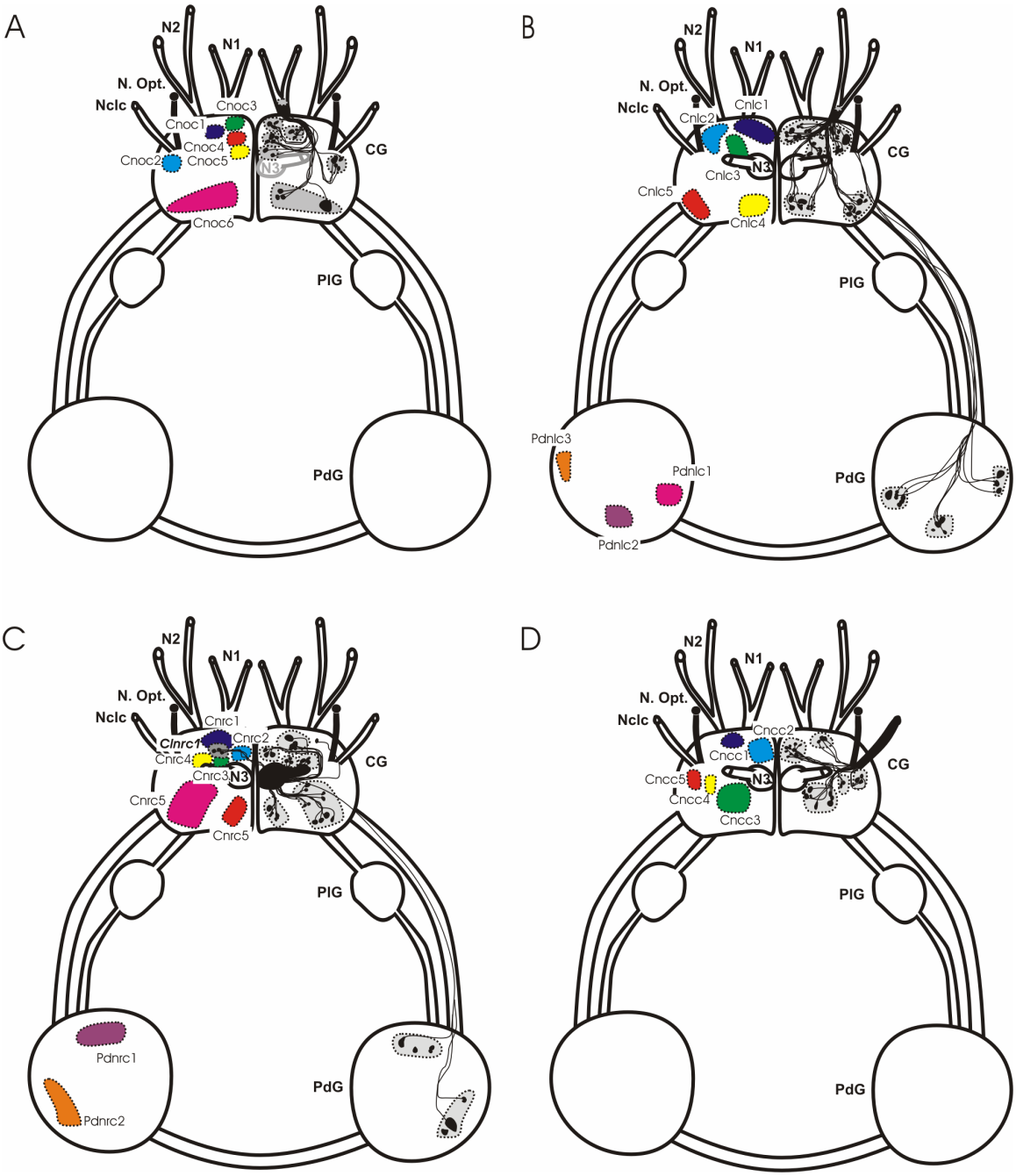


Figure 27: Schematic outline of somata and their axons projecting into the N1 (**A**), N2 **(B)**, N3 (**C**) and Nclc (**D**) of *Pleurobranchaea meckeli***.** The size and position of the somata are digitalized from a camera lucida drawing, the distribution of the axons are averaged over all replicates. N1 - Nervus oralis, N2 - Nervus labialis, N3 - Nervus rhinophoralis, Nclc - Nervus clypei capitis, N. Opt. - Nervus opticus, CG - cerebral ganglia, PlG - pleural ganglia, PdG - pedal ganglia.

3.5.3 Innervation patterns of *Archidoris pseudoargus*

The characteristic cellular innervation patterns of all four cerebral nerves of *Archidoris pseudoargus* are shown in Figure 28A-D. For the N1/Nervus oralis (n = 10) also six cerebral clusters (Cnoc1-6) in each sample (Fig. 28A) are present. Additionally, I found two pedal clusters (Pdnoc1-2) and a cerebral contralateral cluster (Clnoc1). The contralateral cluster is labelled in italics (Fig. 28A). Again the variation between the samples is restricted to very few somata in some clusters. The cerebral clusters are distributed across the entire cerebral ganglion. Due to the fusion of the ganglia in *Archidoris pseudoargus*, the clusters are not well separated, also the pedal cluster Pdnoc1 (abbreviation in blue, Fig. 28A) is located directly underneath the cerebral clusters. The innervation pattern of the N2/Nervus labialis (n = 10) consists of five cerebral clusters (Cnlc1-5), a contralateral cerebral cluster (Clnlc1) and a pedal clusters (Pdnlc1) (Fig. 28B). Here the cerebral clusters show distinct spatial separations and are also easy to identify, like in *Acteon* and *Pleurobranchaea*. The third traced cerebral nerve (n = 10) is the N3/Nervus rhinophoralis. Six cerebral (Cnrc1-6), one contralateral soma and three pedal cluster (Pdnrc1-3) are identified (Fig. 28C). The contralateral soma is located near the base of the N2 like the contralateral cluster Clnrc1 in *Acteon tornatilis* and *Pleurobranchaea meckeli*. Again I only observed slight intraspecific variability between the samples. In the Nclc/Nervus clypei capitis, the innervation (n = 10) pattern consisted of five cerebral clusters (Cncc1-5) (Fig. 28D) and one pedal cluster (Pdncc1).

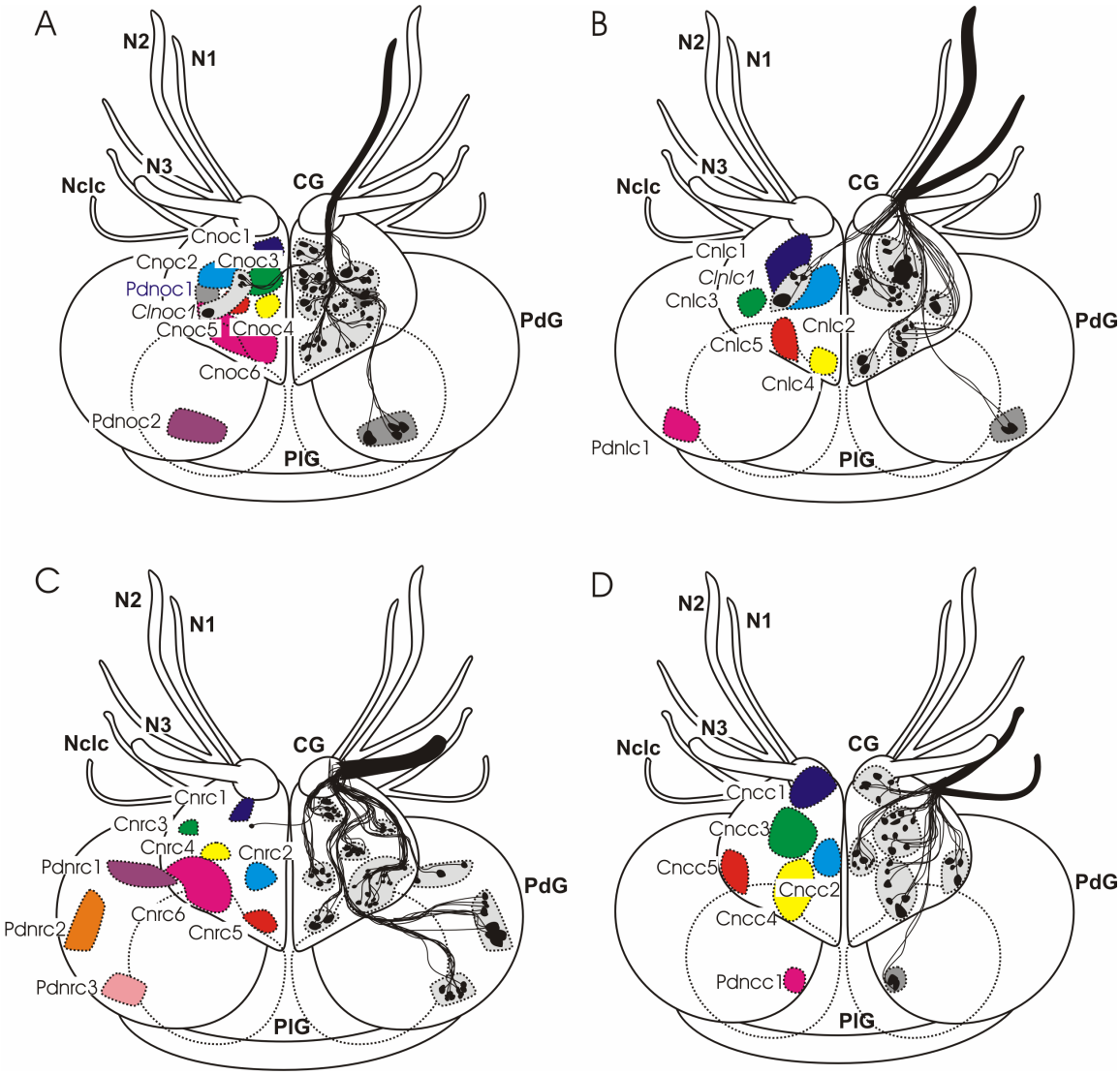


Figure 28: Schematic outline of somata and their axons projecting into the N1 (**A**), N2 **(B)**, N3 (**C**) and Nclc (**D**) of *Archidoris pseudoargus***.** The size and position of the somata are digitalized from a camera lucida drawing, the distribution of the axons are averaged over all replicates. N1 - Nervus oralis, N2 - Nervus labialis, N3 - Nervus rhinophoralis, Nclc - Nervus clypei capitis, CG - cerebral ganglia, PlG - pleural ganglia, PdG - pedal ganglia.

3.5.4 Innervation patterns of *Aplysia* spp.

The tracing patterns of *Aplysia californica* and *Aplysia punctata* show no significant differences, therefore I will describe the innervation patterns of *Aplysia* spp. in this chapter.

All four typical innervation patterns for the cerebral nerves of *Aplysia* spp. are shown in Figure 29A-D. For the N1/Nervus oralis (n = 20) I can characterize six cerebral clusters (Cnoc1-6) in each sample (Fig. 29A). Additionally I found two pedal clusters (Pdnoc1-2). Like in earlier investigated species the variation between the samples is restricted to very few somata in some clusters. The cerebral clusters are distributed over the whole cerebral ganglion. The innervation pattern of the N2/Nervus labialis (n = 20) consists of five cerebral clusters (Cnlc1-5), a contralateral cerebral cluster (Clnlc1) and three pedal clusters (Pdnlc1-3) (Fig. 29B). The innervation patterns for the cerebral clusters show distinct spatial separations and are also easy to identify, like in *Acteon*, *Pleurobranchaea* and *Archidoris*. The third traced cerebral nerve (n = 20) is the N3/Nervus rhinophoralis. Six cerebral clusters (Cnrc1-6), one contralateral soma and three pedal clusters (Pdnrc1-3) are identified, comparable to *Archidoris pseudoargus* (Fig. 29C). I only observed slight intraspecific variability between the samples. In the Nclc/Nervus clypei capitis, the innervation (n = 10) pattern consisted of five cerebral clusters (Cncc1-5) (Fig. 28D) and three pedal cluster (Pdncc1-3).

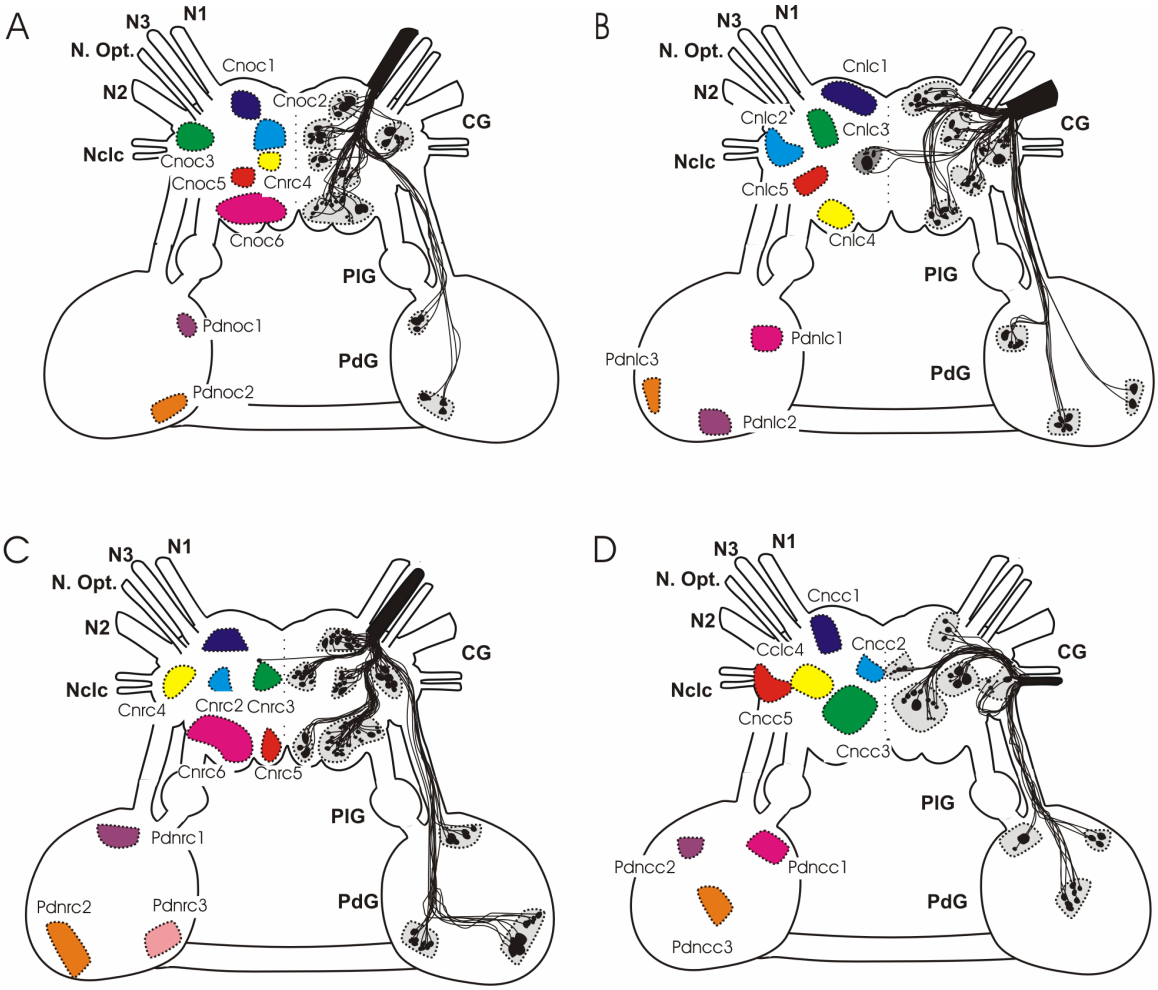


Figure 29: Schematic outline of somata and their axons projecting into the N1 (**A**), N2 **(B**), N3 (**C**) and Nclc (**D**) of *Aplysia***.** The size and position of the somata are digitalized from a camera lucida drawing, the distribution of the axons are averaged over all replicates. N1 - Nervus oralis, N2 - Nervus labialis, N3 - Nervus rhinophoralis, Nclc - Nervus clypei capitis, CG - cerebral ganglia, PlG - pleural ganglia, PdG - pedal ganglia, N. Opt. - Nervus opticus.

3.5.5 Innervation patterns of *Haminoea hydatis*

The innervation patterns of *Haminoea hydatis* have already been described in chapter 3.4, but for an easier comparison of the innervation patterns I will show the figure again.

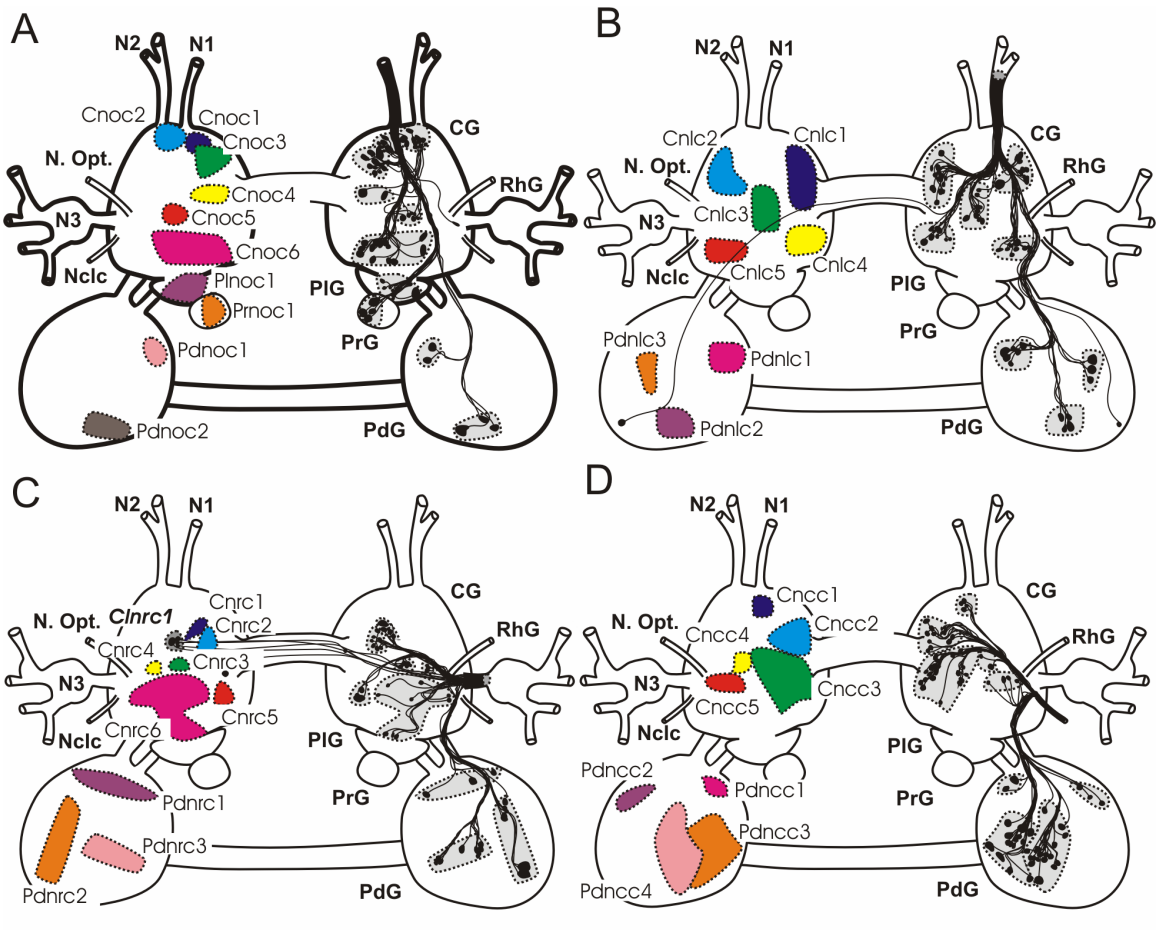


Figure 30: Schematic outline of somata and their axons projecting into the N1 (**A**), N2 **(B**), N3 (**C**) and Nclc (**D**) of *Haminoea hydatis***.** The size and position of the somata are digitalized from a camera lucida drawing, the distribution of the axons are averaged over all replicates. N1 - Nervus oralis, N2 - Nervus labialis, N3 - Nervus rhinophoralis, Nclc - Nervus clypei capitis, CG - cerebral ganglia, RhG - rhinophoral ganglia, PlG - pleural ganglia, PdG - pedal ganglia, N. opt. - Nervus opticus.

3.5.6 Innervation patterns of *Achatina fulica*

The characteristic patterns of labelled somata for all four cerebral nerves of *Achatina fulica* are shown in Figure 31A-D. For the N1/Nervus oralis (n = 4) I can locate six cerebral clusters (Cnoc1-6) in each sample (Fig. 31A), comparable to all species described so far. Additionally, I found two pedal clusters (Pdnoc 1-2). The cerebral clusters are distributed across the complete cerebral ganglion. Here I found a torsion of the first three cerebral clusters (Cnoc1-3) which are located at the lateral and not median margin of the ganglion. However, they are located close to the N1, which is here positioned laterally and not anteriorly like in the other investigated species. The innervation pattern of the N2/Nervus labialis (n = 4) consists of five cerebral clusters (Cnlc1-5), a contralateral cerebral cluster (Clnlc1) and four pedal clusters (Pdnlc1-4) (Fig. 31B). The cerebral clusters show the typical distinct spatial separations and are also easy to identify, like in some of the earlier described species. Again I found a torsion in the position of the cerebral clusters. The third traced cerebral nerve (n = 4) is the N3/Nervus rhinophoralis. Six cerebral (Cnrc1-6), two contralateral clusters (*Clnrc1-2*) and two pedal clusters (Pdnrc1-2) are identified (Fig. 31C). Again I only observed slight intraspecific variability between the samples. In the Nclc/Nervus clypei capitis (n = 4), the innervation pattern consisted of five cerebral clusters (Cncc1-5) (Fig. 31D) and two pedal clusters (Pdncc1-2).

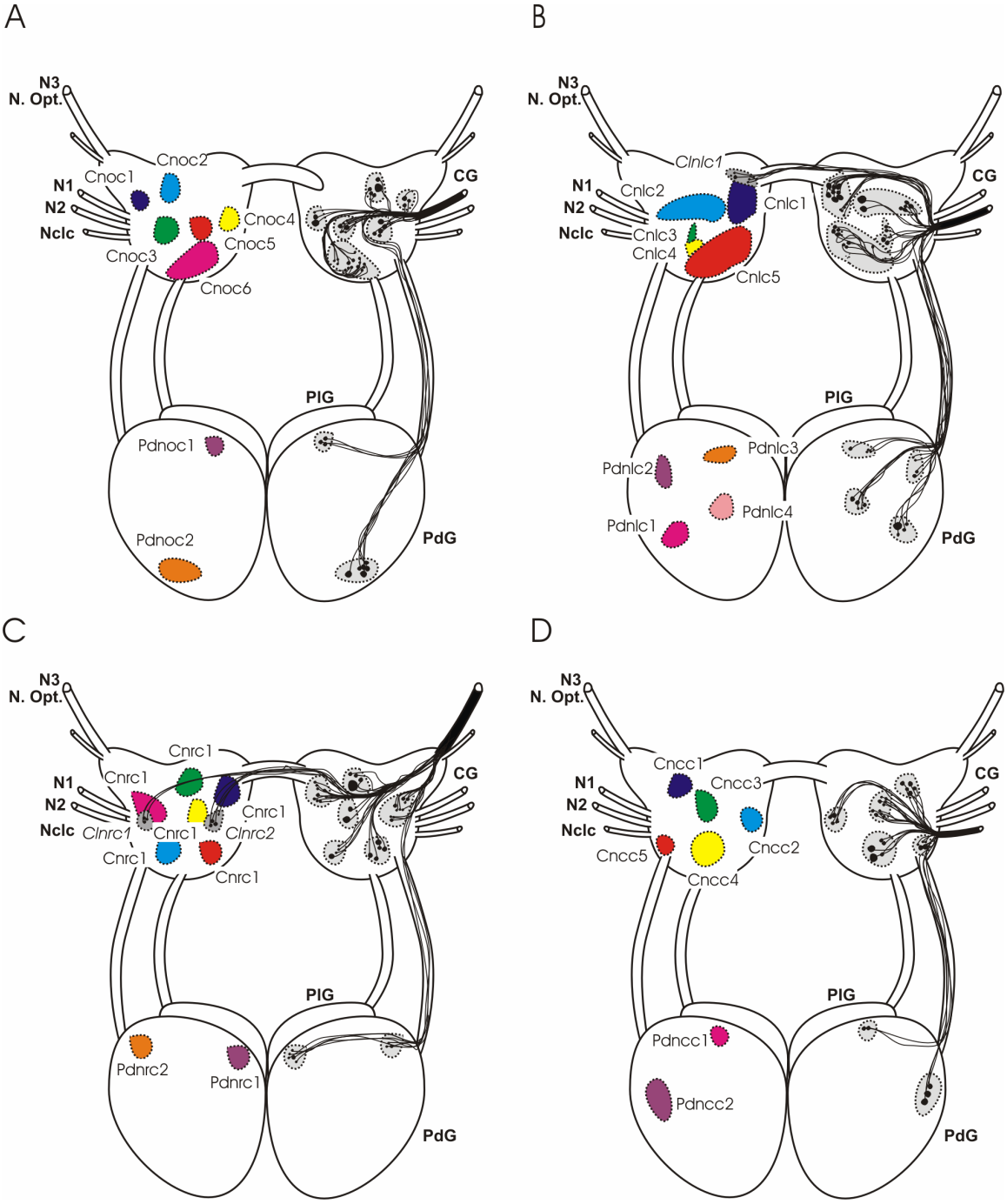


Figure 31: Schematic outline of somata and their axons projecting into the N1 (**A**), N2 **(B**), N3 (**C**) and Nclc (**D**) of *Achatina fulica***.** The size and position of the somata are digitalized from a camera lucida drawing, the distribution of the axons are averaged over all replicates. N1 - Nervus oralis, N2 - Nervus labialis, N3 - Nervus rhinophoralis, Nclc - Nervus clypei capitis, CG - cerebral ganglia, PlG - pleural ganglia, PdG - pedal ganglia, N. Opt. - Nervus opticus.

3.5.7 Innervation patterns of *Littorina littorea*

The innervation patterns of *Littorina littorea* are considerably different from the innervation pattern of the other investigated species. At this point it is important to mention that *Littorina littorea* belongs to the Caenogastropoda and only possesses three cerebral nerves, the N1, the Nervus tentacularis and the Nclc. The innervation patterns for the three cerebral nerves of *Littorina littorea* are shown in Figure 32A-D. For the N1/Nervus oralis (n = 10) I can identify six cerebral clusters (Cnoc1-6) in each sample (Fig. 32A). Additionally I can describe one pedal cluster (Pdnoc1) and two contralateral pedal clusters (*Clnoc 1-2*). The cerebral clusters are distributed more posteriorly within the cerebral ganglion. The innervation pattern of the Nervus tentacularis (n = 10) consists of ten cerebral clusters (Ctent1-10) and a contralateral cerebral cluster (*Cltent1*) (Fig. 32B, D). I could not detect any pedal cluster. The cerebral clusters show no distinct spatial separations and are not easy to identify, unlike in other investigated taxa. For the Nclc/Nervus clypei capitis, the innervation (n = 10) pattern consists of five cerebral clusters (Cncc1-5) (Fig. 32C) and two pedal clusters (Pdncc1-2).

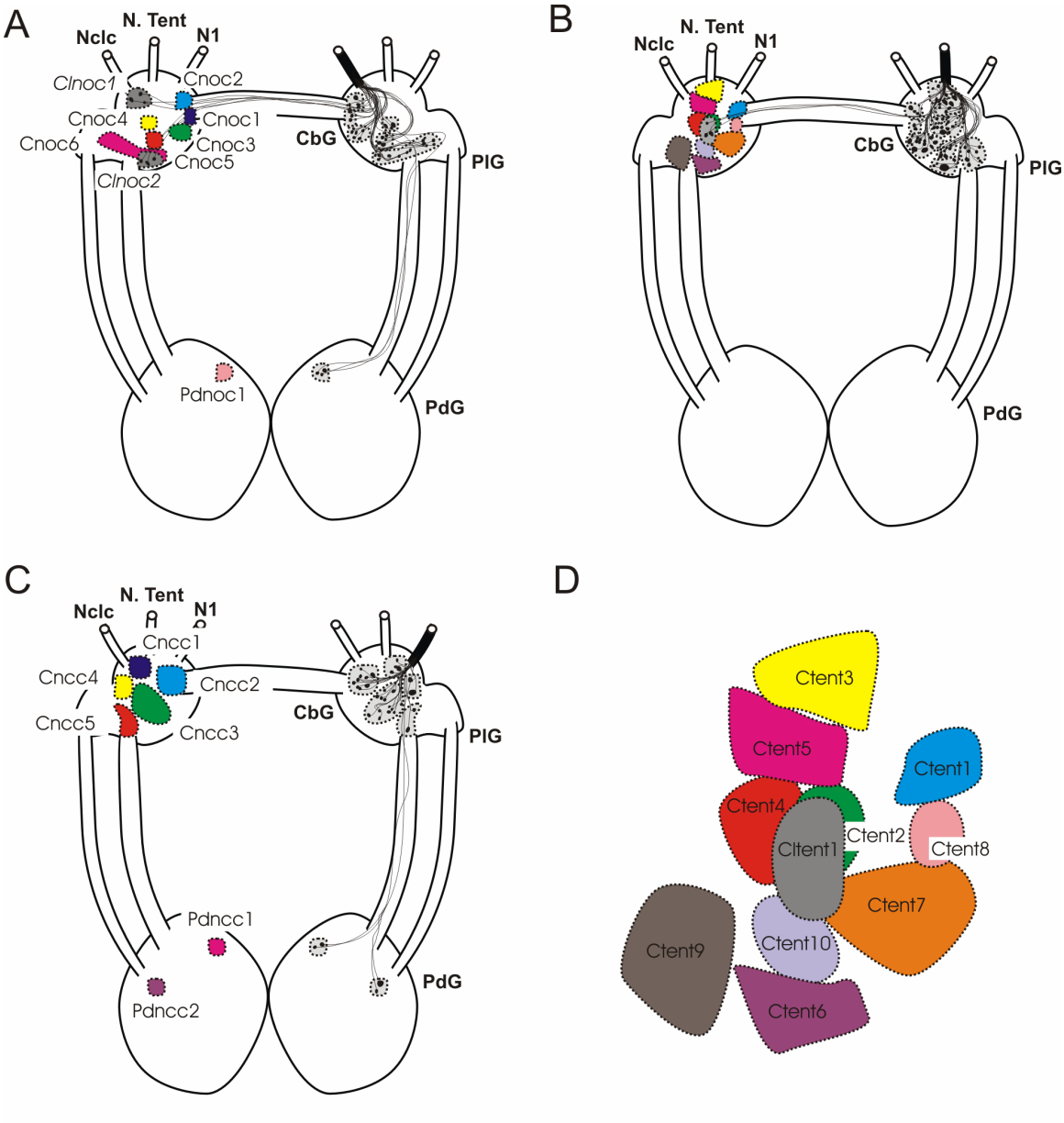


Figure 32: Schematic outline of somata and their axons projecting into the N1 (**A**), Nervus tentacularis **(B)**, and Nclc (**C**) of *Littorina littorea***.** As I found 11 clusters in the Innervation pattern for the Nervus tentacularis, the distribution and abbreviations of the clusters are shown enlarged (**D**). The size and position of the somata are digitalized from a camera lucida drawing, the distribution of the axons are averaged over all replicates. N1 - Nervus oralis, N. Tent - Nervus tentacularis, Nclc - Nervus clypei capitis, CbG - cerebral ganglia, PlG - pleural ganglia, PdG - pedal ganglia.

3.5.8 Distribution of clusters and single somata

The distribution of clusters and single somata over the investigated species is shown in table 3. The clusters for the Nervus tentacularis of *Littorina littorea*, which are highly outnumbered, cover the clusters described in the innervation patterns for the N2 and the N3 of the other investigated species. They are marked by an X and an orange color.

Overall, with exception of *Littorina littorea*, I found an extremely conserved distribution pattern for the cerebral clusters with no variation in the number of clusters across species. The position of the cerebral clusters show some variation, but less within the Opisthobranchia.

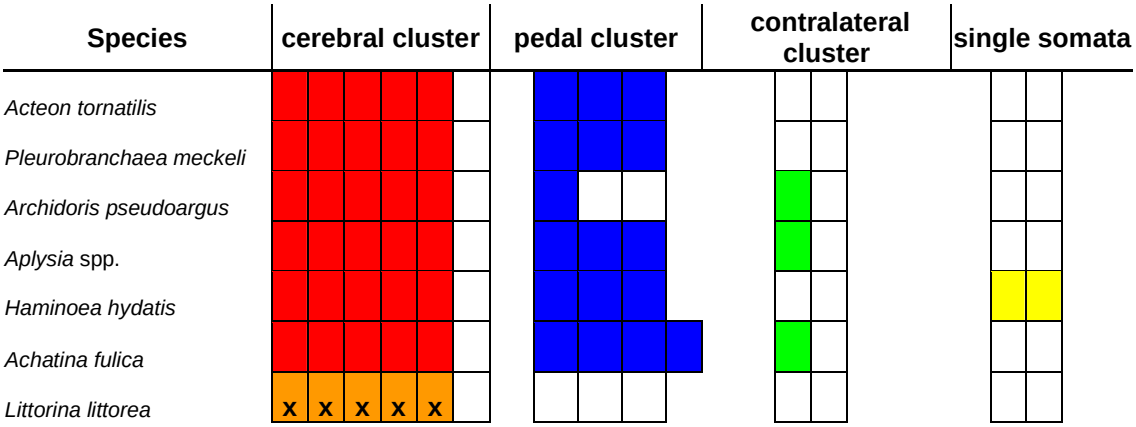
Most variation is found in the two Non-Opisthobranchia, *Achatina fulica* and *Littorina littorea*. The pedal clusters show a higher variation. In the distribution for contralateral clusters it is remarkable, that *Archidoris* and *Aplysia* share their pattern for the N2 and the N3. I found no conserved patterns for the occurrence of single somata.

Table 3: Distribution of clusters and somata

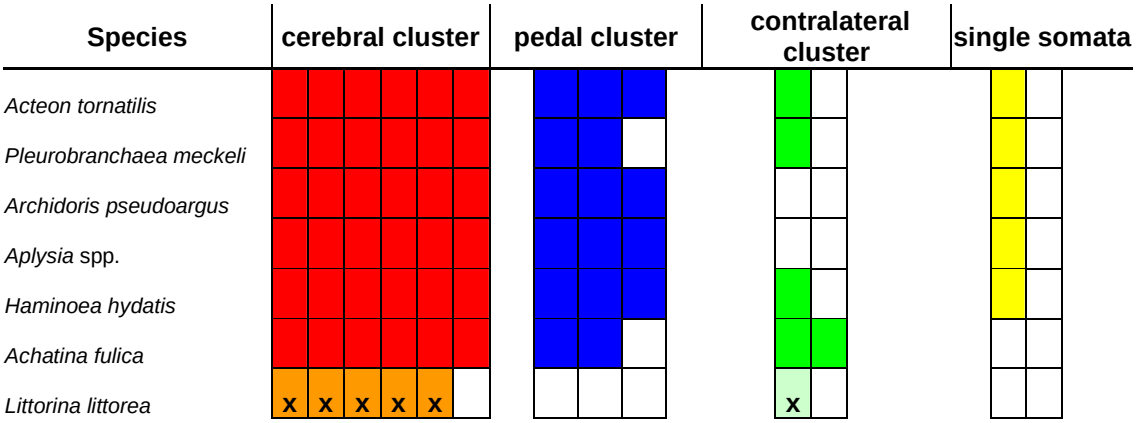
Distribution of cluster and somata for the N1/Nervus oralis

Species	cerebral cluster	pedal cluster	contralateral cluster	single somata
Acteon tornatilis				
Pleurobranchaea meckeli				
Archidoris pseudoargus				
Aplysia spp.				
Haminoea hydatis				
Achatina fulica				
Littorina littorea				

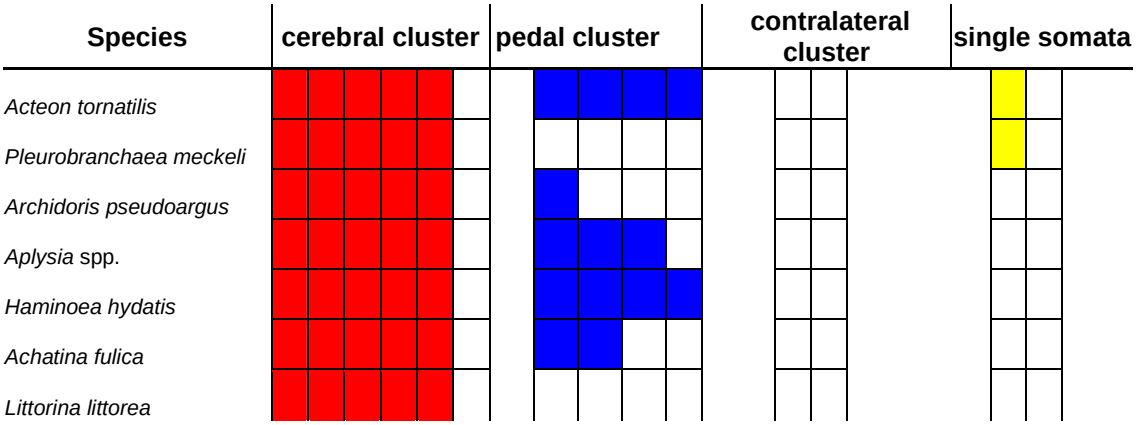
Distribution of cluster and somata for the N2/Nervus labialis



Distribution of cluster and somata for the N3/Nervus rhinophoralis



Distribution of cluster and somata for the N3/Nervus rhinophoralis



3.5.9 Discussion

Here I am going to examine the innervation patterns and cellular origins of the four cerebral nerves which innervate the CSOs in Opisthobranchia / Stylommatophora and the three nerves of the Caenogastropoda. I focused on the tracing patterns of *Acteon tornatilis* (Lower Heterobranchia / Acteonoidea), *Pleurobranchea meckeli* (Pleurobranchomorpha), *Archidoris pseudoargus* (Nudibranchia), *Aplysia punctata/californica* (Aplysiomorpha), *Haminoea hydatis* (Cephalaspidea), *Achatina fulica* (Stylommatophora) and *Littorina littorea* (Littorinimorpha/Caenogastropoda). The primary aim of this chapter is to compare tracing patterns between different orders of Heterobranchia (especially Opisthobranchia) and Caenogastropoda. Secondly I want to postulate preliminary homology hypothesis for the cerebral nerves. Furthermore, I can test if homology hypothesis for the cerebral nerves are consistent with current phylogenetic hypotheses. In molecular and morphological investigations the Nudibranchia and the Pleurobranchoidea are combined to the Nudipleura (Grande et. al 2004, Klussmann-Kolb et.al 2008) as a sister group to the Acteonoidea, and the Cephalaspidea are the sister group of the Aplysiomorpha. If cellular innervation patterns contain a phylogenetic signal this should be visible in the variation of the patterns between these groups.

In this chapter I demonstrate the constancy of nervous structures in the Opisthobranchia. Throughout my investigation of several taxa of Opisthobranchia I could identify uniform innervation/tracing patterns of the head region via four cerebral nerves which can be attributed to characteristic neuronal cell clusters in the CNS. Additionally I investigated the innervation pattern of the Stylommatophora *Achatina fulica* and the Caenogastropoda *Littorina littorea*. The innervation pattern of the Stylommatophora with its four cerebral nerves is congruent to the innervation patterns of the Opisthobranchia. All investigated Euthyneura (Opisthobranchia and Pulmonata) have four cerebral nerves which innervate the head region (optical nerve excluded), these four nerves can be homologised due to the almost identical innervation patterns. In *Littorina littorea* I found a clearly separation from the tracing patterns of the Opisthobranchia. Thus, I postulate a high conservation of tracing patterns within the Euthyneura (and especially the Opisthobranchia). A homologisation of the innervated CSOs can not be postulated upon these data, yet due to the highly conserved nervous structures.

In addition to the constant features of the innervation patterns (see also chapter 3.4), I also found variations in these patterns across the investigated taxa. The number of cells within the clusters and also the size of the somata in each cluster size vary. The position of the clusters in relation to each other and nervous structures seems to be the most useful character to compare the innervation patterns and to identify homologous clusters over different taxa. However, these features are not invariable. This might be caused by the fusion of ganglia and/or the strong variation between the morphology of the central nervous system. The lack of pedal clusters for *Pleurobranchea meckeli* could be explained by the unexpected large size of the CNS, especially the connectives between the cerebral and pedal ganglia.

The axonal tracing technique is very sensitive and a good condition of the investigated species is a presumption. Therefore, it has to be mentioned, that the investigated specimens of *Pleurobranchea meckeli* were not in best shape after collection by fishermen. Furthermore the tracing of pedal clusters predicts a long tracing path due to the extremely large CNS. These factors might cause methodological problems. Pedal clusters could exist but are not stained.
The variation of tracing patterns of the Caenogastropod *Littorina littorea* might be caused by the fact that *Littorina littorea* only has three cerebral nerves. The structure of the CNS of *Littorina littorea* is considerably different to the investigated Euthyneura and this might give us a hint, that neuronal structures (including cellular innervation patterns) at least in some respect reflect the phylogenetic history of Gastropoda.

In the following part of this chapter I am going to postulate a primary homology hypothesis for the cerebral nerves of the investigated species. In all investigated taxa of Euthyneura I found four cerebral nerves innervating the head region (optical nerve excluded). These four nerves innervate a great variety of CSOs, namely different kinds of tentacles, rhinophores and oral veils.

The N1 is dedicated to the lip region which is primarily a contactchemoreceptor. The N2 which has two branches within all Opisthobranchia is related to the category anterior sensory organ (ASO) (see also chapter 3.2 and 3.3). The two branches of the N2 innervate different areas of the ASOs predicted to serve different functions (Murray and Willows 1996): in consequence I distinguish between three types of CSOs: ASOa –

provided by the inner branch of the N2, ASOb – provided by the outer branch of the N2 and the N3, which is related to the posterior sensory organ (PSO) a sensory organ primarily used for olfaction (Chase 2002). The Nclc innervates structures of the head region which are strongly involved in locomotion like the bodywall or the cephalic shield, nevertheless these structures could also perform sensory functions.

Summing up, the conservative innervation patterns of the cerebral nerves of the Opisthobranchia allow me to homologise them. My data confirm the assumption that the posterior Hancocks organ in Cephalaspidea, the rhinophores in Nudipleura (Nudibranchia and Pleurobranchomorpha) and Aplysiomorpha and the ommatophores in the Stylommatophora are innervated by homologous nerves. The same is true for the oral veil (Pleurobranchomorpha), lip organ (Cephalaspidea, Acteonoidea), the labial tentacles of Aplysiomorpha and Nudibranchia and the anterior tentacles ("rhinophores") of the Stylommatophora.

My data do not confirm the hypothesis of a bifurcated N2 as an apomorphy of the Opisthobranchia (Salvini-Plawen and Steiner 1996), since the N2 in *Achatina fulica*, innervating the "rhinophores" and the anterior head region, is also bifurcated. This misinterpretation of nervous structures in *Achatina fulica* could be caused by the fact, that the anterior tentacles (ASO) of *Achatina fulica* have been termed as "rhinophores", a term which in Opisthobranchia is restricted to posterior sensory organs (PSO). Therefore, the undived N3 in *Achatina fulica*, which provides the posterior ommatophores, is confounded with the bifurcated N2 of the Opisthobranchia. Whether the bifurcation of the N2 is an autapomorphy of the Euthyneura (Opisthobranchia and Pulmonata) has to be evaluated by investigations of other Pulmonata and additionally more basal, Heterobranchia.

The innervation patterns of the Caenogastopoda *Littorina littorea* give some indication that the tentacular nerve of the Caenogastropoda might be homologous to the N2 and the N3 of the Euthyneura, since it comprises clusters found in both nerves of Euthyneura. This is also shown in Figure 33. Whether this nerve is fused in *Littorina* or separated in the Euthyneura cannot be conclusively decided here.

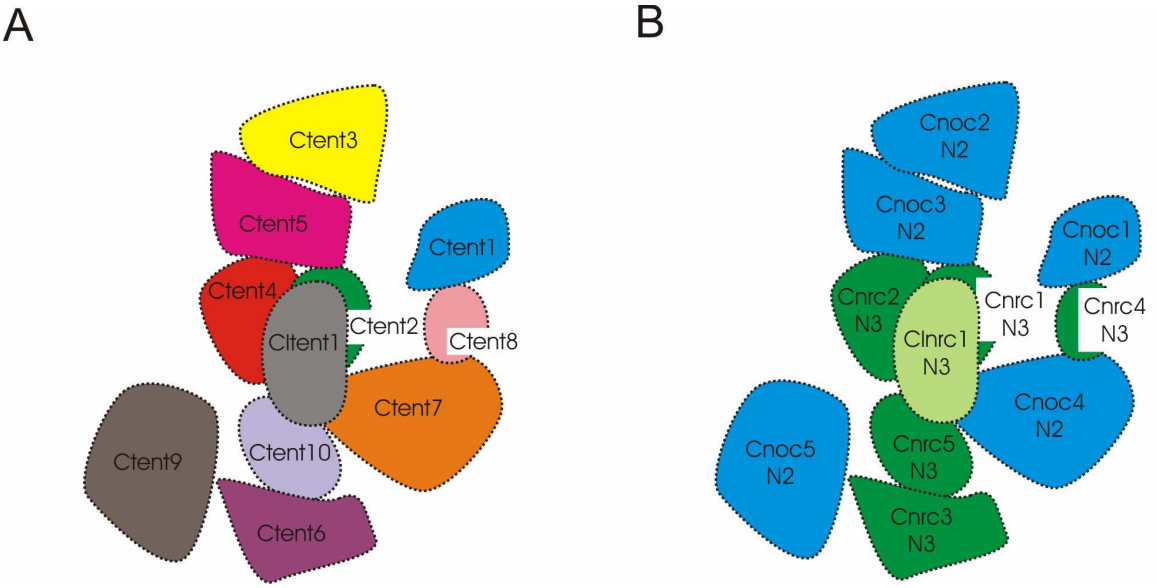


Figure 33: **A:** Positions of the cerebral clusters for the Nervus tentacularis innervation pattern of Littorina littorea. **B:** Correlation to cerebral clusters in innervation patterns for the N2 and N3 of the investigated heterobranch taxa **.**

In conclusion, I postulate the axonal tracing technique primary as a method for the homologisation of nerves between relatively close related species. The axonal tracing technique gives us a morphological character complex to homologise cerebral clusters, and in consequence to describe and identify neuronal structures. This character complex has a higher complexity than ganglionic structures, therefore innervation patterns are more suitable to distinguish between homologous and analogous nerves. This study also confirms investigations (Newcomb et al. 2006, Kutsch and Breitbach 1994) who also postulated, that internal nervous structures are highly conserved during evolution.

Nevertheless the axonal tracing method has its limitations. The first limitation is the size of the species, the species has to be larger than 0.5 cm, as the CNS has to be dissected without damage. The second point is the number of species. 5 to 10 replicates for each nerve plus failure by damaged nerves are needed. This can be a problem as many Opisthobranchia only occur in very small and separated populations. And third, the animals have to be captured alive, which is especially in marine organism's not ever quiet simple as such species are very sensitive to changes in pressure, temperature and salinity.

3.6 Immunohistochemistry of CSOs

In this chapter I will describe the comparative immunohistochemistry for the CSOs of *Pleurobranchaea meckeli*, *Petalifera petalifera* and *Littorina littorea*. This is in addition to the diploma thesis of Simone Faller, who investigated the neurotransmitter contents of the CSOs of *Acteon tornatilis*, *Aplysia punctata*, *Haminoea hydatis* and *Archidoris pseudoargus*. A manuscript titled “Comparative immunohistochemistry of the cephalic sensory organs in Opisthobranchia (Mollusca, Gastropoda)” by Faller et al. is in review at Zoomorphology and will be attached in the Supplement Data.

3.6.1 Tyrosine hydroxilase (TH) – like immunoreactivity

TH-like immunoreactivity (lir) was found in all investigated CSOs of the three investigated species, in accordance to the investigations of Simone Faller. The dominant TH-like immunoreactive structures were bipolar cell somata which had diameters of 5.5 -7 μm and were located subepidermally. These somata possess dendrites which penetrate the epidermis (Fig. 33). The distributions and also the amount of these somata varied within the different CSOs especially between the anterior sensory organs and the posterior sensory organs (Fig. 33A-G).

In *Pleurobranchaea meckeli* TH-lir somata were found in the oral veil (Fig. 33A) and also in the folded rhinophores (Fig. 33B) with a higher concentration in the anterior region, the oral veil, which is also comparable to the results of Simone Faller. In the oral lobe of *Petalifera petalifera* (Fig. 33C) I found the highest number of TH-like immunoreactive somata within this species, meanwhile in the oral tentacles (Fig. 33D) less somata were found and the rhinophores (Fig. 33E) showed the least amount of TH-lir somata. From my observations and the results from Faller et al. (in review) I am able to describe a basic pattern within the Opisthobranchia of a decreasing number of TH containing somata from the anterior to the posterior CSOs.

In *Littorina littorea* I found a completely different pattern of TH-like immunoreactivity. Here I found the highest number of TH-like immunoreactivity in all species in the anterior head region (Fig. 33F) and only few less in the tentacles (Fig. 33G). The decrease of TH containing somata, from the anterior to the posterior CSOs is less in *Littorina littorea* when compared to the Opisthobranchia.

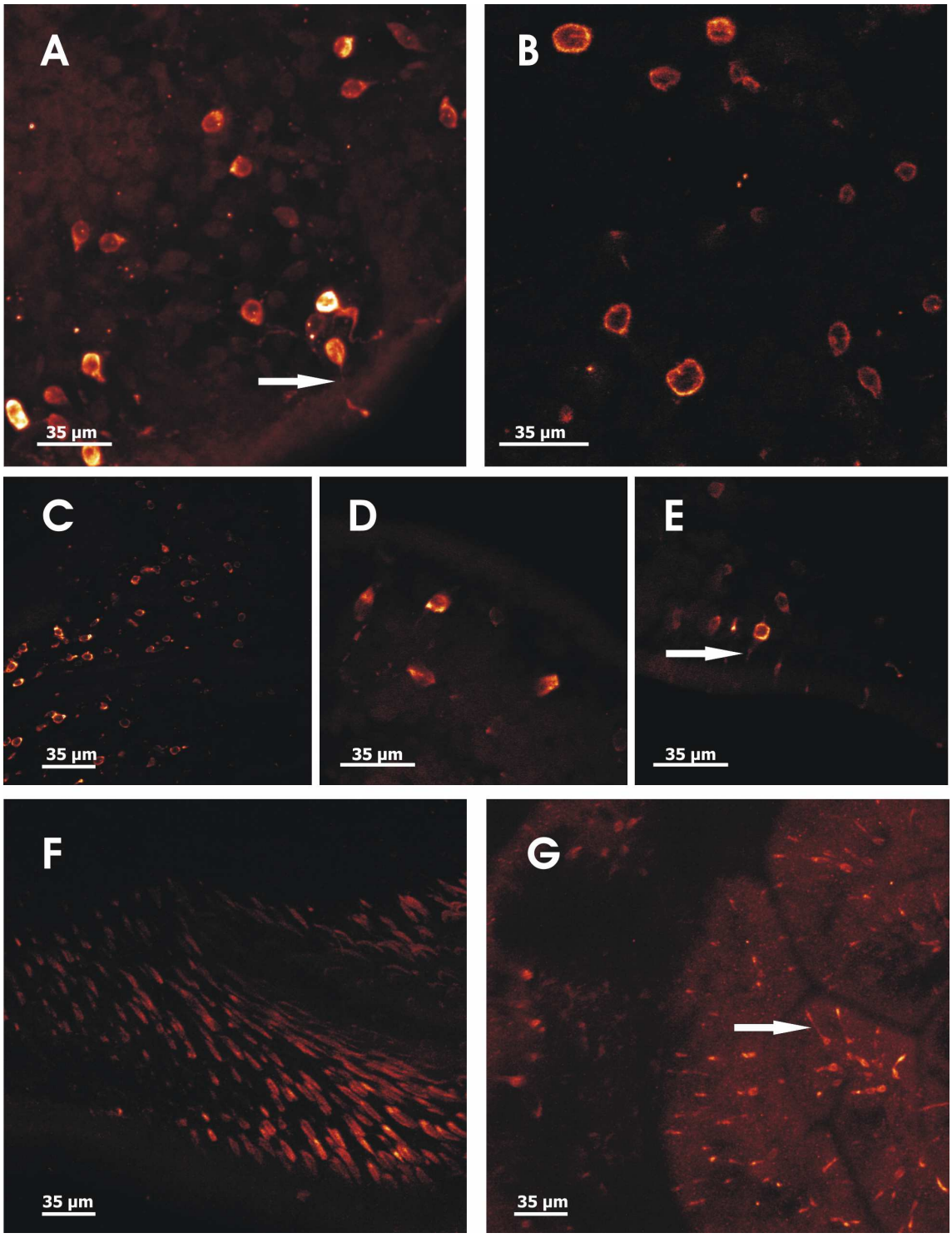


Figure 33: Confocal micrographies of Tyrosine hydroxylase (TH)-like immunoreactivity in the cephalic sensory organs. Denrites are marked with a white arrow. **A:** *Pleurobranchaea meckeli* / oral veil; **B:** *Pleuronbranchaea meckeli* / rhinophores; **C:** *Petalifera petalifera* / oral lobes; **D:** *Petalifera petalifera* / labial tentacles; **E:** *Petalifera petalifera* / rhinophores; **F:** *Littorina littorea* / anterior head region; **G:** *Littorina littorea* / tentacles.

3.6.2 FMRFamide-like immunoreactivity

FMRFamide-like immunoreactivity (lir) was detected in all investigated CSOs of all studied species in diverse structures like nerves, peripheral ganglia, glomerulus-like structures, somata and fibres (Fig. 34A). The dominant peripheral structures which showed FMRFamide-like immunoreactivity were patches of tightly knotted fibres located along the major nerve branches (Fig. 34B, C). The distribution and density of these patches varied within the CSOs of each species as well as between species.

In *Pleurobranchaea meckeli,* such patches, also called glomeruli, could be found in low densities along the major nerve branches of the N2, which provides the oral veil and the labial tentacle, the latter being located at the lateral tip of the oral veil. Moreover, I found glomeruli-like structures along the N3 nerve, which provides the folded rhinophores (Fig. 34B). In *Petalifera petalifera* also both pairs of CSOs, the ASOs with the oral lobes (ASOa) and the labial tentacles (ASOb) and the PSOs as well contained many such glomeruli-like structures (Fig. 34C), with a concentration along the N3 which innervates the spoon like rhinophores. Here the concentration is located within the grooved region at the top of the rhinophores. In contrast, *Littorina littorea* possessed no such tightly knotted fibres in the tentacles or the anterior head region.

In addition to the patches of tightly knotted fibres or glomeruli-like structures, the investigated opisthobranch taxa contained FMRFamide-lir in their cerebral nerves (Fig. 33A-C), FMRFamide-lir could not be detected in the cerebral nerves of *Littorina littorea*. Moreover, the investigated opisthobranch species possessed FMRFamide-lir in peripheral somata. These somata were generally bipolar, located subepidermally and their dendrites penetrated the epidermis. These somata had diameters of 7.5 μm and were distributed in low densities over the whole ASOs and PSOs. They are missing in *Littorina littorea*. Instead, I found relatively large subepidermal somata (up to 12 μm) in the tentacles of *Littorina littorea* with extremely long dendrites (up to 40 μm) penetrating the epidermis (Fig. 34D). Finally, all investigated CSOs of the opisthobranch taxa possessed FMRFamide-lir in a network of subepidermal fibres (Fig. 34A). This network was also absent in *Littorina littorea.*

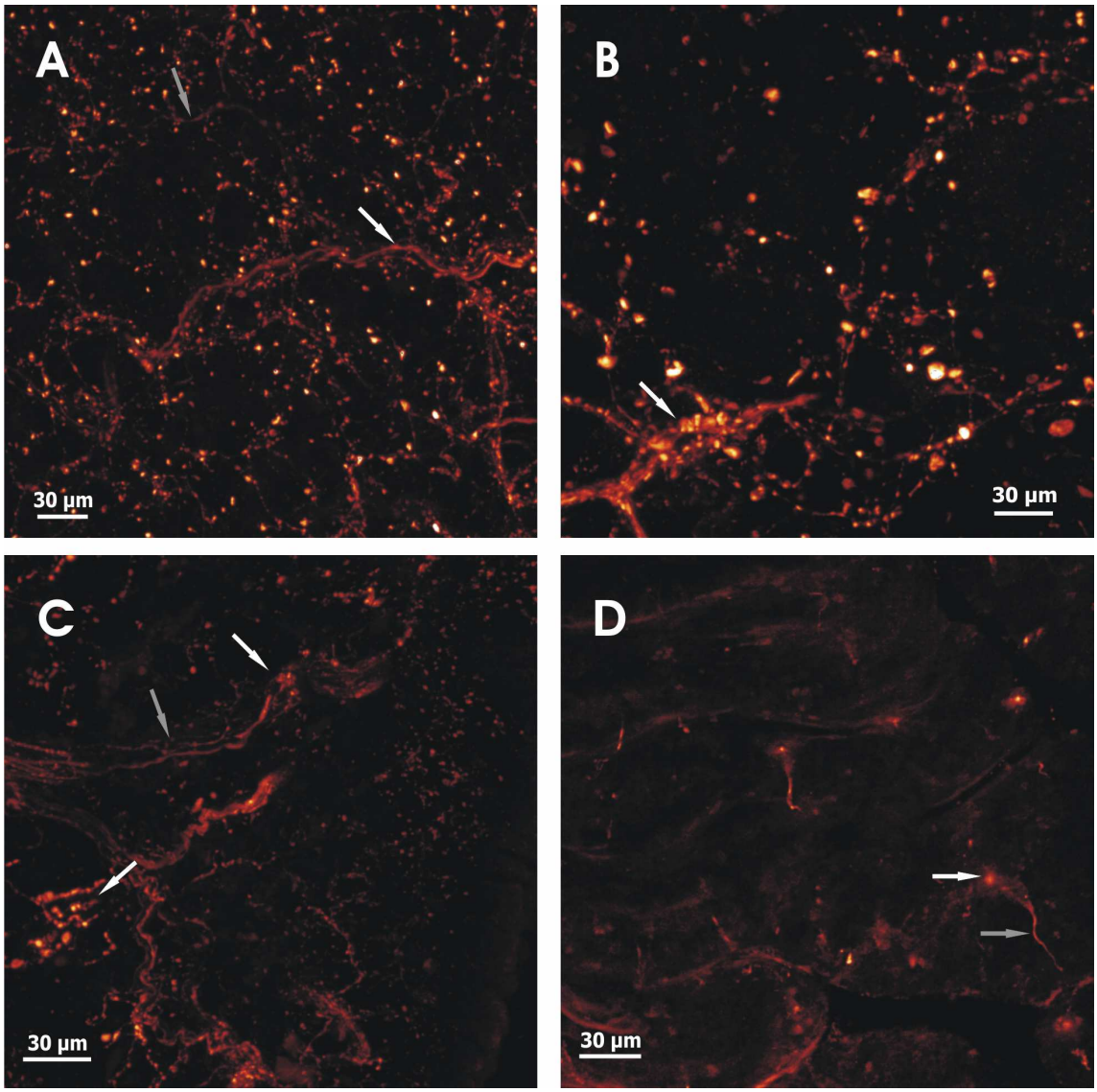


Figure 34: Confocal micrographies of FMRFamide-like immunoreactivity in the cephalic sensory organs. **A:** *Pleurobranchaea meckeli* / oral veil, white arrow cerebral Nerve N2, grey arrow nervous fibres; **B:** *Pleurobranchaea meckeli* / rhinophores, white arrow glomerulus-like structure of knotted fibres; **C:** *Petalifera petalifera* / oral lobes, white arrow - glomeruli-like structures, grey arrow - cerebral nerve; **D:** *Littorina littorea* / tentacles, white arrow large somata, grey arrow dentrite;

3.6.3 Serotonin-like immunoreactivity

Serotonin-lir was predominately detected in a network of subepidermal nerve fibres in all

investigated CSOs of all species. These fibres did not penetrate the epidermis. The density changed minimally between the anterior and posterior CSOs with a minimal higher amount in the ASOs of the Opisthobranchia (Fig. 35A). The oral lobes of *Petalifera petalifera* in particular have a very dense 5HT subepidermal network. The anterior head region including the tentacles of *Littorina littorea* also possessed this subepidermal network, with a comparable density like the one observed in the posterior CSOs of the opisthobranch taxa (Fig. 35B). Additionally, *Littorina littorea* also possessed a network located more deeply in the tissue, comprised of very strong fibres (Fig. 35C). This second network was distributed over the whole anterior head region and the tentacles and could not be found within the other investigated taxa and is unique in *Littorina littorea*. No serotonin-like immunoreactive somata were found within any of the investigated CSOs.

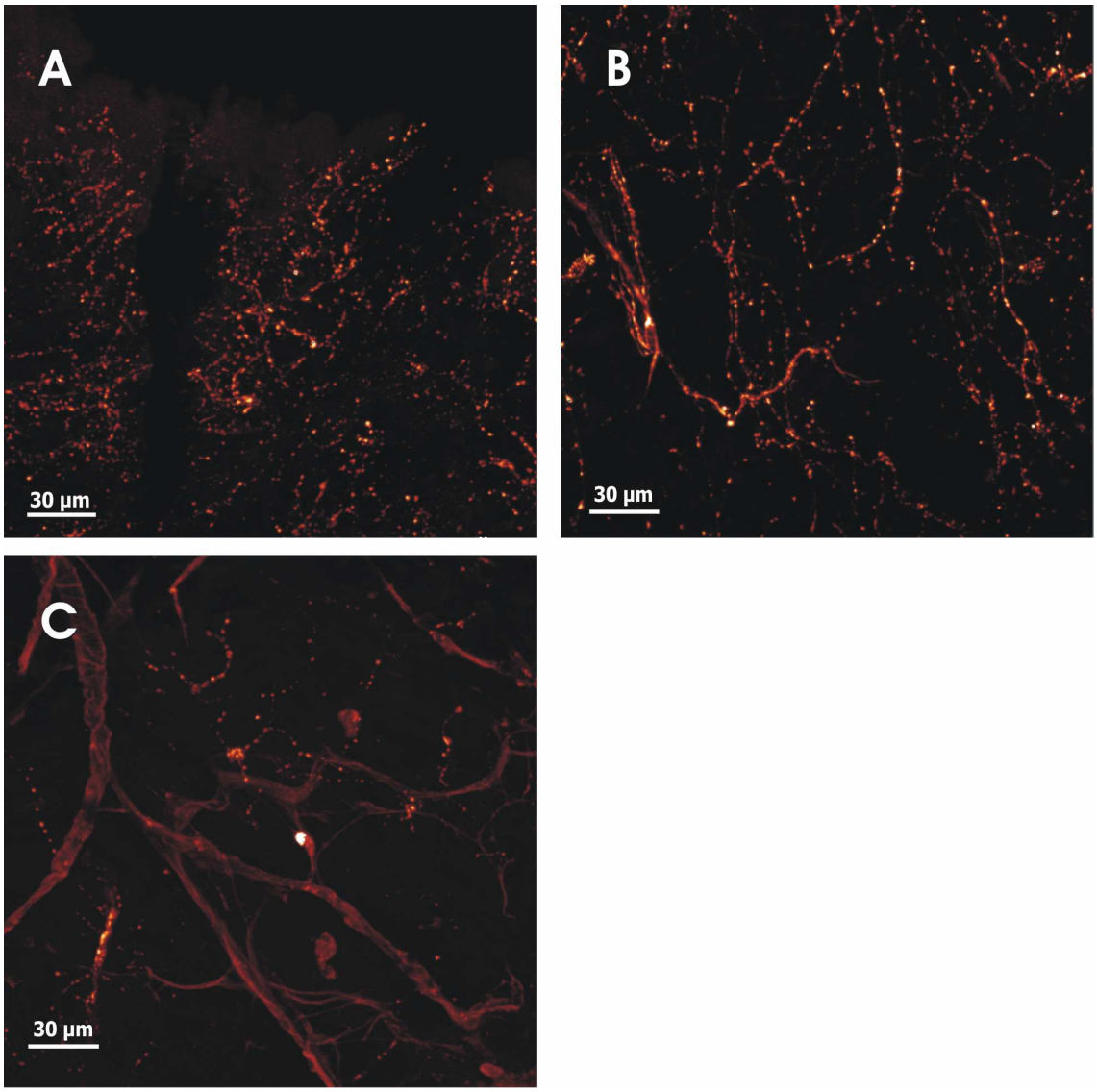


Figure 35: Confocal micrographies of Serotonin (5HT)-like immunoreactivity in the cephalic sensory organs. **A:** *Petalifera petalifera* / oral lobes; **B:** *Littorina littorea* / anterior head region; **C:** *Littorina littorea* / anterior head region.

3.6.4 Discussion

The distribution of TH-lir was very similar within the CSOs of the four taxa investigated by Simone Faller and the two additionally investigated opisthobranch taxa *Pleurobranchaea meckeli* and *Petalifera petalifera* described in the current study. All investigated CSOs possessed subepidermal bipolar TH-like immunoreactive somata. These somata possessed dendrites that penetrated the epidermis and were much more abundant in the ASOs (e.g., the oral tentacles) than in the posterior PSOs (e.g., the rhinophores).

These findings are consistent with those of Croll (2001) in *Aplysia californica* and Croll et al. (2003) in *Phestilla sibogae*. Both *Aplysia californica* and *Phestilla sibogae* possess subepidermal TH-like immunoreactive somata, especially in the anterior CSOs and the dendrites of these cells penetrate the epidermis. These cells are therefore thought to function in contact chemoreception or mechanoreception (Fiedler and Schipp 1991, Croll 2001, Croll et al. 2003). The fact that in *Littorina littorea* reveals a high density of these cells over the whole head reagion, including the tentacles, leads to the conclusion that the anterior head region and the tentacles have a similar function in *Littorina littorea* and that a specialisation in anterior and posterior CSOs is missing.

This can also be seen in the morphology as *Littorina littorea* only has one pair of tentacles and no additionally specialized sensory structures in the anterior head region. In comparison all other investigated taxa possess two pairs of sensory structures/CSOs.
The additional type of TH-like immunoreactive somata found by Simone Faller in *Acteon tornatilis* could not be detected in any of the other investigated taxa. In addition to the TH-like immunoreactive somata, TH-lir was detected in fibres of the nerves innervating the different CSOs. These fibres are presumably the centrally projecting axons of the sensory somata. However, to clearly identify the role of these TH-like immunoreactive somata further studies, especially electrophysiological investigations, are needed.

The distribution of FMRFamide in the peripheral nervous system of opisthobranchs has been investigated by Croll et al. (2003) in the CSOs of *Phestilla sibogae* and by Wollesen et al. (2007a) for *Aplysia californica*. In the investigated opisthobranch taxa of Faller et al. (*in revision*) and the two additional opisthobranch taxa *Pleurobranchaea*

meckeli and *Petalifera petalifera* the dominant features of FMRFamide-lir were patches of tightly knotted fibres. These patches possibly correspond to glomerulus-like structures (Boudko et al. 1999, Croll et al. 2003). Glomeruli have recently been reported in the rhinophores of *Aplysia punctata* (Wertz et al. 2006) and in sensory areas of *Aplysia californica* (Moroz 2006) and are also well-known in the tentacles of land snails (Chase and Tolloczko 1986) as well as in other invertebrates (Kleineidam et al. 2005) and vertebrates (Wachowiak et al. 2004, Chen and Shepherd 2005). Glomeruli are generally considered to be involved in processing of olfactory stimuli. The glomerulus-like structures observed by Faller et al. (*in revision*) and by myself were concentrated in the posterior cephalic sensory organs of the investigated opisthobranch taxa, especially in the rhinophores of *Aplysia punctata, Pleurobranchaea meckeli* and *Petalifera petalifera,* and the Hancocks organ of *Haminoea hydatis.*

This suggests an olfactory role for these posteriorly located sensory organs (PSOs). The rhinophores of *Aplysia punctata* and the Hancock's organ of *Haminoea hydatis* have already been proposed to be involved in chemoreception by Audesirk (1975) and Edlinger (1980). While the rhinophores of *Aplysia punctata* contained numerous glomeruli, the rhinophores of *Archidoris pseudoargus* are lacking glomeruli.

Faller et al. (*in revision*) concluded, that the lack of glomeruli in the rhinophores of *Archidoris pseudoargus* is caused by the fact, that they are not primarily olfactory organs but rather sense other modalities, e.g., detection of water currents. The involvement of the rhinophores of *Archidoris pseudoargus* in rheotaxis has been described by Wolter (1967). Faller et al. (*in revision*) also concluded that the function of the glomeruli was adopted by the large rhinophoral ganglion in *Archidoris pseudoargus*. In this context it is interesting that *Littorina littorea* is also lacking glomeruli. Here I found an unspecific distribution of very large somata with extremely long dendrites penetrating the epidermis, therefore these cells are probably also sensory cells, as suggested for the TH-like immunoreactive cells. The tentacle of *Littorina littorea* also possesses a large tentacle ganglion which additionally provides the eye via a very small nerve. Therefore I come to the conclusion that the function which is performed by glomeruli in other species is processed in this ganglion in *Littorina littorea* and that the tentacle of *Littorina littorea* is not a highly specialized sensory organ primarily restricted to olfaction like the most PSOs of the investigated opisthobranch taxa.

Unlike the distribution of tyrosine hydroxylase and FMRFamides, the distribution of serotonin has already been studied in detail in the peripheral nervous systems of various Opisthobranchia (Moroz et al. 1997, Croll et al. 2003, Wertz et al. 2006, Wertz et al. 2007, Faller et al. *in revision*). In confirmation of the findings of Faller et al. (*in revision*), no peripheral serotonin-like immunoreactive somata were found in any of the investigated CSOs and serotonin was found primarily in subepidermal nerve fibres not penetrating the epidermis. Therefore these fibres appear to be efferent. These findings are consistent with the observation of only efferent fibres in the CSOs of *Aplysia californica* (Wollesen et al. 2007a,b), *Aplysia punctata* (Wertz et al. 2006), *Archidoris pseudoargus* (Wertz et al. 2007), *Phestilla sibogae* (Croll et al. 2003), *Pleurobranchaea californica* and *Tritonia diomedea* (Moroz et al. 1997).

Serotonin-lir was found in the same patches of entangled fibres and peripheral ganglia as FMRFamide. These results agree with Moroz et al. (1997), who suggested that serotonin might play a role in the peripheral modulation of sensory inputs to the CNS. If these entangled fibres indeed correspond to glomeruli-like structures, serotonin might play a role in the efferent control of olfactory inputs. In *Littorina littorea* an additional network of very prominent fibres which might be related to the very large FMRFamide containing bipolar cells was found. Both, the somata of these cells and the secondary network of 5HT containing fibres are located underneath the primary 5HT fibre network. This is also an indication, that the large FRMFamide sensory cells, together with the tentacle ganglion, and the primary and secondary 5HT networks have a similar function to the glomeruli found in the opisthobranch taxa, and that the tentacles of *Littorina littorea* are less specified sensory organs. The CSOs in Opisthobranchia seem to be more sophisticated organs with more specific functions.

In general I follow the conclusions of Faller et al. (*in revision*), that the distribution of sensory structures shows characteristic patterns for different CSOs. In congruence with Faller et al. (*in revision*) I can distinguish between characteristic structures such as bipolar sensory neurons and glomerulus-like structures which can be attributed to different functions like mechanoreception, contact chemoreception and olfaction. The distribution of these structures within the CSOs leads us to the conclusion that the different types of CSOs have different functions, and the neurotransmitter content is

related to the function of the CSOs. The additionally investigated opisthobranch species confirm the conclusions of Faller et al. (*in revision*) that the anterior CSOs (ASOs), i.e. the oral tentacles, the oral veil, the oral lobes, the lip organ and the anterior cephalic shield, comprise numerous bipolar TH containing sensory neurons which are probably involved in contact chemoreception and mechanoreception. Thus the ASOs may play a role in these modalities. Another point which supports a function in contact chemoreception and mechanoreception is that the ASOs are situated close to the substrate.

The posterior CSOs (PSOs), i.e. the rhinophores and the Hancocks organ, generally contain many glomerulus-like structures. Therefore these organs probably primarily fulfill an olfactory function, which is also supported by their posterior location on the head region.

3.7 General homology hypotheses for the cephalic sensory organs

The aim of this PhD study was to reconstruct the evolution of the CSOs within the Opisthobranchia, therefore it was essential to homologise the extreme variable CSOs. This was done by homologising the cerebral nerves via axonal tracing and other approaches, like immunohistochemistry and ontogenetic studies, which have been mentioned earlier. In an earlier chapter (3.5) I have discussed primary homology hypotheses for the cerebral nerves innervating the CSOs. This following chapter will serve to discuss the homology hypotheses for the CSOs themselves. The deduction of a homology hypothesis for the CSOs is based first on my own data (cellular innervation patterns, neuroanatomy and immunohistochemistry) and secondly on data which where produced by several diploma students.

Namely Katrin Göbbeler with investigations on the ultrastructure of CSOs within the Opisthobranchia, Simone Faller, who investigated the neurotransmitter content of the CSOs of several opisthobranch taxa, Tim Wollesen who investigated the ontogeny of *Aplysia californica* (Aplysiomorpha), Alen Kristof studying the ontogeny of *Aeolidiella stephaniae* (Nudibranchia) and Corinna Schulze who studied the ontogeny of *Haminoea japonica* (Cephalaspidea). These additional data will be discussed in context of the homology hypothesis of the CSOs. The data of Tim Wollesen and Katrin Göbbeler have already been published (Göbbeler and Klussmann-Kolb 2007, Wollesen et al. 2007a,b), whereas the data of Alen Kristof and Corinna Schulze are yet unpublished, however manuscripts are in preparation. The data of Simone Faller are currently und review in Zoomorphology (Faller et al. *in revision*).

3.7.1 The CSOs of the Acteonoidea (*Acteon tornatilis*)

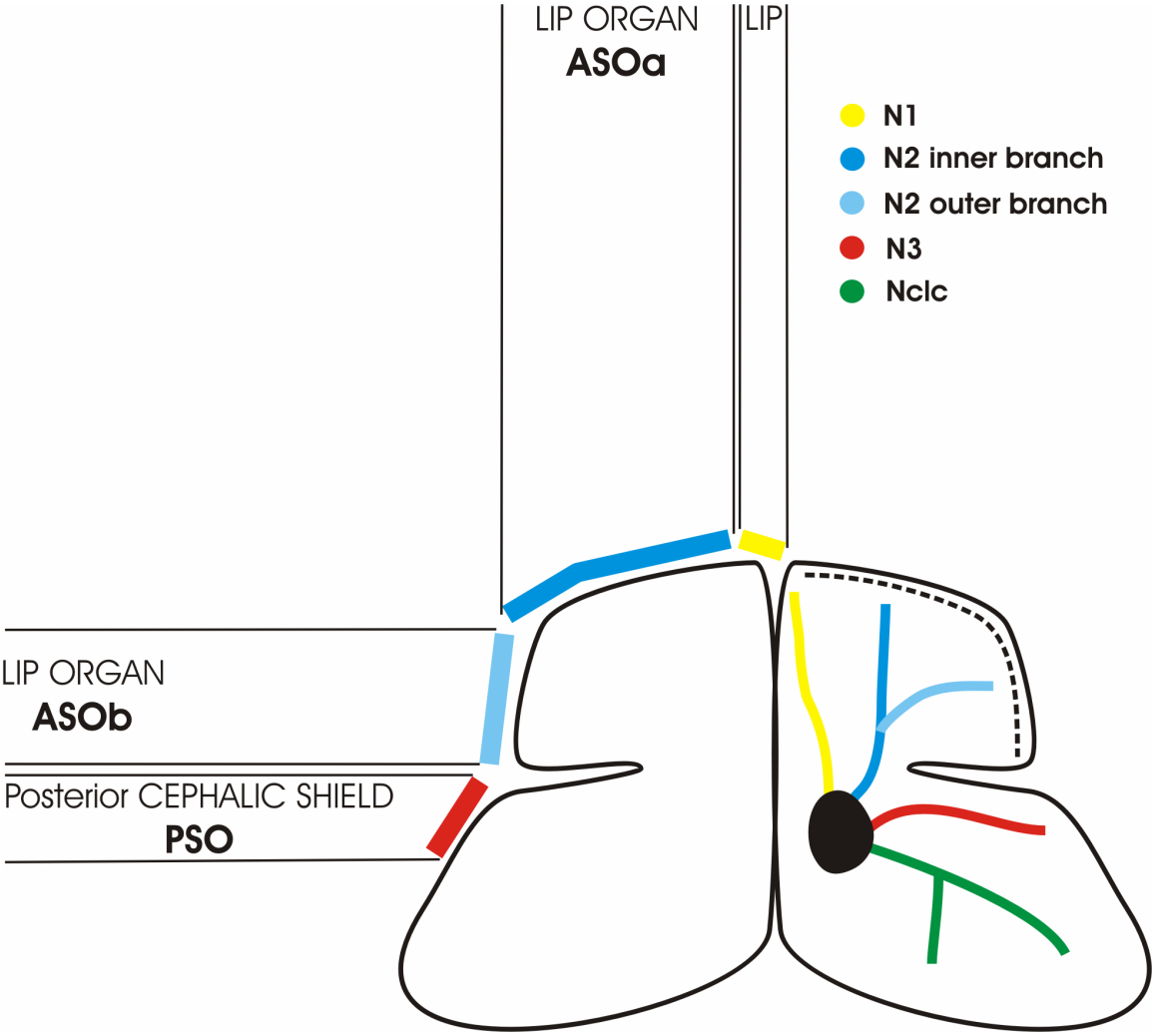


Figure 36: Categories of CSOs for *Acteon tornatilis*. On the right hemisphere the gross morphology of the four cerebral nerves providing the CSOs is shown. On the left hemisphere the categories for the CSOs are shown. The cerebral nerves and their respective CSO categories are marked by the same colour.

The discussion of the CSOs of *Acteon tornatilis* (Fig. 36) is rather extended as I found serious discrepancies to earlier descriptions. Parts of this chapter have been published in a modified form with the title "The cephalic sensory organs of *Acteon tornatilis* (Linnaeus, 1758) (Gastropoda Opisthobranchia) – cellular innervation patterns as a tool for homologisation" in the Bonner Zoologischer Anzeiger (Staubach et al. 2007, see also Supplement Data). *Acteon tornatilis* belongs to the subgroup Acteonoidea, formerly ascribed to the basal Cephalaspidea (Odhner 1939, Burn and Thompson 1998). However, recent investigations have either excluded the Acteonoidea from the

Opisthobranchia (Mikkelsen 1996) or proposed a sister group relationship of Acteonoidea and the highly derived Nudipleura (Vonnemann et al. 2005, Klussmann-Kolb et al. 2008) thus, rendering the phylogenetic position of Acteonoidea within Opisthobranchia unsettled. Acteonoidea are characterised by the presence of a prominent cephalic shield. This structure is also present in Cephalaspidea and has been considered to be an apomorphy of the Cephalaspidea (including Acteonoidea) (Schmekel 1985). However, the structure of the cephalic shields differs considerably in Cephalaspidea and Acteonoidea with the latter possessing two distinct hemispheres while the cephalic shield in the Cephalaspidea possesses a uniform structure. Therefore, common origin of both types of cephalic shields and thus homology is questionable. Further CSOs have been described in Acteonoidea and Cephalaspidea such as lip organ and Hancocks organ (Rudman 1971a,b, Rudman 1972a,b,c, Edlinger 1980). Since the presence of these organs in members of the genus *Acteon* has been disputed by different authors (Edlinger 1980, Schmekel 1985), absolute clarification is certainly necessary. Throughout my investigation of several individuals of the acteonid *Acteon tornatilis* I found uniform innervation patterns of the head region via four cerebral nerves, which can be attributed to characteristic neuronal cell clusters in the CNS. These cellular innervation patterns in *Acteon tornatilis* show an extremely high congruence with the cellular innervation patterns of *Haminoea hydatis.* In the N1, the number of cerebral clusters as well as the position of these clusters to each other is the same in *Acteon tornatilis* and *Haminoea hydatis*. However, I found some differences in the size and number of somata when comparing both species. Additionally, I could not detect a pleural, a parietal and a pedal cluster in *Acteon tornatilis,* which were described for *Haminoea hydatis* in chapter 3.4. This may be due to the differences in the peripheral innervation area of the N1. In *Acteon tornatilis* the N1 only provides the lip and very small parts of the median cephalic shield whereas in *Haminoea hydatis,* it innervates the lip and large parts of the anterior cephalic shield. For the second nerve, the N2 (Nervus labialis), I nearly found no differences between the presence and distributions of the cell clusters for both species.

The only ostentatious difference was the lack of a single pedal soma and its contralateral analogue in *Acteon tornatilis*. In the Nclc (Nervus clypei capitis), the difference between the two species was also reduced to the presence of a single cerebral soma in *Acteon tornatilis*. In contrast to the three nerves described above, I found a prominent difference in the structure of the N3 when comparing *Acteon* and *Haminoea*. On the one

hand, in *Haminoea hydatis* the N3 terminates in a rhinophoral ganglion. Such a ganglion is missing in *Acteon tornatilis*. Hence, I expected considerable differences in the cellular innervation patterns for the N3 of these species. But I only found very small differences. This implies that basic innervation patterns of the N3 are probably the same in both species. Additional functions of the N3 processed in the rhinophoral ganglion can be proposed for *Haminoea hydatis*. These functions are probably related to the Hancocks organ, which is innervated by nerves originating in the rhinophoral ganglion. I was unable to locate such a Hancocks organ in *Acteon tornatilis* in contrast to earlier descriptions (Edlinger 1980). My data cannot support Edlinger´s (1980) description of independent nerves for the lip organ (N1 after Edlinger 1980) and the anterior Hancocks organ (N2 after Edlinger 1980).

Considering the homologisation of the cerebral nerves in light of their neurological origin, neuroanatomics and nervous innervation patterns, I postulate hypotheses of homologies respective of the organs innervated by these nerves. Thus, I consider the lip of *Acteon tornatilis* to be homologous to the lip of Cephalaspideans (Huber 1993) since both organs are innervated by the N1. The same is true for the small median parts of the cephalic shield in *Acteon* and the anterior cephalic shield of *Haminoea hydatis.* I could not find a lip organ in *Acteon tornatilis* as described by Edlinger (1980), but I detected a groove at the ventral side of the anterior cephalic shield. This groove is innervated by the N2 as is the lip organ of Cephalaspidea (Huber 1993). Therefore, I postulate this groove in *Acteon tornatilis* to be homologous to the lip organ of *Haminoea hydatis*. This hypothesis is also supported by data on immunoreactivity against several neurotransmitters (Faller et al. *in revision*). In the groove of *Acteon tornatilis* as well as in the lip organ of *Haminoea hydatis,* characteristic sub-epidermal sensory neurons containing catecholamines could be found in high density indicating that both organs are involved in contact chemoreception.

The N2 of *Haminoea hydatis* is divided into two branches which are described as two single nerves by Edlinger (1980). The first or inner branch provides the lip organ of *Haminoea hydatis* as described earlier. The second, outer branch is related to the anterior Hancocks organ of earlier investigations (Edlinger 1980; Huber 1993) and the posterior lip organ (ASOb) in my definition. In *Acteon* I also found two branches of the N2: the inner one providing the largest part of the groove whereas the outer branch is

restricted to a small region between the anterior and posterior lobe of the cephalic shield. Therefore, this latter region may be homologous to the posterior lip organ of *Haminoea hydatis* and not to the posterior Hancocks organ as described by Edlinger (1980). The N3 of *Acteon tornatilis* provides a large part of the posterior cephalic shield but no identifiable posterior Hancocks organ. Additional immunohistochemical and ultrastructural investigations were also unable to detect a posterior Hancocks organ in *Acteon tornatilis* (Faller et al. *in revision*, Göbbeler and Klussmann-Kolb 2007). The posterior parts of the cephalic shields in *Acteon* and *Haminoea* are probably equally homologous as both where innervated by the Nclc.

The lack of a posterior Hancocks organ in *Acteon tornatilis* might be due to three different reasons:

1. The ancestor of *Acteon tornatilis* never had a posterior Hancocks organ;

2. The posterior cephalic shield of *Acteon tornatilis* may be a homologous structure to the Hancocks organ of *Haminoea hydatis;*

3. The posterior Hancocks organ has secondarily been reduced in *Acteon tornatilis*.

The first hypothesis is rather implausible since I found a distinct N3 with conserved cellular innervation patterns in the central nervous system. If the ancestor of *Acteon tornatilis* never had a posterior Hancocks organ, this nerve and associated neural structures should be lacking. Moreover, a Hancocks organ has been described for other Acteonoidea (Rudman 1971a,b, Rudman 1972a,b,c).

If I consider the second explanation for lack of a posterior Hancocks organ in *Acteon tornatilis,* I imply that the posterior cephalic shield in this species, innervated by the N3, represents a sensory organ like the Hancocks organ in the Cephalaspidea. However, immunohistochemical and ultrastructural investigations of the respective epithelia in *Acteon tornatilis* do not indicate a sensory function at all (Faller et al. *in revision*, Göbbeler and Klussmann-Kolb 2007). I reject this hypothesis of homology of the posterior cephalic shield in *A*cteon *tornatilis* and Hancocks organ in *Haminoea hydatis* since I found no evidence for a function of the posterior cephalic shield as an olfactory

sensory organ. Moreover, the posterior cephalic shield is mostly innervated by the Nclc and not by the N3. The third hypothesis regarding the reduction of a Hancocks organ seems to be the most plausible when the habitat and the food sources of *Acteon tornatilis* in comparison to *Haminoea hydatis* are considered. The Hancocks organ is believed to be an olfactory sensory organ (Audesirk 1979, Emery 1992).

Haminoea hydatis feeds on green algae which occur in patches in open water whereas *Acteon tornatilis* is a predator of soft invertebrates living up to ten centimeters in solid sand (Fretter 1939, Yonow 1989, own investigations). In such an environment, an olfactory sensory organ is not plausible since olfaction or distance chemoreception is generally associated with water currents, which are not substantial in a sandy substrate habitat. Here, a contact chemoreceptor, which is located near the edge of the cephalic shield, is more plausible. This I witnessed in *Acteon tornatilis* via its display of a potentially chemoreceptive groove along the lateral margin of the anterior cephalic shield. This assumption of secondary reduction of the Hancocks organ in the endobenthic *Acteon tornatilis* is also supported by the fact that a Hancocks organ has been described for other epibenthic Acteonoidea (e.g. *Bullina*, *Micromelo*, *Hydatina*) (Rudman 1971a,b, Rudman 1972a,b, Rudman 1972c).

Despite all discussion, homology of the described Hancocks organs in Acteonoidea to those in Cephalaspidea cannot undoubtedly be proposed at this stage, particularly since data on innervation patterns in the epibenthic Acteonoidea are lacking to date. Moreover, current phylogenetic hypotheses (Grande et al. 2004, Vonnemann et al. 2005, Klussmann-Kolb et al. 2008) regarding Opisthobranchia propose an independent origin of Acteonoidea and Cephalaspidea, indicating convergent development of these sensory organs in both evolutionary lineages (see also chapter 4).

3.7.2 The CSOs of the Pleurobranchomorpha (*Pleurobranchaea meckeli and Berthella plumula*)

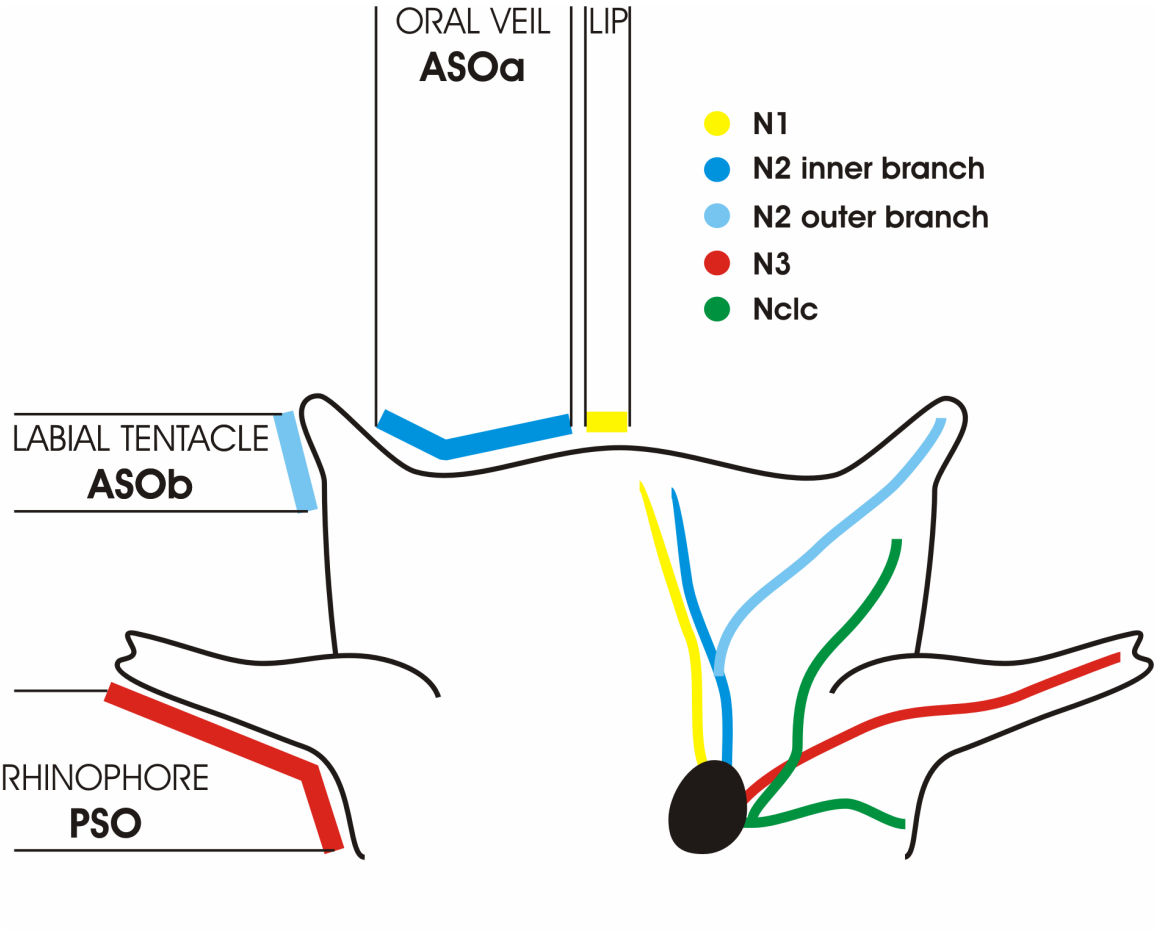


Figure 37: Categories of CSOs for *Pleurobranchaea meckeli*. On the right hemisphere the gross morphology of the four cerebral nerves providing the CSOs is shown. On the left hemisphere the categories for the CSOs are shown. The cerebral nerves and their respective CSO categories are marked by the same colour.

Pleurobranchaea meckeli belongs to the taxon Pleurobranchomorpha. Recent studies (Wägele and Willan 2000, Grande et al. 2004, Vonnemann et al. 2005), combined the taxon Pleurobranchomorpha with the taxon Nudibranchia to the taxon Nudipleura. Within the Pleurobranchomorpha the ASOs (Fig. 37) are regularly expressed as an oral veil. The cellular innervation patterns as well as the immunocytochemistry indicate a primary homology to the lip organ in the Cephalaspidea and Acteonoidea or the labial tentacles in the Aplysiomorpha and Nudibranchia. In *Pleurobranchaea meckeli* the

outer branch of the N2 provides very small folded labial tentacles. In many Pleurbranchomorpha these structures are missing, for example in the investigated pleurobranchomorph *Berthella plumula.*

The immunohistochemistry, especially the high content of TH lir somata indicates a primary function of the oral veil (and also the small labial tentacles) as contact chemo- and mechanoreceptors. The separation between the ASOa and ASOb need further investigations as different functions of these separated structures are not clarified yet. The rhinophores of *Pleurobranchaea meckeli* are believed to be primarily olfactory sensory organs (Hoffmann 1939, Gillette and Yafremava 2005), like the rhinophores of the other opisthobranch taxa and the Hancocks organ of the Cephalaspidea.

Such a separation between the ASOs and the PSOs is very common within the Opisthobranchia. *Pleurobranchaea meckeli* is a predator which feeds on small Crustacea, Bivalvia and other Gastropoda. As light and optical senses are less important in locating the prey, the dominant oral veil with its contact chemo- and mechanoreceptors is very useful to locate prey which is buried in the substrat. The rhinophores might have the function to locate the general direction of food sources.

Like *Pleurobranchaea meckeli, Berthella plumula* belongs to the taxon of the Pleurobranchomorpha. It possesses a very prominent oral veil (Fig. 38) and rhinophores. The main difference to *Pleurobranchaea meckeli* is the lacking of labial tentacles. Instead *Berthella plumula* has a groove at the lateral margin of the oral veil. This structure is provided by the outer branch of the N2 like the labial tentacles of *Pleurobranchaea meckeli*, the posterior lip organ in *Haminoea hydatis*, the groove on the anterior cephalic shield in *Acteon tornatilis* or the labial tentacles in *Aplysia* or *Archidoris.* This groove is very similar to a structure described as a Hancocks organ for *Tritonia diomedea* by Murray and Willows (1996).

The nudibranch *Tritonia* has a very similar gross morphology in comparison to the Pleurobranchomorpha (Wyeth and Willows 2006, Wyeth et al. 2006), with a prominent oral veil and rhinophores. However, the so called Hancocks organ of *Tritonia diomedea* is also provided by the outer branch of the N2 (Murray and Willows 1996), like the labial tentacles or the lip organ of other taxa within the Opisthobranchia and therefore

should be renamed, since a Hancocks organ is a CSO of the posterior category as mentioned earlier (chapter 3.2). The term Hancocks organ in *Tritonia diomedea* may be caused by the fact that the Hancocks organ of earlier investigations (Edlinger 1980) was divided into an anterior and posterior part. The Hancocks organ of *Tritonia diomedea* represents only the anterior Hancocks organ of earlier descriptions. The rhinophores of *Berthella plumula* are folded like in *Pleurobranchaea meckeli*, and there is no doubt that they are homologous structures within the Pleurobranchomorpha. Like in the earlier investigated species the cerebral nerve Nclc is not related to a specialised sensory organ.

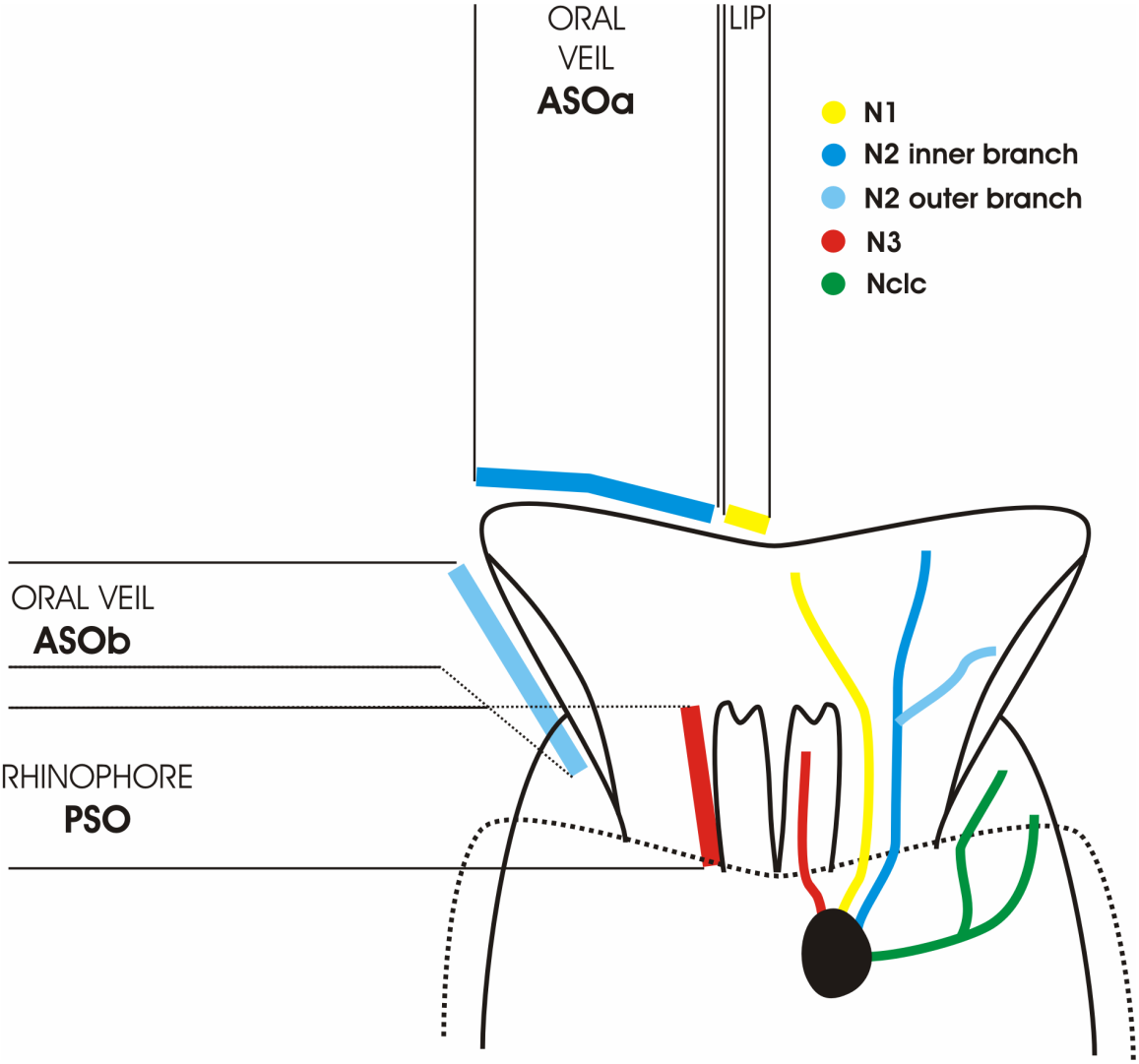


Figure 38: Categories of CSOs for *Berthella plumula*. On the right hemisphere the gross morphology of the four cerebral nerves providing the CSOs is shown. On the left hemisphere the categories of the CSOs are shown. The cerebral nerves and their respective CSO categories are marked by the same colour.

3.7.3 The CSOs of the Nudibranchia (*Archidoris pseudoargus*)

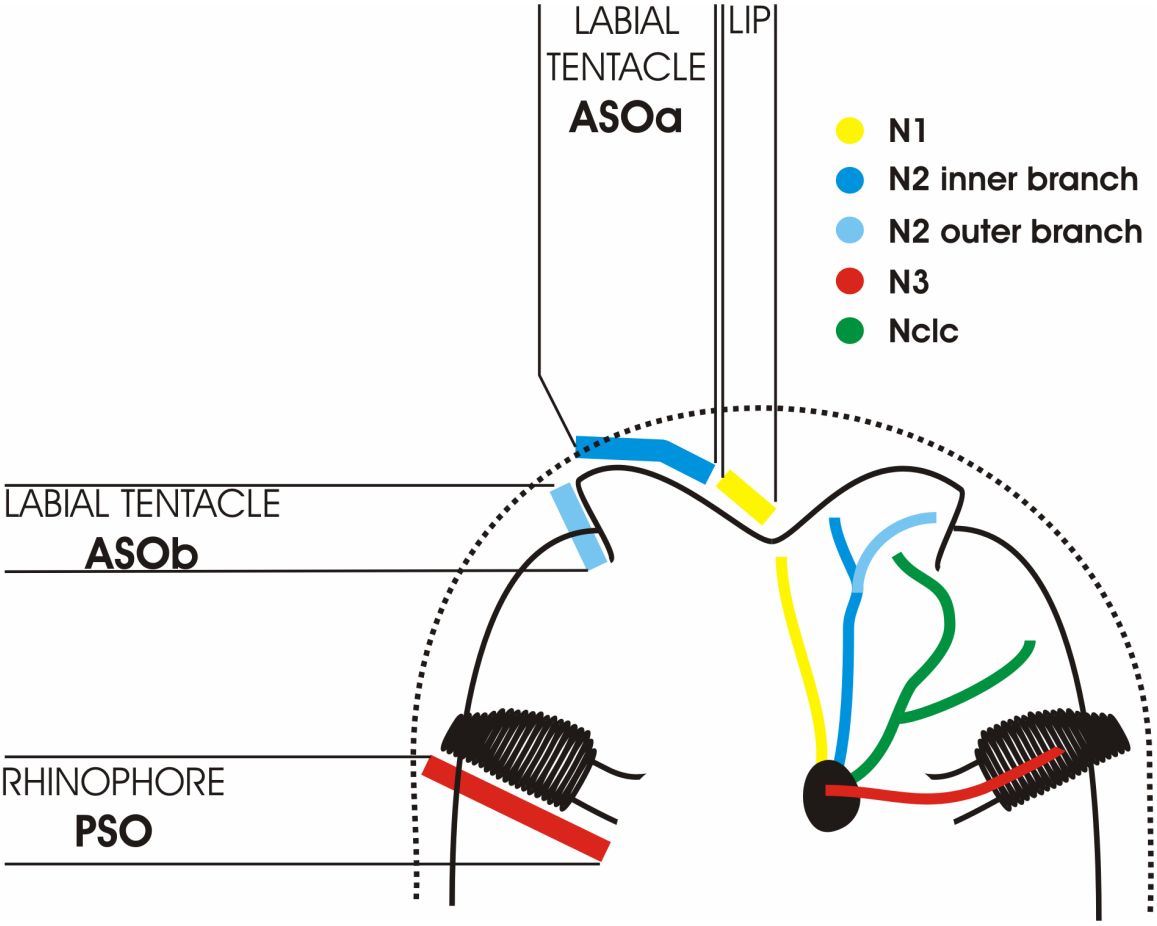


Figure 39: Categories of CSOs for *Archidoris pseudoargus*. On the right hemisphere the gross morphology of the four cerebral nerves providing the CSOs is shown. On the left hemisphere the categories of CSOs are shown. The cerebral nerves and their respective CSO categories are marked by the same colour.

Archidoris pseudoargus belongs to the taxon Nudibranchia, the sister group of the Pleurobranchomorpha. The small labial tentacle of *Archidoris pseudoargus* also has a very small groove which is innervated by the outer branch of the N2 (Fig. 39). This is a structure very similar to the groove on the oral veil in *Berthella plumula* or the Hancocks organ of *Tritonia diomedea*. The oral veil of *Pleurobranchaea meckeli* with its small labial tentacles could be regarded as an intermediate form between the oral veil of *Berthella plumula* with no labial tentacles and the small labial tentacles of *Archidoris pseudoargus*. In other nudibranch taxa, the labial tentacles are very prominent unlike in *Archidoris pseudoargus* whose labial tentacles have a gross morphology which is very similar to the oral veil of the Pleurobranchomorpha. Therefore, I postulate homology of

the oral veil of the Pleurobranchomorpha and the labial tentacles of the Nudibranchia. Labial tentacles are also very prominent CSOs within the Aplysiomorpha, but the ontogentic investigations of Alen Kristof and Wollesen et al. (2007a,b) have shown, that the labial tentacles of the Nudibranchia and the Aplysiomorpha show strong differences in their ontogentic development. This may implicate homoiology of the labial tentacles of the Nudipleura to the labial tentacles of the Aplysiomorpha. The gross morphology of rhinophores of the Nudibranchia (in the current study *Archidoris pseudoargus* with massive rhinophores) differs completely from the rhinophores of the Pleurobranchomorpha (rolled rhinophores) and the Aplysiomorpha (rhinophores folded at the tip).

However, innervation patterns and immunohistological investigations, which implicate a primary olfactory function of the rhinophores, indicate homology of rhinophores in the Nudibranchia to the rhinophores of all other Opisthobranchia, and the ommatophores of the Stylommatophora.

3.7.4 The CSOs of the Aplysiomorpha (*Aplysia californica/punctata, Petalifera petalifera*)

The two investigated species of the genus *Aplysia*, *Aplysia californica* and *Aplysia punctata* show no differences within their CSOs, considering structure, innervation patterns and immunohistochemistry. *Aplysia* spp. belong to the Aplysiomorpha. The labial tentacles are very prominent with a broad base innervated by the inner branch of the N2 and a folded tip provided by the outer branch of the N2 (Fig. 40). This differention is also found within the oral veil of the Pleurobranchomorpha and the labial tentacles of the Nudibranchia.

The ontogenetic investigations of Wollesen et al. (2007a,b) have shown that the ASOs develop first in *Aplysia californica*. This may be caused by the life history of this species. The metamorphosis from a veliger larvae to the juvenile form is triggered by the occurrence of the nutrition, green and red algae. Thus it is more important for the postmetamorphic juvenile animal to discriminate these algae. The importance of the olfactory sense, which is correlated to the rhinophores, becomes more important when the animal is growing and needs to locate other patches of algae. Therefore in the juvenile form, the contact chemoreceptors in the ASOs are more important, whereas for adult animals olfaction via rhinophores is more important to locate the algae.

Whereas in *Aplysia* spp. the labial tentacles with the folded tip (ASOb) and the broad basis (ASOa) seem to constitute a single structure, in *Petalifera petalifera* I found a clear separation into oral lobes (ASOa) and folded labial tentacles (ASOb) (Fig. 41). Here I postulate homology for the broad basis of the labial tentacles in *Aplysia* to the oral lobes in *Petalifera petalifera*. Furthermore, the oral lobes which are innervated by the inner branch of the N2 have an extremely high amount of TH-lir somata like the oral veil of the Pleurobranchomorpha or the anterior lip organ of *Haminoea hydatis*, indicating that the oral lobes are primary contact chemo- and mechanoreceptors.

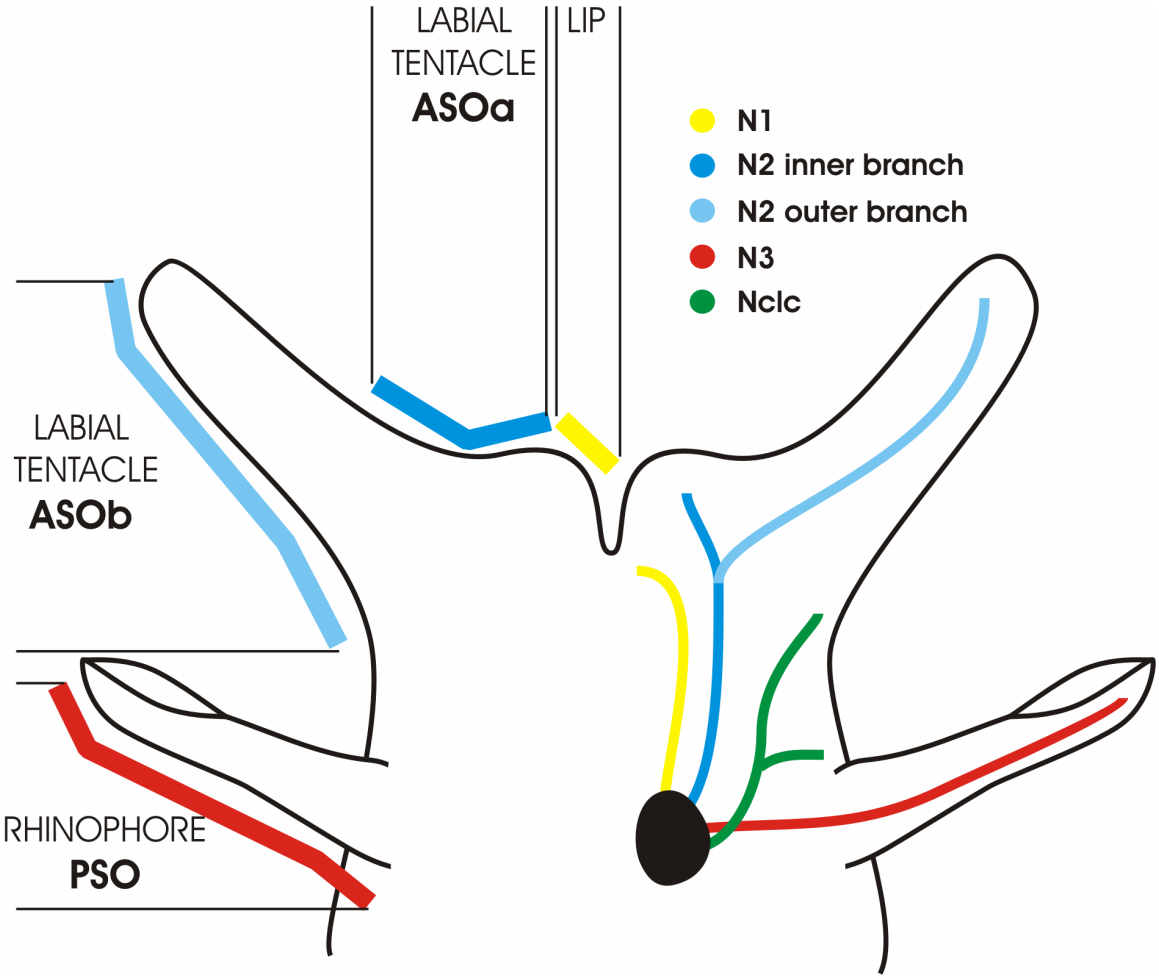


Figure 40: Categories of CSOs for *Aplysia* spp. On the right hemisphere the gross morphology of the four cerebral nerves providing the CSOs is shown. On the left hemisphere the categories of the CSOs are shown. The cerebral nerves and their respective CSO categories are marked by the same colour.

The innervation patterns of the cerebral nerves indicate a homology for the labial tentacles of the Aplysiomorpha and the labial tentacles or oral veil of the Nudipleura, the posterior lip organ of the Cephalaspidea and the groove/lip organ of the Acteonoidea, as well as a homology of the rhinophores of the Nudipleura and the Aplysiomorpha. Nevertheless it has to be regarded that the ontogenetic development indicates homoiology between the CSOs of the Nudipleura and the Aplysiomorpha.

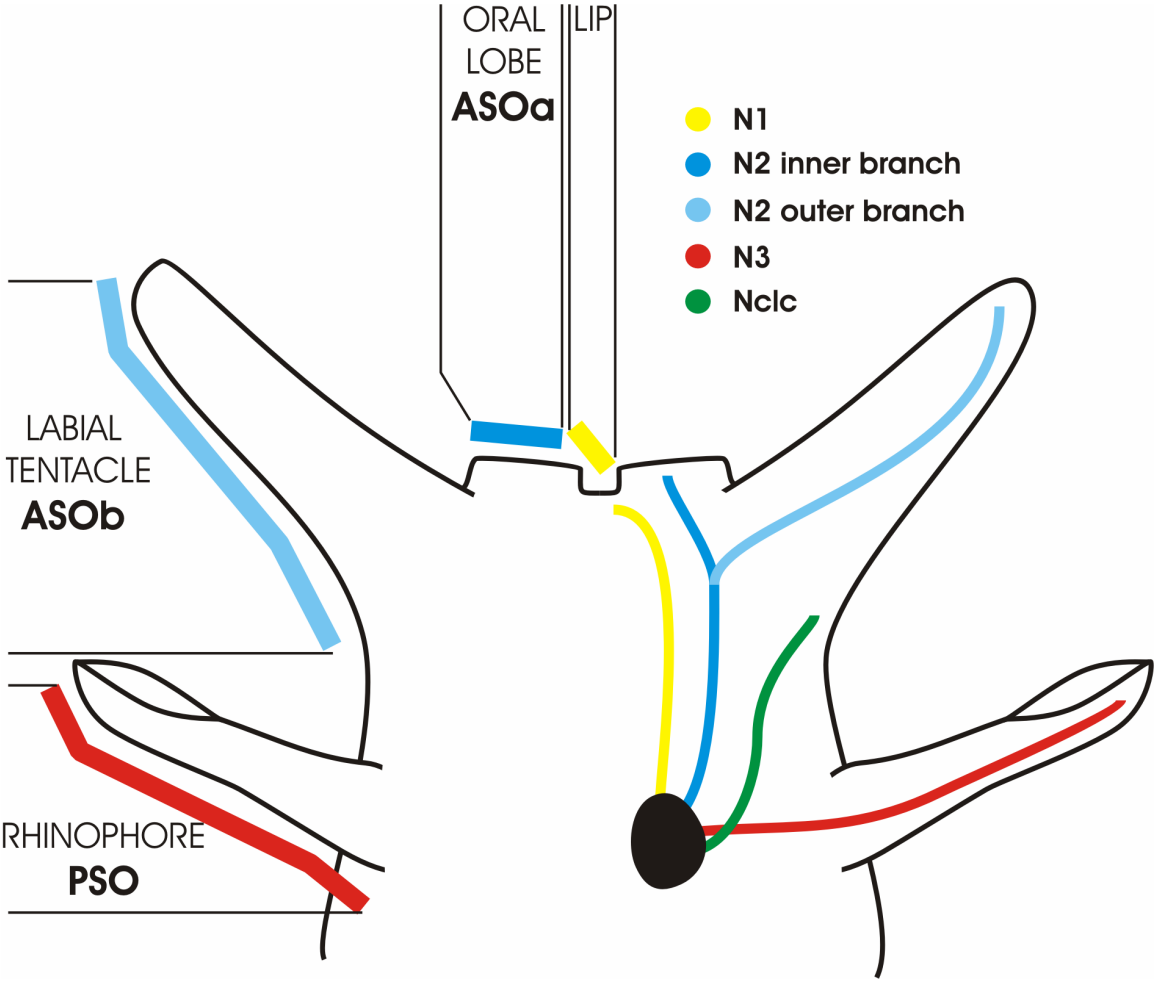


Figure 41: Categories of CSOs for *Petalifera petalifera*. On the right hemisphere the gross morphology of the four cerebral nerves providing the CSOs is shown. On the left hemisphere the categories of the CSOs are shown. The cerebral nerves and their respective CSO categories are marked by the same colour.

In follow I postulate, that the labial tentacles of *Aplysia* spp. are the basal form of the ASOs within the Aplysiomorpha, and that the more differentiated ASOs of *Petalifera petalifera* represent a derived form. A hypothesis which is also supported by the derived phylogenetic position of *Petalifera petalifera* within the Aplysiomorpha (Klussmann-Kolb 2004).

3.7.5 The CSOs of the Cephalaspidea (*Haminoea hydatis and Scaphander lignarius*)

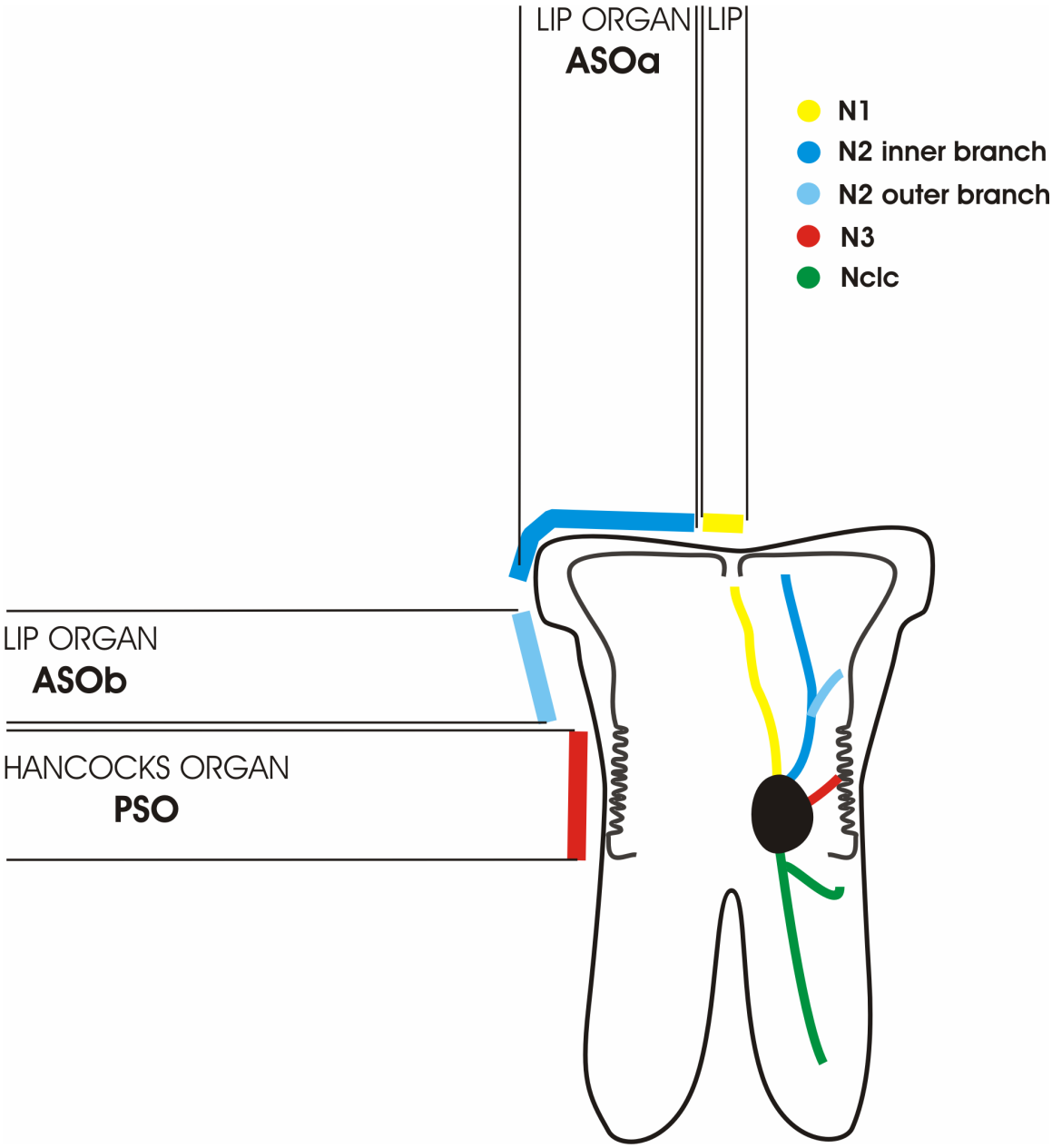


Figure 42: Categories of CSOs for *Haminoea hydatis.* On the right hemisphere the gross morphology of the four cerebral nerves providing the CSOs is shown. On the left hemisphere the categories are shown. The cerebral nerves and their respective CSO categories are marked by the same colour.

The CSOs of *Haminoea hydatis* (Fig. 42) have been discussed in detail in a previous chapter (3.4). *Scaphander lignarius*, the second investigated Cephalaspidea shows the same set of cephalaspidean CSOs: lobe like structures besides the mouth and a

Hancocks organ on the ventral side of the cephalic shield/disc (Fig. 43). These correspond to the ASOs and PSOs respectively. Edlinger (1980) has described the same set of CSOs for the Acteonoidea but as I have discussed earlier (chapter 3.2, 3.3, 3.4, 3.7) this could have been a misinterpretation by Edlinger (1980) since *Acteon tornatilis* has reduced the PSO.

The groove (ASO) in *Acteon tornatilis* and the Hancocks organ (PSO) in *Scaphander lignarius*, are not homologous (as implied by Edlinger 1980), but have probably evolved independently in both species due to similar life history. In follow I postulate primary homology hypothesis for the anterior lip organ, which is innervated by the inner branch of the N2 (Fig. 41, 42) of the Cephalaspidea with the oral lobes and the broad basis of the labial tentacles of the Aplysiomorpha, whereas the posterior lip organ is a homologous structure to the folded labial tentacles of the Aplysiomorpha and the groove on the oral veil of the Pleurobranchomorpha.

As mentioned earlier it has to be discussed if they are homoiologous to the labial tentacles of the Nudibranchia and the groove of the Acteonoidea. In my opinion the lip organ of the Cephalaspidea and the groove of the Acteonoidea are homoiologous structures as they are an adaptation to the convergent life history. *Haminoea hydatis* is burying into the sand to avoid predation, meanwhile *Scaphander lignarius* buries in the sand for predation. Therefore extended structures like labial tentacles or rhinophores are not useful. It has been mentioned earlier, that the Hancocks organ of the Cephalaspidea is a homologous structure to the rhinophores of other Opisthobranchia (Hoffmann 1939).

I agree with this hypothesis if the term Hancocks organ in the Cephalaspidea is confined to the CSO provided by the N3. Furthermore ontogentic investigations of Corinna Schulze (pers. comm.) have shown that the lip organ of *Haminoea japonica* which is very close related to *Haminoea hydatis* develops first in the juvenile animal like the labial tentacles in *Aplysia*. This was also mentioned earlier and is an indication for a homoiology of the lip organ of the Cephalaspidea and the labial tentacles of Aplysiomorpha with the labial tentacles of the Nudibranchia, which develop as second CSOs (Alen Kristof pers. comm.).

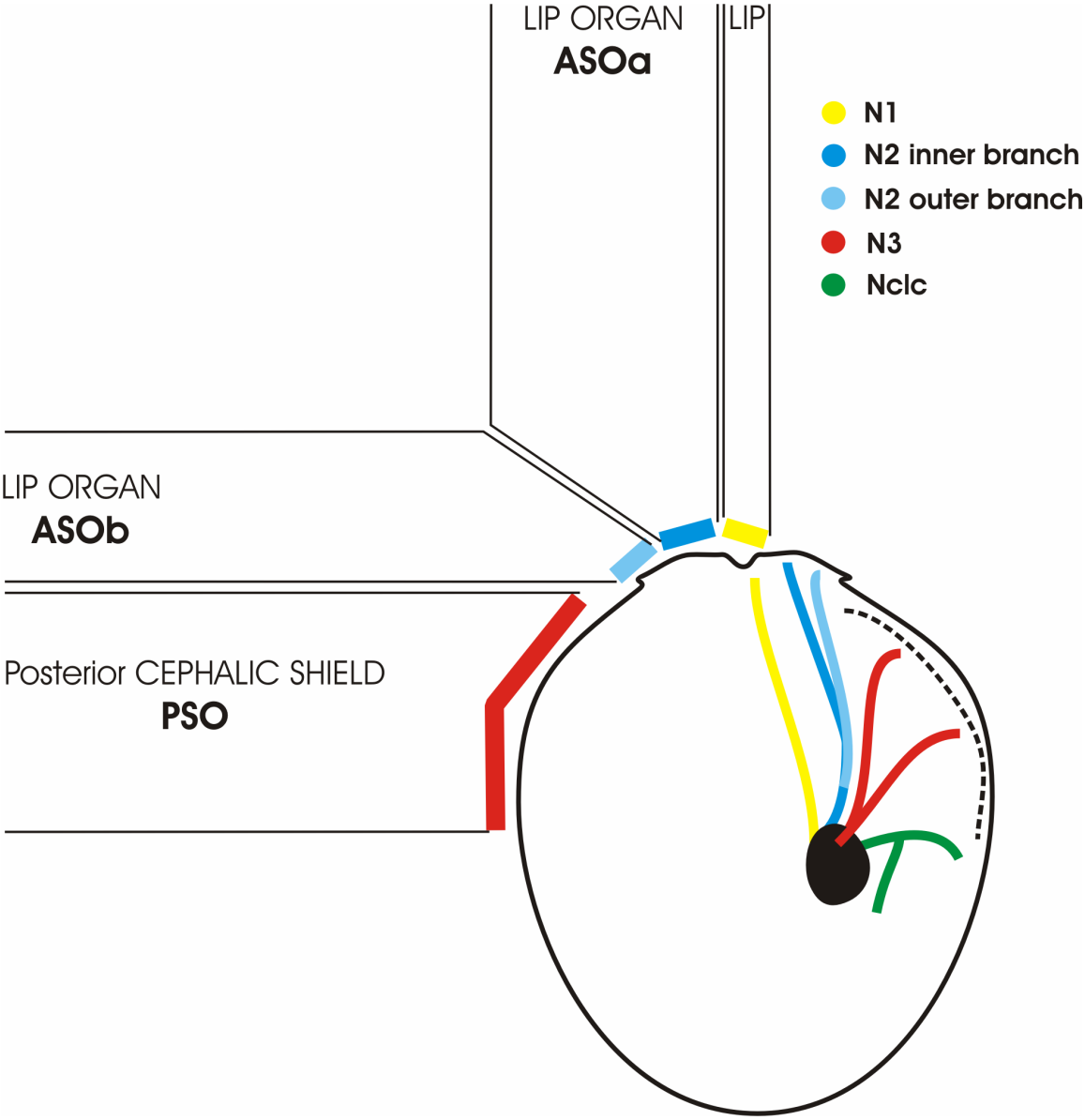


Figure 43: Categories of CSOs for *Scaphander lignarius*. On the right hemisphere the gross morphology of the four cerebral nerves providing the CSOs is shown. On the left hemisphere the categories of the CSOs are shown. The cerebral nerves and their respective CSO categories are marked by the same colour.

3.7.6 The CSOs of *Achatina fulica*

The terrestrial snail *Achatina fulica* belongs to the taxon Stylommatophora. *Achatina fulica* also has four cerebral nerves, with a bifurcated N2 which provides the ASOa and ASOb (Fig. 44). The small anterior tentacles were termed as “rhinophores” in earlier investigations (Zaitseva 1992), but in accordance to the cellular innervation patterns and the neuroanatomy, I postulate a primary homology hyphothesis of these “rhinophores” to the labial tentacles, the oral veil or the lip organ of the Opisthobranchia.

In follow the large posterior tentacles, with the eye on the tip, called ommatophores (Zaitseva 1992) are considered to be homologous structures to the rhinophores or the Hancocks organ of the investigated Opisthobranchia. Investigations of Ierusalimsky and Balaban (2007) and Chase and Tolloczko (1986, 1989) have shown, that the ommatophores of another stylommatophoran, *Helix pomatia*, have a high number of glomeruli like structures, like the rhinophores of the Opisthobranchia (Croll 2000, Wertz et al. 2007, Faller et al. *in review*).

Furthermore, Ierusalimsky and Balaban (2007) came to the conclusion that the ommatophores of Stylommatophora are primarily involved in olfaction and the anterior tentacles (“rhinophores”) more commonly respond to tactile stimuli or chemoreception. This specialization is a general pattern I also assumed for the CSOs of the investigated Opisthobranchia. Moreover, it makes also sense considering functional aspects, as the anterior tentacles of the Stylommatophora are located close to the substrate, whereas the posterior tentacles are raised into the air and probably explore the olfactory environment, while rarely touching the surface.

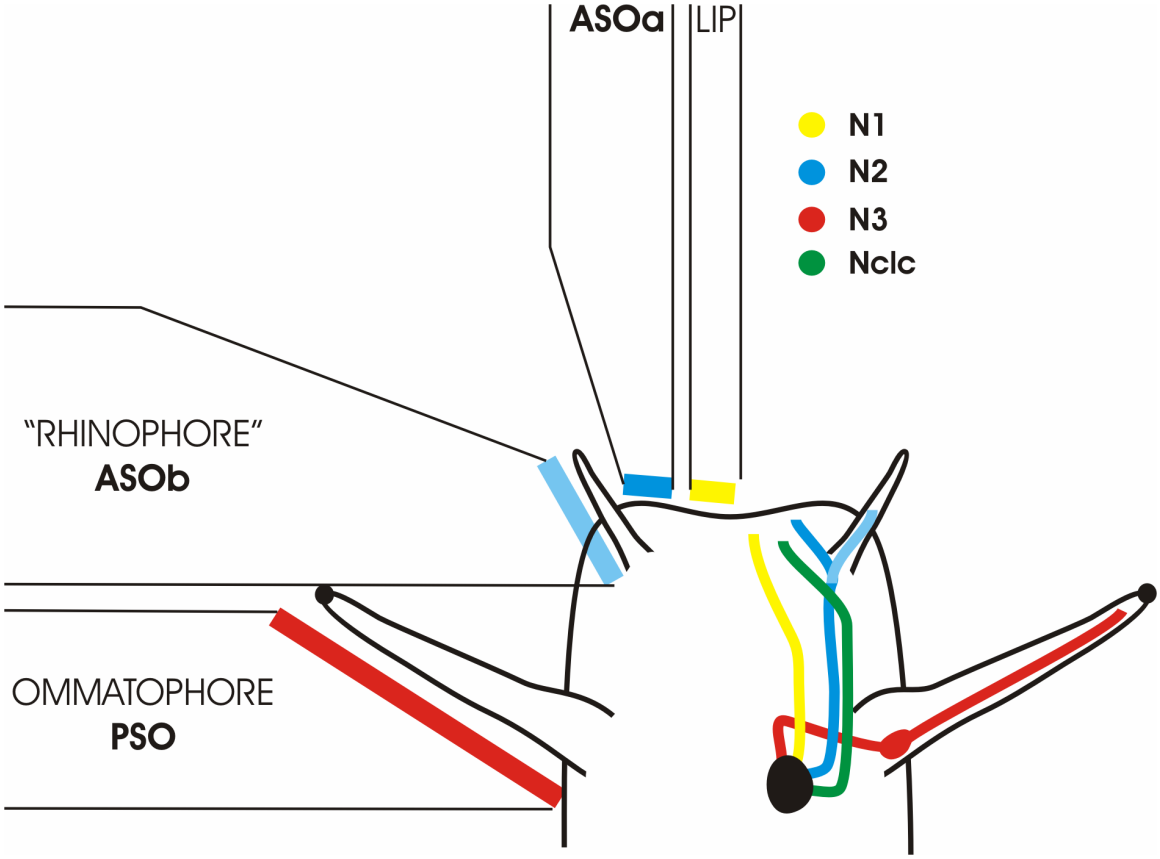


Figure 44: Categories of CSOs for *Achatina fulica*. On the right hemisphere the gross morphology of the four cerebral nerves providing the CSOs is shown. On the left hemisphere the categories of the CSOs are shown. The cerebral nerves and their respective CSO categories are marked by the same colour.

3.7.7 The CSOs of *Littorina littorea*

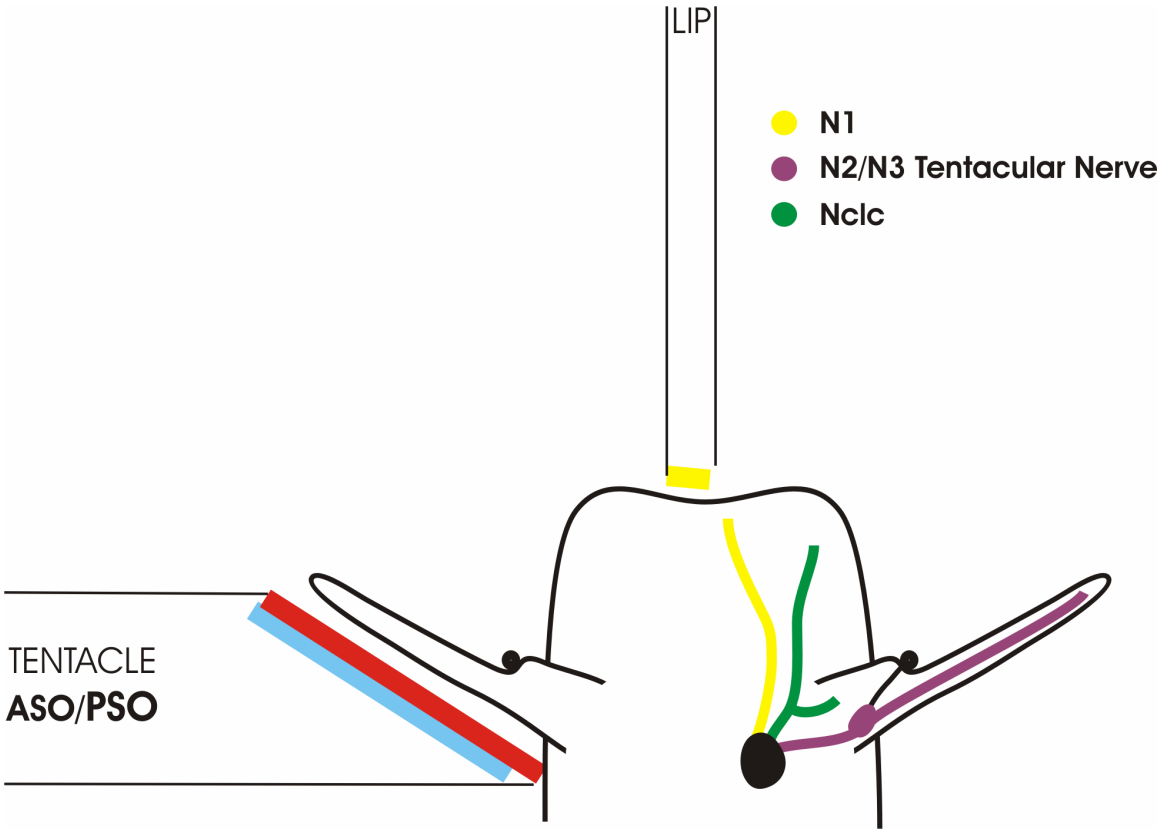


Figure 45: Categories of CSOs for *Littorina littorea*. On the right hemisphere the gross morphology of the four cerebral nerves providing the CSOs is shown. On the left hemisphere the categories of the CSOs are shown. The cerebral nerves and their respective CSO categories are marked by the same colour.

Littorina littorea belongs to the taxon Caenogastropoda and its investigated CSOs as well as their neuroanatomy differ completely from the investigated Euthyneura. *Littorina littorea* only has three cerebral nerves and only one pair of tentacles (Fig. 45). My investigations on the cellular innervation patterns of the cerebral nerves indicate that the lip, provided by the N1 and the anterior head region, provided by the Nclc are homologous structures to the lip and the anterior head region of the Euthyneura. This is different for the tentacles, here the innervation patterns of the tentacle nerve show combined patterns for the N2 and the N3 of the investigated Euthyneura. I can give two explanations for this finding: first, the tentacular nerve in *Littorina littorea* is a plesiomorphic structure which is divided into two nerves in the investigated Euthyneura

or secondly the tentacular nerve is a derived structure and represents a fusion of the nerves N2 and N3. The phylogentic position of the Caenogastropoda prefers the first assumption. Marshall and Hodgeson (1990), came to the conclusion that the tentacles of "Prosobranchia" (a former taxon which includes the Caenogastropoda), react to mechano- and chemoreception. This indicates that the tentacles are less specific than the ASOs and PSOs of the investigated Euthyneura (Storch 1972, Thollesson 1999, Dayrat and Tillier 2002). My immunohistochemical investigations support this assumption as a clear differentiation in the neurotransmitter content between the anterior head region and the tentacles is lacking. Under functional aspects, this also makes sense as *Littorina littorea* like many Caenogastropoda is a grazer on mikroalgae (Moran 1999, Edwards and Davies 2002) a food source which is very common in their habitat, therefore a specialisation of CSOs is redundant and the tentacles may have other functions like avoidance of predators or mating. Furthermore it has to be mentioned, that *Littorina littorea* shows a territorial behaviour and the tentacles are used to follow mucus trails (Edwards and Davies 2002).

3.7.8 Categories of CSOs

As described in chapter 3.2, I divided the CSOs into the categories Lip, ASOa and ASOb and PSO, innervated by three homologous nerves (N1-N3). The homologised nerves, the head regions of their innervation and their categories are shown as an overview in table 4. Based on the innervation patterns I postulate homology of the lip, which is innervated by the N1.

As second I postulate homology of the ASOs, this includes labial tentacles, oral veils, oral lobes, oral tentacles, lip organ, “rhinophores” of the Stylommatophora and the anterior part of the Hancocks organ as it was defined by Edlinger (1980). The cerebral nerve Nclc does not seem to correspond to a primary sensory organ. As discussed earlier I distinguish between two types/parts of the ASOs provided by the two different branches of the N2. Although homologies of types ASOa on the one hand and ASOb on the other hand are most likely, axonal tracing of the single branches of the N2 is necessary in order to clarify homology of innervation patterns.

In the current study I only traced the entire nerve. The third postulated homology is between the PSOs which are innervated by the N3. In my opinion, Hancocks organ and rhinophores are homologous throughout the Opisthobranchia. Homology of these structures has been postulated earlier (Hoffmann 1939, Edlinger 1980, Huber 1993) and can be confirmed by my data as I found similar tracing patterns for the rhinophores of Nudipleura (Nudibranchia and Pleurobranchomorpha) and Aplysiomorpha, and the Hancocks organ of the Cephalaspidea. The homologisation of Hancocks organ in different opisthobranch taxa (as e.g. Cephalaspidea and Acteonoidea) is more difficult and will be discussed in a following chapter.

Table 4: Categories of cerebral nerves and their innervation targets (CSOs)

Nerve	*Acteon tornatilis*	*Pleuro-branchaea meckeli*	*Archidoris pseudoargus*	*Aplysia* spp.	*Haminoea hydatis*	*Achatina fulica*	*Littorina littorea*	CSO cate-gories
N1	lip	lip	lip	lip	lip / anterior cephalic shield	lip	lip / anterior head region	Lip
N2 inner branch	anterior groove among the anterior cephalic shield	oral veil	inner part of the oral tentacle/oral lobe	basal part of the labial tentacle	lip organ	anterior head region	tentacle N2 and N3 possibly fused	ASOa
N2 outer branch	posterior groove among the anterior cephalic shield	labial tentacle	outer part of the oral tentacle/oral lobe	tip of the labial tentacle	anterior Hancocks organ	"rhinophore" (anterior tentacle)	tentacle N2 and N3 possibly fused	ASOb
N3	possibly reduced in *Acteon*	rhinophore	rhinophore	rhinophore	posterior Hancocks organ	ommatophore (postterior tentacle)	tentacle N2 and N3 possibly fused	PSO
Nclc	posterior cephalic shield	anterior/ lateral body wall	anterior/lateral body wall	anterior/ lateral body wall	posterior cephalic shield	anterior/lateral body wall	anterior/ lateral body wall	no category

3.7.9 The Hancocks organ

The Hancocks organ needs a special discussion, as in earlier studies it was described to be innervated by two nerves (Edlinger 1980, Huber 1993) and was also divided into two parts: an anterior part, which is innervated by the outer branch of the N2 and a posterior part innervated by the N3. Additionally, the Hancocks organ occurs in several orders of the Opisthobranchia. At this point I want to propose a redefinition of the Hancocks organ, as it is the only CSO which is innervated by two different nerves within the old definition (Edlinger 1980).

From my point of view, the term Hancocks organ should be restricted to the posterior Hancocks organ (Edlinger 1980) which is innervated by the N3 (category PSO). Also the term Hancocks organ is used with levity within the Opisthobranchia, there is also a Hancocks organ described for the Nudibranchia (*Tritonia*) (Murray and Willows 1996), the Acochlidiacea (Sommerfeldt and Schrödl 2005, Neusser and Schrödl 2007) and the Aplysiomorpha (*Akera*) (Hoffmann 1939, Edlinger 1980). *Tritonia* also has rhinophores, since the Hancocks organ of *Tritonia* and *Akera* (James Murray pers. com., own investigations) is innervated by the outer branch of the N2 it should be called a CSO of the category ASOb. Currently, I cannot postulate a hypothesis for the Hancocks organ of the extremely small Acochlidiacea possessing a very compressed nervous system.

However, to redefine the term Hancocks organ overall, more investigations, also about the morphology and neuroanatomy of other Cephalaspidea, Acteonoidea and Acochlidiacea are needed. Moreover, additional species especially like *Akera*, *Tritonia*, Acholidiacea and other taxa within the Opisthobranchia could give us more information about the homology of nerves and Hancocks organs within the Opisthobranchia.

4. The evolution of the CSOs within the Opisthobranchia

In this chapter, I will reconstruct the evolution of the CSOs within the Opisthobranchia, which is one of the primary aims of my PhD thesis. I will trace my investigations about the homology of CSOs on a molecular phylogeny published by Klussmann-Kolb et al. recently (2008) (Fig. 46). Before I trace my own data several aspects raised by the investigations of Klussmann-Kolb et al. (2008) have to be mentioned. First, the Caenogastropoda (here *Littorina littorea*) represents the outgroup. Second, the taxon Opisthobranchia is polyphyletic and not monophyletic. Third, within the investigated Opisthobranchia two major clades can be distinguished. The first one includes the Acteonoidea and the Nudipleura (Pleurobranchomorpha and Nudibranchia). The second clade is formed by the Cephalaspidea, the Pteropoda, Umbraculida and the Aplysiomorpha. Furthermore, it has to be mentioned that the Sacoglossa and the Acochlidiacea are grouping with the Pulmonata and not with the Opisthobranchia as previously assumed. In follow, I will discuss the ground patterns for the nodes 1 to 4 marked in Figure 46.

In the following chapter a definition of the term homoiology is needed. A basic assumption for a homologisation of morphological structures is, that an increasing similarity is caused by increasing evolutionary relationship. To exclude convergency (similar morphological structures but different evolutionary lineages) and divergency (the same evolutionary lineages but different morphological structures) an abstract ground pattern with abundance of convergent and divergent traits will be created. The term homoiology (similar to parallelism) describes a convergent development of homologous structures. So, for example the lip organ of the Cephalaspidea and the labial tentacles of the Nudibranchia are homologous structures as ASOs, meanwhile the cephalic shield of the Acteonoidea and the Cephalaspidea evolved independently as a convergent adaptation to their life habitat. Homoiology does not exclude homology.

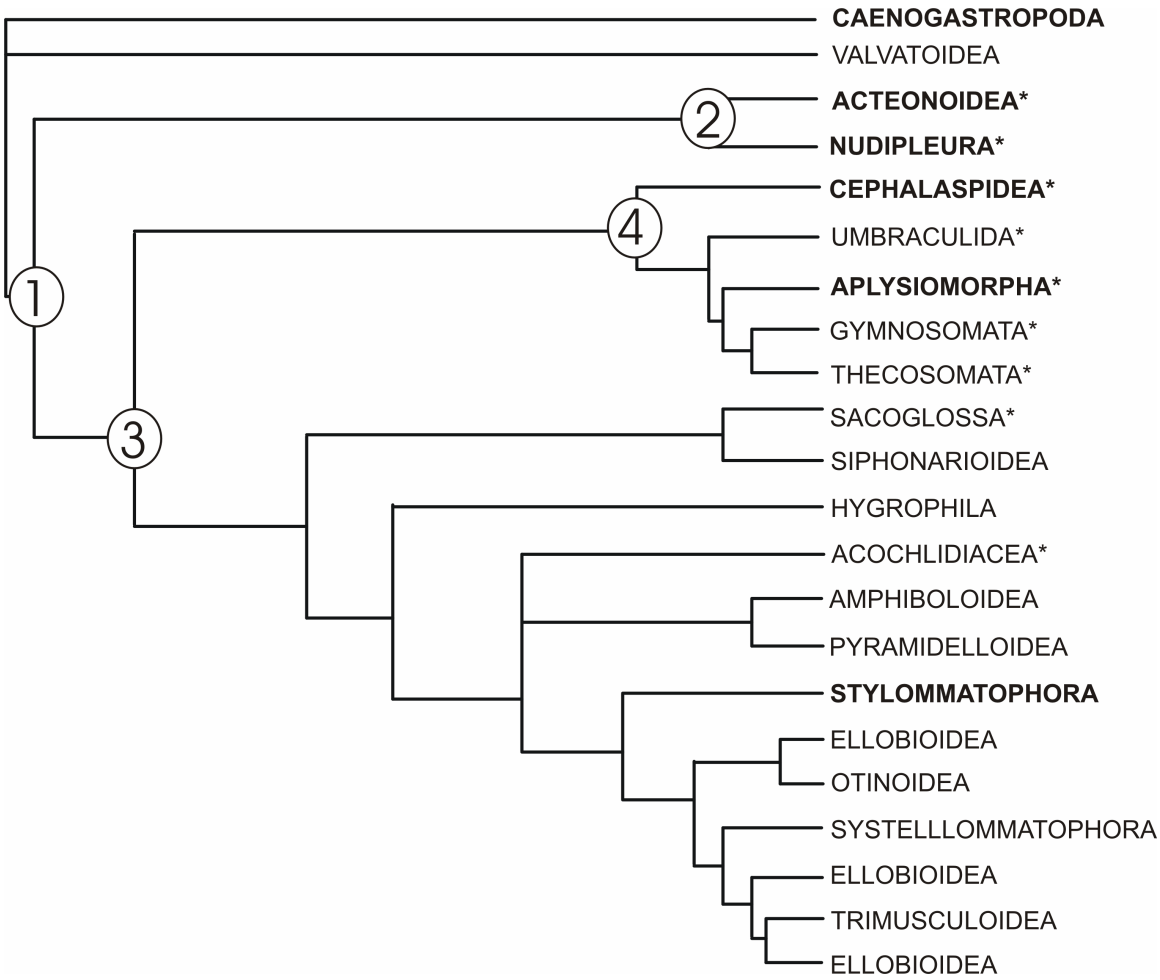


Figure 46: A reduced molecular cladogram after Klussmann-Kolb et al. 2008 of the Euthyneura. The evolution of the CSOs will be reconstructed based on this cladogram. The nodes marked by numbers (1-4) will be discussed in detail. The taxa marked with an * belong to the Opisthobranchia and the bold taxa have been investigated in the current study.

4.1 Node 1

Node 1 represents the ground pattern for all investigated species including the Opisthobranchia and the Stylommatophora (Figs. 46, 47) except for the outgroup *Littorina littorea*. Here I postulate two pairs of cephalic structures which evolved out of the tentacles of the Caenogastropoda, this was indicated by the innervation pattern of the tentacular nerve of *Littorina littorea*.

I postulate two sets of cephalic structures as I found this separation within nearly all Opisthobranchia, except for the Sacoglossa which are not investigated in this study, furthermore two pairs of cephalic structures occur quite often within the Eupulmonata. Meanwhile these cephalic structures are expressed as ASOs and PSOs within the investigated Opisthobranchia and the Stylommatophora, in other taxa of the Pulmonata especially the anterior set of cephalic structures is a less specialised structure.

In follow I will list the taxa which where not investigated in this study but also have two pairs of cephalic structures:

Umbraculida – small reduced labial tentacles and massive rolled rhinophores (Hoffmann 1939, Thompson 1976, Willan and Burn 2003);

Pteropoda – labial tentacles and rhinophores, labial tentacles missing within the Thecosomata (Hoffmann 1939, Thompson 1976);

Sacoglossa – only one set of CSOs but it is proposed based on the neuroanatomy that these tentacles are fused structures (Huber 1993);

Hygrophila – the most Hygrophila only have one pair of tentacles but the anterior head region often shows some kind of bifurcation. Within the Basommatophora it had also been shown that this bifurcated head region has sensory functions (Chase 2002, Croll et al. 1999);

Acochlidiacea – labial tentacles and a Hancocks organ (Neusser and Schrödl 2007,

Hochberg 2007);

Pyramidelloidea – posterior tentacles and a bifurcated anterior cephalic structure (Huber 1993, Wise 2001);

Otinoidea - posterior tentacles and a bifurcated anterior cephalic structure (Powell 1979);

Systellommatophora - posterior tentacles and a bifurcated anterior cephalic structure (Powell 1979);

From the above mentioned outline of CSOs in Euthyneura and Caenogastropoda, it can be deduced that the ground pattern for node one has a bifurcated cephalic structure which will evolve towards the ASOs of the Opisthobranchia and posterior massive tentacles which will evolve towards the PSOs. Within the Opisthobranchia the ASOs are extremely specialised, such a specialisation is often missing within the Pulmonata, which in general show only one pair of tentacles.

Here the ground pattern cannot be defined more clearly. This is caused by the concept of this study, as the reconstruction of the CSOs was restricted to the Opisthobranchia, but node 1 includes the Pulmonata and Pyramidelloidea. Therefore to define the ground pattern for node one in detail, further investigations of the CSOs of non-opisthobranch euthyneuran taxa are needed.

4.2 Node 2

As second node I will discuss the node for the major clade comprising the Acteonoidea and the Nudipleura. The investigation of Klussmann-Kolb et al. (2008) positioned the Acteonoidea as sister group to the Nudipleura and not closely related to the Cephalaspidea. Furthermore the Cephalaspidea form a sister group to the Anaspidea and Umbraculida and are a derived taxon. The assumption that the Acteonoidea are not basal within the Opisthobranchia, leads to the hypothesis, that the set of CSOs, including the groove, the reduced Hancocks organ and the cephalic shield overall are an adaptation to living in the sand. In conclusion the CSOs of the Acteonoidea are very derived structure and not plesiomorph, like mentioned earlier by Edlinger (1980). Therefore I postulate the ground pattern for node 2 (Figs. 46, 47) with two sets of CSOs, the ASOs and the PSOs as the Acteonoida and the Nudipleura have also two types of CSOs. I have shown that the Hancocks organ has been reduced in *Acteon tornatilis* (Staubach and Klussmann-Kolb 2007) but it is existent in other Acteonoidea (Rudmann 1972a,b). Furthermore I postulate the ASOs at node 2 as small lobe like labial tentacles which were fused with the cephalic shield to form the groove at the anterior cephalic shield of the Acteonoidea. This groove but also the cephalic shield is an adaptation to the life habitat of the Acteonoidea. Within the Nudipleura these small lobe like labial tentacles evolve towards extended labial tentacles and oral veils. This is supported by the oral veil of *Pleurobranchaea meckeli* which has small labial tentacles and could be an intermediate form between the labial tentacles of the Nudibranchia and the oral veil of *Berthella plumula* which has no labial tentacles. In follow I postulate massive rhinophores for the ground pattern of node 2. Within the Acteonoidea these rhinophores are reduced to the Hancocks organ as an adaptation to life history. Within the Nudibranchia the massive rhinophores can show extensions like disc, meanwhile the rhinophores of the Pleurobranchomorpha are rolled. In both cases this is an extension of the surface area. I consider the rhinophores of the Nudibranchia and the Pleurobranchomorpha as homologous structures. Furthermore I assume that the Hancocks organ in the Acteonoidea is a homologous structure to the rhinophores of the Nudipleura. I also assume that the groove along the anterior cephalic shield is a homologous structure to labial tentacles of the Nudipleura or the lip organ of the Cephalaspidea.

4.3 Node 3

This node represents the ground pattern for the second major clade comprising the investigated Cephalaspidea, Aplysiomorpha and Pulmonata (Figs. 46, 47). I postulate the same ground pattern as described for node 1, relatively undifferentiated bifurcated anterior cephalic structures and posterior tentacles. I come to this conclusion as the most taxa included in this clade have two sets of paired cephalic structures which include posterior tentacles. At the level of ASOs and PSOs the anterior tentacles ("rhinophores") of the Stylommatophora are homologous to the labial tentacles of the Aplysiomorpha, here I postulate homoiology for these structures as both structures develop out of the bifurcated anterior cephalic structures in the ground pattern of node 3.

The same is true for the Hancocks organ of the Cephalaspidea, the rhinophores of the Aplysiomorpha and the posterior tentacles (ommatophores) of the Stylommatophora as all structures develop in my opinion out of the massive posterior tentacles in the ground pattern of the node 3. Here again, the ground pattern cannot be defined more clearly. This is also caused by the concept of this study as mentioned earlier, as the reconstruction of the CSOs was restricted to the Opisthobranchia, but node three also includes the Pulmonata. Therefore to define the ground pattern for node 3 in detail, further investigations of the CSOs of the pulmonate taxa are needed.

4.4 Node 4

Node 4 represents the second major clade within the Opisthobranchia, which includes the investigated Cephalaspidea and the Aplysiomorpha but also the Umbraculida and the Pteropoda. Altough at the higher level of ASOs and PSOs the CSOs are homologous throughout the Opisthobranchia, neither the lip organ, nor the Hancocks organ or the cephalic shield of the Cephalaspidea are homologous to the lip organ, the Hancocks organ and the cephalic shield of the Acteonoidea.

These structures are homoiologous (to remember: convergent development of

homologous traits) as they are an adaptation to life in the sand. This assumption is supported by the facts, that the cephalic shield of the Cephalaspidea shows a different gross morphology, furthermore the ontogenetic investigations of Wollesen (2007a,b) and Schulze (unpublished data) have shown that in this major clade, the ASOs develop first meanwhile in Nudibranchia the PSOs develop first (Kempf et al. 1996, Kristof, unpublished data). In conclusion, this indicates, that the rhinophores of the Nudipleura and the Aplysiomorpha are also homoilogous structures. For node 4 I postulate the following ground pattern (Fig. 47): for the ASOs clearly differentiated bifurcated cephalic structures more related to the lip organ of the Cephalaspidea, and very small massive rhinophores as PSOs. This is supported by the fact that the Umbraculida and Pteropoda also show massive rhinophores (Hoffmann 1939).

In follow I postulate homology of the rhinophores of the Aplysiomorpha and the Hancocks organ of the Cephalaspidea. I came to this hypothesis, as the sensory function of the aplysiomorphan rhinophores is restricted to a folded groove at the top of the rhinophores. So it can be imagined that the base of the basal rhinophores in the ground pattern of node 4 were extended within the Aplysiomorpha and reduced, in order to the life habitat, within the Cephalaspidea. This reduction of the base of the small rhinophores in the ground pattern of node 4 leads to the gross morphology of the recent Hancocks organ of the Cephalaspidea, under the assumption, that the cephalic shield is also an adaptation and developed out of the lateral body wall. Moreover, the lip organ of the Cephalaspidea and the labial tentacles of the Aplysiomorpha are homologous structures, the same is true for the oral lobes of *Petalifera petalifera* and the broad basis of the labial tentacles of *Aplysia*.

4.5 Summary

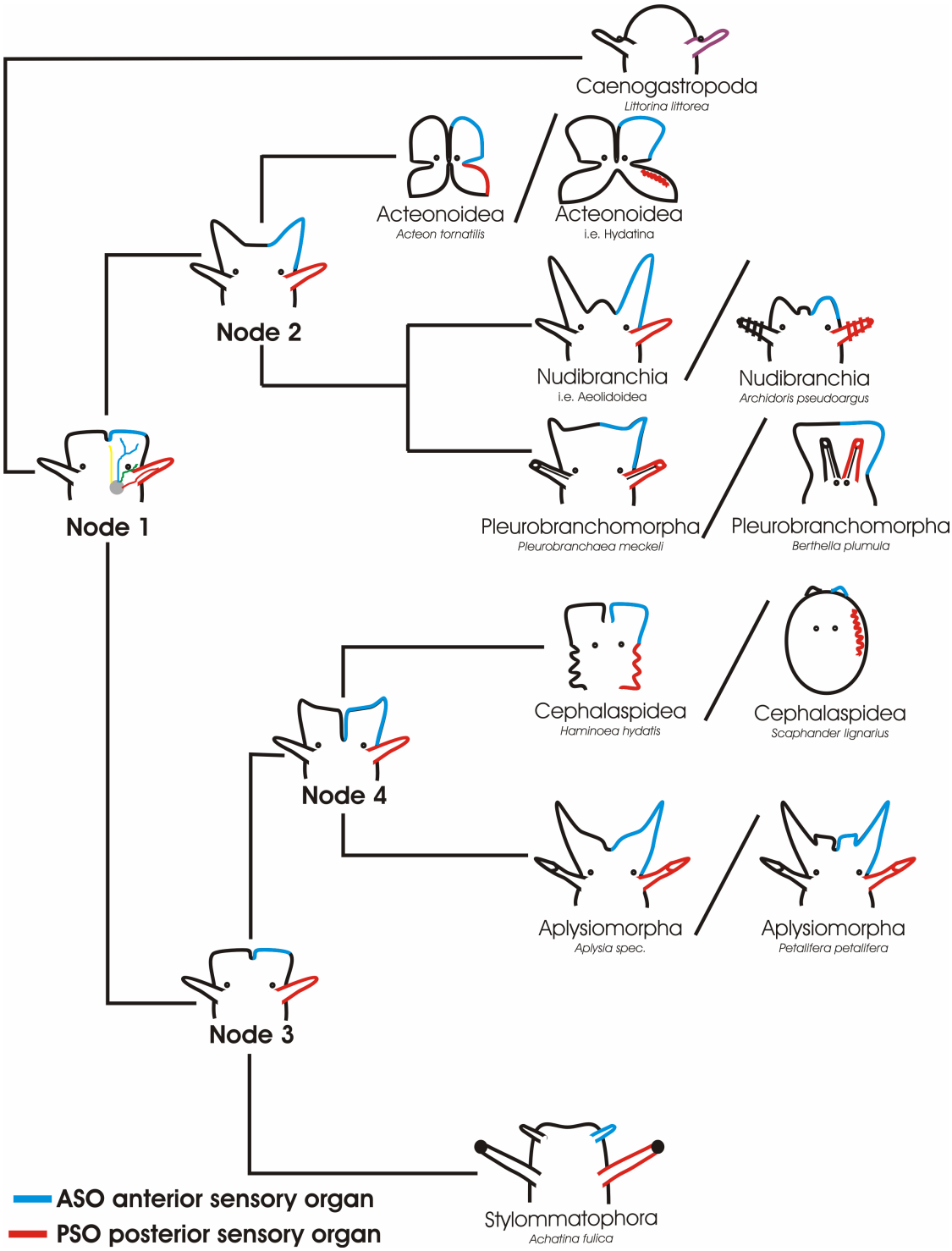


Figure 47: Schematic illustration of the evolution of the CSOs, for the earlier discussed nodes and the investigated taxa. The anterior cephalic structures are marked with a blue line, the posterior cephalic structure with a red line.

4.6 General ground patterns

My PhD thesis and the taxon sampling were designed under the assumption that the Opisthobranchia are a monophyletic taxon but recent investigations (Klussmann-Kolb et al. 2008) recovered the Opisthobranchia to be polyphyletic or paraphyletic. Some opisthobranch taxa are missing in my investigations (Umbraculida, Pteropoda, Sacoglossa and Acochlidiacea). This was caused by the difficulty to get a high number of living animals of relevant species which are suitable for my methods. In this chapter I will discuss my postulated ground patterns in relation to these missing opisthobranch taxa and the fact, that the Opisthobranchia are not monophyletic. This will be done by a comparision with data from the literature.

The opisthobranch taxon of the Umbraculida is possibly closely related to the Aplysiomorpha and the Cephalaspidea (Fig. 45). Umbraculida have extremely small labial tentacles and short and massive rolled rhinophors (Hoffmann 1939). This supports the ground pattern for node 4 with a lip organ-like structure as ASO, as the labial tentacles of the Umbraculida show a higher similarity to the lip organ of the Cephalaspidea then to the labial tentacles of the Aplysiomorpha. The ground pattern of small basal rhinophores at node 4 is also supported by the taxa of this major clade, as most of these taxa have rhinophores.

The Pteropoda, another opisthobranch taxon, are also within a clade including the Cephalaspidea, Aplysiomorpha and Umbraculoidea, but the taxa of the Pteropoda are very derived (Klussmann-Kolb and Dinapoli 2006, Klussmann-Kolb et al. 2008). Within the Pteropoda two taxa have been described, first the Gymnosomata, which have two sets of CSOs, mentioned to be homologous to the labial tentacles and the rhinophores of the Aplysiomorpha (Hoffmann 1939) and second the Thecosomata, which have only one pair of tentacles, assumed to be homologous to the rhinophores of the Aplysiomorpha, showing a rudimental form (Hoffmann 1939). As the Thecosomata are a highly derived taxon, it can be assumed, that the ASOs are reduced. Nevertheless the Pteropoda also support rhinophores in the ground pattern of node four.

The next taxon, which I will discuss are the Sacoglossa which have formerly been

believed to belong to the Opisthobranchia. However the investigations of Klussmann-Kolb et al. (2008) indicate that this taxon is more closely related to the Pulmonata. The Sacoglossa have only one pair of tentacles, but this tentacle pair is provided by the three cerebral nerves N2, N3 and Nclc, (Hoffmann 1939, Huber 1993, own investigations), this indicates a fusion of the ASOs and the PSOs in the Sacoglossa, which was also mentioned earlier (Huber 1993).

In the investigation of Klussmann-Kolb et al. (2008) the formerly opisthobranch taxon of Acochlidiacea is closer related to the Pulmonata then to the rest of the Opisthobranchia. The CSOs of the Acochlidiacea have been described in detail by Sommerfeld and Schrödl (2005) and Hochberg (2007), but due to the compression of the CNS and the CSOs (due to extremely small species which live in the interstitial system) I am not able to postulate homology hypotheses for these organs within the Acochlidiacea.

In summary I postulate the following homology hypotheses (see also Fig. 46):

- The lip in all investigated Gastropoda is homologous.
- The ASOs as well as the PSOs of the investigated Euthyneura are homologous structures.
- The labial tentacles of the Nudipleura and the Aplysiomorpha are homoiolog.
- The lip organ, the Hancocks organ and the cephalic shield of the Acteonoidea and the Cephalaspidea are also homoiologous structures.
- The groove of the Acteonoidea is homologous to the labial tentacles of the Nudipleura.
- The rhinophores, as posterior tentacles, are homologous within all investigated taxa as they are included in the ground pattern of node one.
- The oral veil of the Pleurobranchomorpha might be homoiolog to the oral velum of the Dentronotoidea (i.e. *Tritonia diomedea* / Nudibranchia) as the oral veil is not included in the ground pattern for node 2.
- The tentacles of the Sacoglossa are derived fused structures which need further investigations

5. Outlook

As I have shown, it is possible to use neurobiological methods to answer evolutionary questions. Nevertheless new phylogenetic investigations postulate the Opisthobranchia as non-monophyletic, which is slightly supported by my investigations, as I rejected one of the last autapomorphies, the bifurcation of the N2, supporting a monophyletic taxon Opisthobranchia. Therefore the reconstruction of a ground pattern for the CSOs of the Opisthobranchia is theoretically impossible, as the taxon Opisthobranchia might not exists. Several opisthobranch taxa are missing in my investigations. The lack of these opisthobranch taxa is primary caused by methodological problems which are also relevant for other heterobranch taxa. The first problem is the number of replicates needed, as many euthyneuran taxa are cryptic species, and as second the axonal tracing technique is restricted to living species with a minimum size.

In detail I suggest to investigate the cellular innervation patterns for the cerebral nerves, and the neurotransmitter content for the CSOs of the following taxa. First of all, the Sacoglossa, as this opisthobranch taxon only has one pair of tentacles in the head region, and the earlier mentioned methods could be used to homologise them with the CSOs of other opisthobranch taxa. However, the phylogenetic investigations of Klussmann-Kolb et al. (2008) postulate that the Sacoglossa do not belong to Opisthobranchia but are more closely related to the Pulmonata. Therefore the investigations of the Sacoglossa might be very useful to confirm my hypotheses about the evolution of the CSOs.

As second I will suggest a detailed investigation of the Hancocks organ in different opisthobranch taxa which were not included in this study. I postulated that the term Hancocks organ should be restricted to the posterior part of the Hancocks organ of earlier studies. Therefore it would be useful to investigate taxa of the Acteonoidea, where the Hancocks organ is not reduced like in *Acteon tornatilis*. Furthermore *Tritonia diomeda* (Nudibranchia) should be investigated, as I have mentioned that the Hancocks organ of this species might only represent the anterior part of the Hancocks organ of earlier investigations and should be renamed as ASO. In addition, the other opisthobranch taxa with a Hancocks organ like the Aplysiomorpha (i.e. *Akera bullata*)

or the Acochlidiacea should be investigated to clarify the term and the definition of the Hancocks organ. Here it should be mentioned that this might cause some difficulties, as many Acteonoidea only show a scattered occurance. Furthermore, the Acochlidiacea are in general too small for the axonal tracing and only some rare indo-pacific species might reach the needed minimum size.

Other methods might be helpful to confirm or deny the postulated hypothesis for the evolution of the CSOs. Double labeling, which combines the axonal tracing with the neurotransmitter content (Chiasson et al 1994, Ierusalimsky and Balaban 2005, Kononenko and Zhukov 2005), might create an additional homology criterion for the cellular innervations patterns. But my own investigations (data not shown) indicate that the fluorescent marker Biocytin, which is used instead of Nickel-Lysine shows less cluster in smaller species, therefore these differences between these two markers which should show identical results need further empirical investigations.

Other kind of data, like the muscle structure (which could be shown with the fluorescent marker phalloidin) of the CSOs or the ontogeny of the CSOs might also be helpful. Ontogeny of several opisthobranch taxa (Aplysiomorpha - Wollesen et al 2007a,b, Nudibranchia – Carroll and Kempf 1994, Kristof et al. *in preparation*, Cephalaspidea – Schulze et al. *in preparation*) using immunohistological methods have been investigated until now. In future, other opisthobranch taxa should be added, but is has to be mentioned that ontogenetic studies are very time intensive and often complicated for marine gastropod species with a veliger larvae. Another approach, which is also very time intensive and expensive until now, might be neuronal transcriptomes, which have been described for *Aplysia californica* by Moroz et al. (2006). Neuronal transcriptomes represent expression patterns of single morphological or cellular structures and not of the whole animal (in the investigations of Moroz et al. (2006) neuronal transcriptomes of nervous structures were described). Therefore comparisons of the neuronal transcriptomes of other opisthobranch taxa might also create an independent data set useful for the homologisation of morphological structures. Here it has to be mentioned, that many Opisthobranchia are model organisms within the neurobiology, therefore it could be expected, that further describtions of neuronal transcriptomes for Opisthobranchia will follow.

At last, I want to mention that the approach of my study, as well as the methods mentioned earlier, which were commonly used for functional questions, could also be used for other questions, besides the evolution of the CSOs within the Opisthobranchia. I have shown that cellular innervation patterns within the CNS are very conserved structures, therefore they could be used to clarify homology hypotheses of other morphological structures besides the CSOs. In the Gastropoda, the CNS is clearly separated into different kind of ganglia which innervate defined body parts of the Gastropoda. Therefore cellular innervation patterns but also neurogenesis of buccal, pleural or pedal nerves could be used to homologise the organs and structures innervated by these nerves, e.g. structures the foot (parapodia).

Another usage of the cellular innervation pattern and the neurotransmitter content might be given within the central nervous system and not in the periphery. The CNS of the Gastropoda shows a high variation at the gross anatomical level. Ganglia are often fused or sometimes lacking when comparing different gastropod taxa. As for example, within the Opisthobranchia, the cerebral ganglion is often fused with the pleural ganglion, forming the cerebropleural ganglion, but also the occurrence of peripheral ganglia, like the rhinophoral ganglion is very variable. The most variation, regarding the CNS of Gastropoda is found within the visceral loop, which ganglia vary in numbers from two to five. Regarding the high conservation of cellular nervous structures, the cellular innervation patterns, but also the neurotransmitter content and the ontogeny might be used to define the ganglia at a cellular level and to reconstruct the evolution of the CNS within the Gastropoda, which is done until now primarily based on the gross anatomy.

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7. Acknowledgements

There are really a lot of people I need to thank, and as my English isn't the best, I will make it short.

First of all I want to thank all my scientific advisors, and I will start with Professor Dr. Annette Klussmann-Kolb, my supervisor and the chief of our workgroup Phylogeny and Systematic. I think it was not ever easy with me (especially in the end) and I am very grateful for her support. I thank "Mr. Neuron", Roger P. Croll who was one of the designers of this PhD study and supported me over the whole time. Furthermore, I would like to thank Prof. Dr. Heike Wägele for her critics and the organisation of the fantastic Opisthobranchia workshop in Bonn.

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Claudia Nesselhauf, the good soul of our workgroup, mistress of aquaria, technical assistance and secretary, Claudia, thank you (and she also proof-read the whole manuscript). Furthermore I thank Adrienne Jochum also for proof-reading.

Before I will thank several people, who supported me in field sampling or with the fantastic atmosphere in our institute, I will place my family here, my mom and my sisters and brother, who supported me over the whole time, yes I am alive and I love you all. My sister Tabea Staubach will be named here especially, as she is also a member of the small club which proof-readied my manuscript……The Dark Lords were greeting.

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So to finish, I thank all the people who have supported me, especially Christian Vogt, Christian Gerlinger, Martin Wagner, Marc Hasenbank, Benedikt and Cynthia (the games group).

To all the people I forgot…….Sorry.

V. Supplement Data

Table: I Used protocols for the Immunohistochemistry

5HT	**FMRFamide**	**TH**
Fixation: 4% PFA in 0,1 M PBS overnight at 4° C	**Fixation**: 4% PFA in 0,1 M PBS overnight at 4° C	**Fixation**: 99% Methanol, 1% Acetic acid-30 min at -18° C
Washing: 3xPBS (5`5`60 min)	**Washing**: 3xPBS (5`5`60 min)	**Bewässern:** 70/50/30% Methanol /(10`10`10 min)
		Washing: 3xPBS (5`5`60 min)
Permilisation and Blocking: 4% Triton, 1% **NGS**	**Permilisation and Blocking**: 4% Triton, 1% **NGS**	
in PBS overnight at 4°C	in PBS overnight at 4°C	**Permilisation and Blocking**: 0,2-1% Triton, 0,5-1% **NSS**
		in PBS overnight at 4°C
Washing: **3xPBS (5`5`60 min)**	**Washing:** **3xPBS (5`5`60 min)**	**Washing:** **3xPBS (5`5`60 min)**
PA and Blocking: 1/500-1/1000 PA;	**PA and Blocking:** 1/500-1/1000 PA;	
1% NGS; 0,2% Triton; overnight at RT	1% NGS; 0,2% Triton; overnight at RT	**PA and Blocking:** 1/50-1/250 PA;
		1% NSS; 0,2% Triton; overnight at RT
Washing: **3xPBS (5`5`60 min)**	**Washing:** **3xPBS (5`5`60 min)**	
		Washing: **3xPBS (5`5`60 min)**
SA: 1/500-1/1000	**SA:** 1/500-1/1000	
1% NGS; 0,2% Triton; overnight at RT	1% NGS; 0,2% Triton; overnight at RT	**SA:** 1/50-1/250
		1% NSS; 0,2% Triton; overnight at RT
Washing: **3xPBS (5`5`60 min)**	**Washing:** **3xPBS (5`5`60 min)**	**Washing:** **3xPBS (5`5`60 min)**
Mounting: 3/1 Glycerol in 0,5 TRIS	**Mounting:** 3/1Glycerol in 0,5 TRIS	**Mounting:** 3/1 Glycerol in 0,5 TRIS
pH 8,0 + 2% Propyl Gallate (anti fading agent)	pH 8,0 + 2% Propyl Gallate (anti fading agent)	pH 8,0 + 2% Propyl Gallate (anti fading agent)

Table IV: Table of the number of specimen, shell size calculated by the product of length and breath 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	14	11	12	5
5,67	9	4	6		
	8				
2	12	8	9	25	21
7,4	14	11	14	11	19
	9				
3	17	13	21	14	29
7,6	21	12	14	16	21
				17	8
4	18	31	19	17	24
7,82	19	13		18	27
		14			6
5	21	29	24	21	36
8,25	19	17	9	19	31
	18	18			12
6	22	31	26	22	31
10,08	21	19	17	23	34
		16	8	19	21
7	22	32	28	24	34
10,53	24	26	21	16	24
	18		15	19	21
8	13	33	22	20	36
10,92	15	28	21		21
		10	24		19
					24
					23
9	20	17	13	19	20
11,02	19		15	20	18
	13			21	17
	14				
	12				
10	23	17	21	23	21
12,71					
11	19	21	25	21	34
15,04	24	19		19	33

		23		19	29
					12
12	17	32	16	29	26
15,18	12	8	4	16	14
	8		7	9	3
	4				
13	17	23	11	26	23
17,5	18	23	21	12	17
	13	26		4	19
		15			
14	18	29	12	19	18
25,8		27	15	17	10
		7	9		9
		8	9		
		5	8		
		10	11		
15	24	8	14	24	27
17,5	13	12	17	28	18
	11	26	19		12
	6				
16	15	33	20	25	33
21,45	17	30	19		21
	16	23			23
	15	8			26
					28
17	36	12	12	17	14
25,8	21	15	15	16	12
	15	29	6	6	28
	14			24	
18	26	41	20	41	41
26,46	24	17	23	24	
	23	24	31		
	22	27	33		
		28	38		
19	9	21	11	22	28
34,3	12	23	12	19	22
	18	17	9	18	24
	16		25	13	
			16		
			14		
20	13	26	18	20	27
35,77	17	28	18	21	28
	18	27	16		22

	12		12		29
	16				9
	15				
	7				
21	22	31	33	24	40
37,63	17	30	26	22	22
	24	29	24	18	21
	29		22	16	
			17		
			16		
			15		
22	23	54	30	30	31
57,42	31	36	28	22	33
	29	32	27	21	32
	23	31	12	27	29
	22	29	15	28	28
	21	28			34
	20	24			35
	19	23			32
	17	19			
	24	18			
	19				
	24				
	23				
	12				
	27				
23	36	59	24	24	29
65,1	35	48	23	26	28
	34	36	27	27	27
	37	34	31	31	26
	38	24	37	41	32
	35	23	38	44	35
	28	22	39	42	41
	26	27	36	49	44
	27	18			
	29	17			
	29	18			
	28				
	26				

The supplement data also include three manuscripts, which could be found on the Supplement Data CD-Rom. These manuscripts are in follow:

1. Staubach S, Schützner P, Croll R P, Klussmann-Kolb, A (*in press*)
   Innervation patterns of the cerebral nerves in *Haminoea hydatis* (Linnaeus, 1758) (Gastropoda: Opisthobranchia) – A test for intraspecific variability.
   Zoomorphology, (DOI: 10.1007/s00435-008-0064-6)

2. Staubach S, Klussmann-Kolb A (2007)
   The cephalic sensory organs of *Acteon tornatilis* (Linnaeus, 1758) (Gastropoda Opisthobranchia) – cellular innervation patterns as a tool for homologisation.
   Bonner Zoologischer Beiträge 55: 311-318

3. Faller S, Staubach S, Klussmann-Kolb A (Zoomorphology – *in revision*)
   Comparative immunohistochemistry of the cephalic sensory organs in Opisthobranchia (Mollusca, Gastropoda)

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Frankfurt am Main, 30.05.2008

Eidesstattliche Versicherung

Ich erkläre hiermit an Eides Statt, dass ich die vorgelegte Dissertation über

"The Evolution of the Cephalic Sensory Organs within the Opisthobranchia"

selbständig angefertigt und mich anderer Hilfsmittel als der in ihr angegebenen nicht bedient habe, insbesondere, dass aus Schriften Entlehnungen, soweit sie in der Dissertation nicht ausdrücklich als solche mit Angabe der betreffenden Schrift bezeichnet sind, nicht stattgefunden haben.

Frankfurt am Main, den 02.06.2008 .....

(Unterschrift)