Streptoneury is independent from ontogenetic torsion in the caenogastropod snail *Marisa cornuarietis*

J. Anton Morath¹#, Stefan Fischer²,³#, Leonie Hannig¹, Simon Schwarz¹,⁴, Rita Triebskorn¹,⁵, Oliver Betz², Heinz-R. Köhler¹*

¹ Animal Physiological Ecology, Institute of Evolution and Ecology, University of Tübingen, Auf der Morgenstelle 5, D-72076 Tübingen, Germany
² Evolutionary Biology of Invertebrates, Institute of Evolution and Ecology, University of Tübingen, Auf der Morgenstelle 28, D-72076 Tübingen, Germany
³ Present address: Tübingen Structural Microscopy Core Facility, Applied Geosciences, University of Tübingen, Schnarrenbergstrasse 94-96, D-72076 Tübingen, Germany
⁴ Present address: German Environment Agency, Wörlitzer Platz 1, D-06844 Dessau-Roßlau, Germany
⁵ Steinbeis-Transfer Center Ecotoxicology and Ecophysiology, Blumenstrasse 13, D-72108 Rottenburg, Germany

# joint first authorship

* Corresponding author: heinz-r.koehler@uni-tuebingen.de

A hallmark in snails’ anatomy is the conspicuous crossing of the pleurovisceral nerve cords present in all but the most derived gastropod clades. This feature is called streptoneury and hitherto near-universally believed to derive from the process of torsion which is, ontogenetically, visible by a 180° rotation of the visceral sac relative to the cephalopodium, being also responsible for the formation of a cranially bent gut and the location of gills in a mantle cavity that opens to the anterior. However, the mechanical link between the ontogenetic rotation of the visceropallium and streptoneury has never been demonstrated directly. After suppressing ontogenetic torsion in the freshwater apple snail *Marisa cornuarietis*, we could show in a 3D reconstruction based on serial sectioning that the nervous system of the non-torted snail almost identically mirrored the classical organization of a normally developed individual and showed all features of streptoneury in this species. Furthermore, confocal laser scanning microscopy revealed the pleurovisceral cords not to be fully shaped after completion of ontogenetic torsion. We therefore conclude that, ontogenetically, and potentially also phylogenetically, torsion is not an implicit prerequisite for streptoneury, thereby fundamentally challenging a century-old ‘certainty’ in molluscan developmental biology and evolution.
The origin of the various body plans of molluscs has been a matter of speculation for a long time. More than 130 years ago, Lankester proposed the anatomy of a 'schematic mollusc' which later has been adopted as a blueprint for the 'ancestral mollusc's' body plan that has formed the basis for generations of textbooks. Today, there is almost unanimous agreement that the supposedly single-shelled unsegmented ancestral mollusc’s mantle cavity, or bilaterally positioned mantle cavities, together with the ctenidia (gills) and the anus were confined to the posterior end of the body, and the pleurovisceral nerve cords did not cross (Fig. 1A). Present-day snails show an anterior position of the mantle cavity, ctenidia and anus, and feature a crossing of the pleurovisceral nerves, the so-called streptoneury, in all but the most derived extant gastropod classes. This is explained by a hypothetical phylogenetic process, called 'torsion', that rotated all anatomical components of the visceropallium by 180° relative to the cephalopodium. To harmonize this theory with embryological observations of gastropod early development from the early 1900s Garstang proposed an evolutionary saltation by a macromutation that altered the embryonic development of an ancient, pre-gastropod mollusc and gave rise to the 'ontogenetic torsion' still preserved in the developmental programme of today’s gastropods. According to this theory, the ontogenetic torsion is represented by the clearly visible 180° counterclockwise rotation of the visceropallium, directly resulting in the establishment of streptoneury, together with a U-shaped gut and an anteriorly positioned mantle cavity in extant snails. However, more than a decade ago this causal relation, as well as the view on torsion as a uniform process have been questioned based on embryological observations in the vetigastropod Haliotis kamtschatkana as not all components of the visceropallium were found to rotate synchronously in this species. In consequence, Page proposed the formation of the gastropod’s anterior mantle cavity from only a single cavity on the right of, originally, a bilateral set of mantle cavities ('asymmetry hypothesis'). Nevertheless, also this hypothesis involves a rotation of visceropallial structures by 90°-180° which, according to figure 6 in that publication, supposedly leads to streptoneury. However, it has never been directly demonstrated that ontogenetic torsion mechanically twists the pleurovisceral nerves and thus leads to streptoneury, because nerve cords that join the posterior ganglia in adult snails could not be visualized in gastropod embryos or larvae. Even though several serotonin-, FMRFamide- and catecholamine-immunoreactive cells have been found in early embryonic and larval stages of snails they seem to 'disappear' later and are replaced by an 'adult' nervous system after a short time of coexistence and cooperation. Thus, despite of its implied conclusiveness, the proof for a causal linkage of ontogenetic torsion and streptoneury in the adult gastropod has not yet been provided.
Figure 1. Organisation of the gastropod nervous system, schematic. Portions held in orange point to the observer. (A) Situation proposed for the ancestral, non-torted pre-gastropod mollusc, dorsal view. The pleurovisceral nerve cords connecting the pleural ganglia and the visceral ganglion (VG) extend over both lateral sides of the visceropallium and do not cross. (B) Situation in extant caenogastropods, after torsion, dorsal view. Left: Prototype. The proximal portions of the two pleurovisceral nerve cords cross the longitudinal body axis dorsal (red) and ventral (blue) of the intestine, forming streptoneury. Right: The situation in ampullariids, such as *Marisa cornuarietis*: As the subintestinal ganglion (SBG) has fused with the right pleural ganglion (RPlG), the connecting nerve cord, the subintestinal nerve (SBN, blue) still comes to lie ventral of the intestine but has moved to the anterior, masking the pleurovisceral nerve crossing. Nevertheless, the transversal course of the supraintestinal nerve (SPN, red) across the longitudinal body axis indicates the persistence of streptoneury in this derived situation. (C) Anatomy of the main components of the nervous system in *M. cornuarietis*, redrawn after Demian & Yousif\textsuperscript{15}. Right: Dorsal view, corresponding to the scheme displayed in B, left side. Left: Lateral view from the left. Labels: BG: buccal ganglion; CG: cerebral ganglion; CPeN = cerebro-pedal connective; CPIN: cerebro-pleural connective; LPaG: left parietal ganglion; LPeG: left pedal ganglion; LPlG: left pleural ganglion; LPz: left zygosis; OSG: osphradial ganglion; RPaG: right parietal ganglion; RPeG: right pedal ganglion; RPlG: right pleural ganglion; SBG: subintestinal ganglion; SBVN: subintestinal-visceral connective; SPG: supraintestinal ganglion; SPVN: supraintestinal-visceral connective.
In the caenogastropod apple snail, Marisa cornuarietis (Linnaeus, 1758), the classical anatomy of the nervous system, including streptoneury, is secondarily modified (Fig. 1B). In the apple snail family, Ampullariidae, the right pallial ganglion, called subintestinal ganglion after torsion, has moved to the anterior and fused with the right pleural ganglion, thus disguising the crossing of the pleurovisceral cords to some extent. Nevertheless, the presence of the supraintestinal nerve transversely crossing the longitudinal midline body axis clearly demonstrates streptoneury also in ampullariids. On the left side of the body, a secondary nerve, the left zygosis, connects the left pleural ganglion with the supraintestinal ganglion which, in turn, is connected to the osphradial ganglion which develops at a later embryonic stage by proliferation of the tissue below the osphradium itself. The anatomy of the nervous system of M. cornuarietis has been described in detail by Demian and Yousif15 (Fig. 1C).

To clarify whether, in M. cornuarietis, streptoneury is a direct mechanical consequence of ontogenetic torsion we used two approaches. First, we investigated whether the pleurovisceral cords are already present prior to the rotation of the visceropallium, so that they have a chance to be twisted during this process or whether they are formed for the first time after ontogenetic torsion. Second, we used a methodology to block ontogenetic torsion during the embryonic development of M. cornuarietis and 3D reconstructed the nerve system of a non-torted individual after completed embryonic development. The latter was possible by chemically preventing the TGF-β cytokine-dependent16 differential outgrowth of the mantle and thus the counter-clockwise rotation of the visceropallium which results in individuals with a posterior ctenidium, lacking both mantle cavity and external shell17,18 (Fig. 2A). Instead of torsion, these non-torted individuals showed a slight, at most 90° horizontal rotation of the visceral sac along the longitudinal axis to the left19.

Results

Using confocal laser scanning microscopy (CLSM), we could visualize all prominent ganglia and present nerve cords by 5-hydroxytryptamine (serotonin)-like immunolabelling in situ in an individual 4 days post fertilization (dpf) which had completed torsion (Fig. 2B). At this developmental stage, several connectives and commissures were still incomplete. Particularly the formation of the supraintestinal nerve had just started with tiny portions of nervous tissue protruding from both the supraintestinal ganglion on the left and the ganglionic mass formed by the fusion of the pleural ganglion and the subintestinal ganglion on the right. The incompleteness of connectives revealed by immunolabelling even after completion of the rotation of the visceropallium contradicts the assumption that fully developed pleurovisceral nerve cords are mechanically twisted by ontogenetic torsion. Nevertheless, at a later stage, at 56 dpf, a three-dimensional reconstruction of the nervous system of a normally developed, torted individual20 revealed the establishment of all the ganglia and commissures described for the adult nerve system before15 (Fig. 3A).
Figure 2. Habitus and organization of the nervous system. (A) Habitus of *Marisa comuarietis*. Two-months-old non-torted individual (left), Torted, non-transformed individual prior to hatch, 10 dpf (middle) and hatched non-torted individual, 14 dpf (as analysed in this study, right). Labels: CT: ctenidium (gill); F: foot; G: gut; H: head; VP: visceropallium. Scale bars: 500 µm. (B) Organisation of the nervous system of a normally developed, torted *M. comuarietis* individual, 4 dpf. Confocal laser scan microscopy, nerve cells labelled by an anti-serotonin antibody (rabbit-anti-serotonin whole antiserum). Visualised as maximum intensity projection of the image stack (left) and processed model of the stained areas (right). Artificial colouration of the nerve system in yellow/orange shades of colour. Portions held in orange point to the observer. Non-labelled body parts kept semi-transparent (left) or excluded from visualization (right). Labels as in Fig 1.
Figure 3. Three-dimensional reconstructions of the nervous system of *M. cornuarietis*, based on serial sections. (A) Normally developed, torted individual, 56 dpf, dorsal view. The supraintestinal nerve (SPN) and the supra- and subintestinal-visceral connectives (SPVN, SBVN) are not fully visualized due to limitations in resolution (section thickness 5 µm, taken from Hannig20). (B-E) Non-torted individual, 14 dpf, that exhibits streptoneury as indicated by the transversal course of the supraintestinal nerve (SPN) across the longitudinal body axis. Nervous system in yellow, intestinal tract including hepatopancreas in blue and eyes in red and purple. (B) Dorsal view. (C) Lateral view from the left. (D) View obliquely from the left above. (E) View obliquely from the front above. Labels as in Fig 1.

The three-dimensional reconstruction of the nervous system, together with the intestinal tract in a non-torted individual 14 days post fertilization (Figs. 3B-E) almost identically mirrors the neuroanatomy described by Demian & Yousif15 for torted *M. cornuarietis*. The definitive type of the nervous system of *M. cornuarietis*, independent whether undergone torsion or not, is of the streptoneurous hypoathroid kind, i.e. with the pleural ganglia (LPIG and RPIG) adjacent to the pedal (LPeG and RPeG) rather than to the cerebral ganglia (CG)8. Paired cerebral, pedal and pleural ganglia are arranged in a circumenteric ring. Distant from that ring are the sub- (SBG) and supraintestinal (SPG) ganglia and the unpaired visceral ganglion (VG). The connection between supraintestinal ganglion and right pleural/subintestinal ganglionic mass, the supraintestinal nerve (SPN), crosses over the digestive tract and the longitudinal midline body axis and is indicative for fully established streptoneury. The intercrossing connection between the left pleural ganglion and the subintestinal ganglion, the subintestinal nerve (SBN), also passes the midline body axis, but ventral of the digestive tract (Fig. 4A-B) as required for streptoneury. The secondary connection between the left pleural and supraintestinal ganglia, i.e. the left zygosis (LPRN) is also present, as in torted ampullariids. There are only two slight modifications in the neuronal anatomy of the untorted snail: the supraintestinal and subintestinal ganglia are at approximately equal height and the osphradial ganglion (OSG) is not located at the height of the supraintestinal ganglion but more to the ventral (Fig. 3C-D). Both modifications very reasonably result from the ≤90° horizontal leftward movement of the visceral sac in artificially created non-torted individuals (Fig 4C).

Discussion

Our results lead to the following conclusions and proposals:

1. Previous failures to visualize pleurovisceral nerve cords in pre-torsion stages of snail larvae are likely not to be attributed to inadequate staining techniques, but rather to the absence of them in these stages. Despite its high resolution and potential to visualize single neurons, also the CLSM method used here did not provide any evidence for the presence of pleurovisceral nerve
Figure 4. Posterior view on the nervous system and axes of rotation. (A) Non-torted individual, 14 dpf, posterior view of the anterior part of the nervous system. Intestinal tract electronically removed. (B) Identical view, electronic sectioning reveals streptoneury as the subintestinal nerve (SBN) transverses across the longitudinal body axis, ventral of the intestine. Nervous system in yellow, sectional plane in dark yellow, intestinal tract in blue and eyes in red and purple. OSN: osphradial nerve. Other labels as in Fig 1. (C) Rotation axes and direction of rotation of the visceropallium (VP) versus the cephalopodium (CP) in normal, torted *M. cornuarietis* individuals (180°, top) and in non-torted individuals (90°, bottom).
cords that could have been mechanically twisted during ontogenetic torsion. Decades ago, Demian & Yousif\textsuperscript{15} described the cerebral, intestinal, pleural and pedal ganglia of \textit{M. cornuarietis} to arise and develop simultaneously and separately by delamination from the ectoderm at an early embryonic stage. The visceral ganglion develops later, by delamination from the right side of the visceral sac. Already this study has proposed a secondary formation of commissures and connectives developing as 'extensions' from the periphery of the ganglia. As well in \textit{H. kamtchatkana}, the first two neurites of a neuron that was formed before ontogenetic torsion and appeared to delineate the trajectory of the future pleurovisceral nerve cords did not cross over during torsion because this neuron's soma was shifted in the same direction as the rotating visceropallium\textsuperscript{14}. Nevertheless, also in that case a full crossing of the pleurovisceral connectives occurred secondarily at later stages in ontogeny. In the pulmonate snail, \textit{Lymnaea stagnalis}, the pathways of embryonic neurites seem to exhibit streptoneury and, later, also detorsion (a feature typical for Pulmonata) but these structures did not appear to join the ganglia of the future adult nerve system, ceased to express immunoreactivity and disappeared after hatching\textsuperscript{12,13}. We therefore must conclude that the establishment of streptoneury in the post-embryonic nerve system is a secondary process and, at most, triggered by the early neurons’ pioneer pathways and their signals which may act as guidance for the neuronal growth of the developing adult nerve system\textsuperscript{11}.

(2) The development of streptoneury is independent of whether or not the parietal ganglia have changed places during ontogenetic torsion, at least in \textit{M. cornuarietis}. Plausibly, any rotation of the visceropallium may mechanically relocate structures. This is confirmed by our study as the observable 90° movement of the visceropallium in the non-torted individual\textsuperscript{19} (Fig 4C), which most likely is due to the increasing weight of the growing shell that pulls down the left side of the body went along with a shift of the position of at least 3 posterior ganglia. On the one hand, the osphradial ganglion that develops by proliferation directly below the osphradium was shifted ventrally (Fig. 3D) and, on the other hand, the arrangement of the supra- and subintestinal ganglia (which are considered homologous to the parietal ganglia; cf. Fig. 1A-B) relative to one another was tilted in the same direction and came to lie at almost the same height (Fig. 3E). Even though torsion likely is not a uniform process\textsuperscript{9,17} and thus may result in gradual interspecific variation in the degree to which different organs are displaced\textsuperscript{9}, we do not question the general possibility that the right and the left parietal ganglion change places in the course of the rotation of the visceropallium. However, in the non-torted (torsionally suppressed) \textit{M. cornuarietis} individual, we assume that the two parietal ganglia have not been interchanged because the visceropallium does not show any horizontal movement (which is supposed to be the driving process behind this exchange). Although the sub- and supraintestinal ganglia of the non-torted individual almost certainly originate from the right and the left parietal ganglia, respectively – and not, \textit{vice versa}, from the left and the right parietal ganglia (as traditionally assumed in torted snails) --, the key criterion of streptoneury, i.e. the transversal crossing of the midline axis by the supra- and
subintestinal nerves (as shown in Fig. 1B: right, C), was nevertheless established also in the non-torted snail. Our findings allow the explanation that, in torted *Marisa* snails, the pleurovisceral connectives, which are formed after ontogenetic torsion, grow crosswise towards the opposite ganglia across the central axis. The same occurs when ontogenetic torsion has been blocked: also in this case, the pleurovisceral connectives connect the opposite ganglia in a crosswise manner. This suggests that, independent of a rotation of the visceropallium, the ultimately relevant information for the formation of the adult nervous system, including streptoneury, is established at the earliest at a point in time after the ontogenetic torsion has taken place in torted individuals.

(3) We propose that, in *M. cornuarietis*, streptoneury is determined not concurrently with but rather subsequent to – or even independent of – the rotation of the visceropallium; potentially by differential expression of attractive or repulsive molecular signalling factors and their transmembrane receptors that direct the outgrowth of neurons from one ganglion to another across the midline body axis. On the basis that the mechanical twisting of connectives and the formation of crossed pleurovisceral nerve cords within the developing visceropallium have never been confirmed experimentally, Haszprunar has proposed that cytochemical markers may guide pioneer neurites of the pleurovisceral connectives to their final positions and that, eventually, spatial coordinates of these markers have been preserved from a pretorsional ancestor. Indeed, FMRF amide-positive neuron-like cells that occur very early in pretorsional states of the caenogastropod *Crepidula fornicata* and the pulmonate *Lymnaea stagnalis* seem to delineate the course of the future pleurovisceral cords. Furthermore, the expression pattern of *Has-Hox 3* in pretorsional larvae of *Haliotis asinina* has been interpreted as a possible template for the arrangement of the pleurovisceral connectives. We know that asymmetric expression of genes of and upstream the Nodal signalling pathway (*Nodal, Pitx, Ldia2*) establish chirality in a number of snail species at the molecular level long before detectable morphological asymmetries occur. Thus, it may well be possible that gradients of signalling factors are established very early in snail embryonic development, remain unaffected by ontogenetic torsion, and later guide connections between ganglia according to their positions given at that time. It is also possible that the expression patterns of those genes responsible for these signalling factors were already present in pretorsional gastropod ancestors in the Devonian – after all, we do not know whether streptoneury had been established in these ancient organisms. Although the results of our study cannot shed light on this, they clearly show that directional growth of nerve fibers across the midline axis is independent of ontogenetic torsion. In non-molluscan taxa, which do not show developmental processes comparable to torsion, the interactions of biochemical signal molecules, transcription factors and receptors that regulate axon guidance across the midline axis are well known. To give just two examples, the Netrin-Frazzled/DCC and Slit-Robo pathways organize the formation of the numerous commissures in the nervous systems of insects, and Slit2 together with the Sonic Hedgehog signalling cascade and other factors (Gli2, Lhx2, FoxG1, FoxD1) are responsible for the crossing of commissures in the optic chiasm of vertebrates. Thus, as implicated by the present study, further research should be conducted to elucidate the
biochemical signal transduction processes responsible for nerve growth across the midline axis in molluscs.

The present demonstration of the independence of streptoneury and torsion in the apple snail *M. cornuarietis* refutes, despite individual case, the universality of the assumption that these two criteria are necessarily based on each other, at least from the view of ontogeny (Popper’s black swan paradigm\(^30\)). The fact that streptoneury in molluscs is also possible without rotation of the visceropallium does not necessarily refute the generally accepted phylogenetic view of the mechanisms behind this distinctive feature of the Gastropoda, but it fundamentally challenges it. Thus, it needs to be discussed whether either the developmental programme of present-day gastropods does not necessarily reflect the phylogenetic origin of gastropods or whether the phylogenetical torsion hypothesis used to explain the formation of streptoneury in due conjunction with the rotation of the visceropallium appearing for the first time at the rise of the Gastropoda in the Devonian needs to be reconsidered. Further research on the ontogeny of the nervous system in more basal taxa of gastropods, in which developmental processes are specifically suppressed, should provide evidence in this regard in the future.

**Materials and methods**

**Test animals.** We used the caenogastropod species *Marisa cornuarietis* (Linnaeus 1758) [Ampullariidae], because previous research has enabled us to block the process of ontogenetic torsion by high concentrations of PtCl₂ using the protocols of Osterauer et al.\(^{17}\) and Marschner et al.\(^{18}\). The rearing conditions of the lab stock culture were the same as in their work (17, 18, 20). The embryonic development and organogenesis of *M. cornuarietis* has been described in detail in a series of papers by Demian and Yousif\(^{15,31}\). The nomenclature used in their papers was adopted in the present work to simplify comparison between the normal development involving torsion and the artificially altered development in which torsion was blocked in focus of this work.

**Confocal laser scanning microscopy.** Normally developed embryos (4 days post fertilisation) were removed from the egg capsule and anesthetized in acidulated mineral water (Aqua culinaris, Hansa-Heemann AG, Rellingen, Germany) for 15 to 30 min. After relaxation, the embryos were fixed in 4% paraformaldehyde containing 0.01% Triton X overnight. Samples were washed 4 x 5 min in 0.01 M phosphate-buffered saline (PBS) containing 0.1 % sodium azide. This was followed by 4 h blocking in blocking buffer (1% Triton X, 3% horse serum and 0.1 % sodium azide in PBS) at 6°C and subsequent 96 h incubation with the primary antibody (rabbit-anti-serotonin whole antiserum (Sigma-Aldrich, St. Louis, MO, USA)) 1:200 in blocking buffer at 6°C. Incubated samples were washed 8 x 45 min in PBS containing 0.1% sodium azide, and afterwards incubated for 96 h in secondary antibody (goat-anti-rabbit IgG, fluorophor Alexa 488® (Invitrogen, Eugene, OR, USA)) 1:200 in blocking buffer at 6°C. This and all following steps took place in darkness. Stained samples were washed 4 x 30 min PBS. Samples were cleared at room temperature for 7 weeks in ScaleB4\(^{32}\), which was also used as mounting medium during microscopy.
The samples were analysed using a confocal laser scanning microscope (Leica TCS SPE, Leica Microsystems, with Leica Application Suite - Advanced Fluorescence (LAS-AF, Version 2.6.0.7266); illumination wavelength: 488 nm, emission wavelength: 519 nm (fluorescence) and 488 nm (transmission), 10 x air objective, 1024 x 1024 pixels, 400 Hz, phase correction: -31.83, pinhole size: 94.34 µm). The obtained image stacks were processed by 3D deconvolution to remove noise and improve image quality. Images were evaluated using FIJI-ImageJ (fiji.sc33). The overview image shown in Fig. 2B (left) was generated using maximum intensity projection. The structure of the stained areas was retraced in Amira 5.2.1 to generate a 3D model of the nervous system, shown in Figure 2B (right).

Blocking ontogenetic torsion. Egg clutches of *M. cornuarietis* were scraped off the aquarium wall with a razorblade and extracted from the clutch with pipette tips, as in Osterauer et al.17,34. Treatment with PtCl₂ was executed according to Osterauer et al.17. In that same paper as well as in the present work, glass Petri dishes and filtered tap water from the aquaria for raising the lab stock culture were used and the medium was exchanged daily; however, different concentrations were necessary in the present study. PtCl₂ concentrations (400 µg/l) were higher than in Osterauer et al.17, as it was the aim to obtain non-torted individuals as reliably as possible. The individual used for serial sectioning was approximately 1 mm long after 14 days post-fertilization (dpf). Note that PtCl₂, apart from having the desired effect of interfering with torsion also decelerates growth35.

Histological fixation and embedding. After 14 dpf, the individual used in this experiment was near the maximal size that would still allow 3D reconstruction using the ultramicrotome approach at our disposal. The sample was fixed in 2% glutaraldehyde buffered in 0.01 M phosphate buffered saline (PBS) at pH 7.4 and 5°C for one week. The organism was then washed three times for 15 minutes with 0.01 M PBS, pH 7.4 before it was postfixed in 1% osmium tetroxide (OsO₄) solution buffered with 0.1 M cacodylate (pH 7.4). Postfixation with osmium tetroxide, not mandatory for light microscopy, was chosen to allow for re-sectioning and reassessing the same samples with electron microscopy, if light microscopy proves insufficient for answering the present questions (36). After washing the sample first in PBS, then in H₂O, 2x10 minutes respectively, the individual was dehydrated in an ascending series of ethanol dilutions (10 min in 30%, 15 min in 50%, 15 min in 70%) and decalcified for 3 hours with 98% formic acid mixed 1:1 with 70% ethanol to dissolve the internal shell the snail was known to produce after PtCl₂ treatment (18). The ascending ethanol series was then completed with two steps of 10 minutes in 90% and 100%, before the sample was passed through increasing concentrations of Spurr’s embedding resin in acetone (3:1, 1:1, 1:3, 100% Spurr). All steps were performed at room temperature, unless stated otherwise. To centre the probe in the resin block, a thin ’ground-layer’ of resin was pre-polymerised (4.5 hours at 70 °C) in the embedding moulds. The sample was then oriented in the moulds to produce transversal slices from anterior to posterior in fresh pure resin (formulated for hard blocks) and was then polymerized at 70 °C for 10 hr.
Series sectioning and digitalization. Serial sections of 600 nm thickness were cut with a Jumbo-Histo-diamond knife on a Leica Ultracut Microtome, collected on glass slides and then stained with Toluidine blue for 30 s at 60 °C on a hot plate. The image series was generated at a Zeiss Axioplan microscope, equipped with a Nikon D7100 camera, using a 10x Plan-Neofluar objective and Helicon Remote 3.6.2.w software. After BW-conversion the images were aligned in FIJI\textsuperscript{33} using TrakEM2\textsuperscript{37} first by a rigid alignment followed by a second alignment using the 'Elastic Stack Alignment' plugin\textsuperscript{38}. The stack was exported to Amira 6.0 for the actual reconstruction. Structures of interest, such as the thick outer nerve cell regions and central fibrous core zones comprised of nerve fibres mentioned in Demian & Yousif\textsuperscript{15}, were outlined by hand using a Wacom drawing tablet or by using the semi-automatic segmentation tools in Amira 6.0. Additionally, easily visible structures like the alimentary tract and the eyes were labelled for better orientation. Mesh generation (creating a digital surface) and surface smoothing was performed cautiously with the integrated smoothing option in Amira’s Generate Surface module in order to preserve the more delicate protrusions of the nervous system of \textit{M. cornuarietis} and to maintain a realistic picture of where the limits of the used methods lie in terms of Z-resolution (= section thickness). Smoothing was performed independently on the different labels (nervous system, alimentary tract, retina and vitreous body of the eyes). Except for the nervous system, a second smoothing step with the smooth surface module was performed. Visualization of the final meshes for the image plates was done in Amira 6.0.

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**Author contributions**

J.A.M. and H.-R.K. wrote the main manuscript text. S.F., S.S. and H.-R.K. prepared the figures. J.A.M., S.F. and L.H. performed the histological analyses and compiled the data, partly supervised by R.T. and O.B.; S.S. performed the cytochemical labelling and the CLSM analysis. H.-R.K. designed the study and interpreted the data. All authors reviewed the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

**Correspondence** should be addressed to H.-R.K. All images and AMIRA visualization are available from S.F. or H.-R.K.