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Original Article

Streptoneury does not necessarily require horizontal torsion of the visceropallium during ontogeny: A singular observation of existence in the caenogastropod snail *Marisa cornuarietis*

J. Anton Morath[1,#], Stefan Fischer[2,$,#], Simon Schwarz[1,§], Rita Triebskorn[1,3], Oliver Betz[2] and Heinz-R. Köhler[1,*]

[1]Animal Physiological Ecology; [2]Evolutionary Biology of Invertebrates, Institute of Evolution and Ecology, University of Tübingen, Auf der Morgenstelle 5/28, D-72076 Tübingen, Germany; [3]Steinbeis-Transfer Center Ecotoxicology and Ecophysiology, Blumenstrasse 13, D-72108 Rottenburg, Germany.

ABSTRACT

A hallmark in snails' anatomy is the conspicuous crossing of the pleurovisceral nerve cords present in most basal 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° horizontal rotation of the cephalopodium relative to the visceral sac, 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, a mechanical link between the ontogenetic rotation of the visceropallium and streptoneury which is implied in many textbook presentations has never been demonstrated directly. After suppressing ontogenetic torsion and replacing it with a 90° vertical tilt of the visceropallium in an individual of 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 mirrored the classical organization of normal, torted individuals and showed all features of streptoneury in this species. Furthermore, immunolabelling provided no indication that the pleurovisceral cords were fully shaped after completion of ontogenetic torsion. This singular observation provides an experimental proof for the doubts that have recently arisen about the association between streptoneury and 180° horizontal ontogenetic torsion.

KEYWORDS: chiastoneury, embryology, Gastropoda, nervous system, snail, streptoneury, torsion.

INTRODUCTION

The origin of the various body plans of molluscs has been a matter of speculation for a long time. More than 140 years ago, the importance of torsion and the structure of the gastropod nervous system in the context of the monophyly of Mollusca has been recognised [1]. In 1883 Lankester [2] proposed the anatomy of a 'schematic mollusc' which later

*Corresponding author
heinz-r.koehler@uni-tuebingen.de
[#]joint first authorship; the authors contributed equally to this work
[$]Present address: Tübingen Structural Microscopy Core Facility, Applied Geosciences, University of Tübingen, Schnarrenbergstrasse 94-96, D-72076 Tübingen, Germany.
[§]Present address: German Environment Agency, Wörlitzer Platz 1, D-06844 Dessau-Roßlau, Germany.

has been adopted as a blueprint for the 'ancestral mollusc's' body plan [3, 4] that has formed the basis for generations of textbooks. Today, there is almost unanimous agreement that the supposedly single-shelled unsegmented [5] 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 most basal extant gastropod classes. This is hypothetically explained by a phylogenetic process, called 'torsion', that rotated all anatomical components of the visceropallium by 180° relative to the cephalopodium [3, 6, 7, 8]. To harmonize this theory with embryological observations of gastropod early development from the early 1900s Garstang [9] 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 horizontal 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 [5, 10, 11].

Since ontogenetic torsion and streptoneury occur simultaneously in many basal extant snails such as Patello-, Veti- and Caenogastropoda, and as this connection seems plausible, repeated observations led to the general view expressed in numerous textbooks that streptoneury is always coupled to ontogenetic torsion which manifests itself through the counter-clockwise rotation of the visceropallium *vs.* the cephalopodium (or clockwise rotation of the head-foot vs. the visceral hump [11]) and, even more, that ontogenetic torsion is the only mechanistic cause and prerequisite for streptoneury. Although Fitzhugh [12] pointed out that phylogenetic reconstructions are neither testable nor falsifiable because they are based on unique historical events and falsificationism should be considered inappropriate as an approach for phylogenetic studies [13], the extrapolations of a phylogenetic concept into developmental biology, however, are potentially falsifiable and can come in conflict with observations of counterexamples. According to the logic of critical rationalism [14], falsification of these statements will be achieved, if a single snail that has not been torted by 180° horizontal rotation but nevertheless exhibits streptoneury is found. The singular observation of streptoneury without preceding ontogenetic torsion will thus serve to show that the universal statement 'horizontal ontogenetic torsion is the only mechanistic cause and prerequisite for streptoneury' is wrong (*modus tollendo tollens*). This would not, of course, prove an alternative universal principle that supersedes the previous doctrine, but merely show that horizontal ontogenetic torsion is not a prerequisite for streptoneury in all cases.

More than a decade ago, the causal relation between ontogenetic torsion and streptoneury, as well as the view on torsion as a uniform process have been questioned [15] based on embryological observations in the vetigastropod *Haliotis kamtschatkana* [16] as not all components of the visceropallium were found to rotate synchronously in this species. In consequence, Page [15] 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 [17]. 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 [17, 18, 19, 20]. Thus, despite of its implied conclusiveness, the proof for a causal mechanical linkage of ontogenetic torsion and streptoneury in the adult

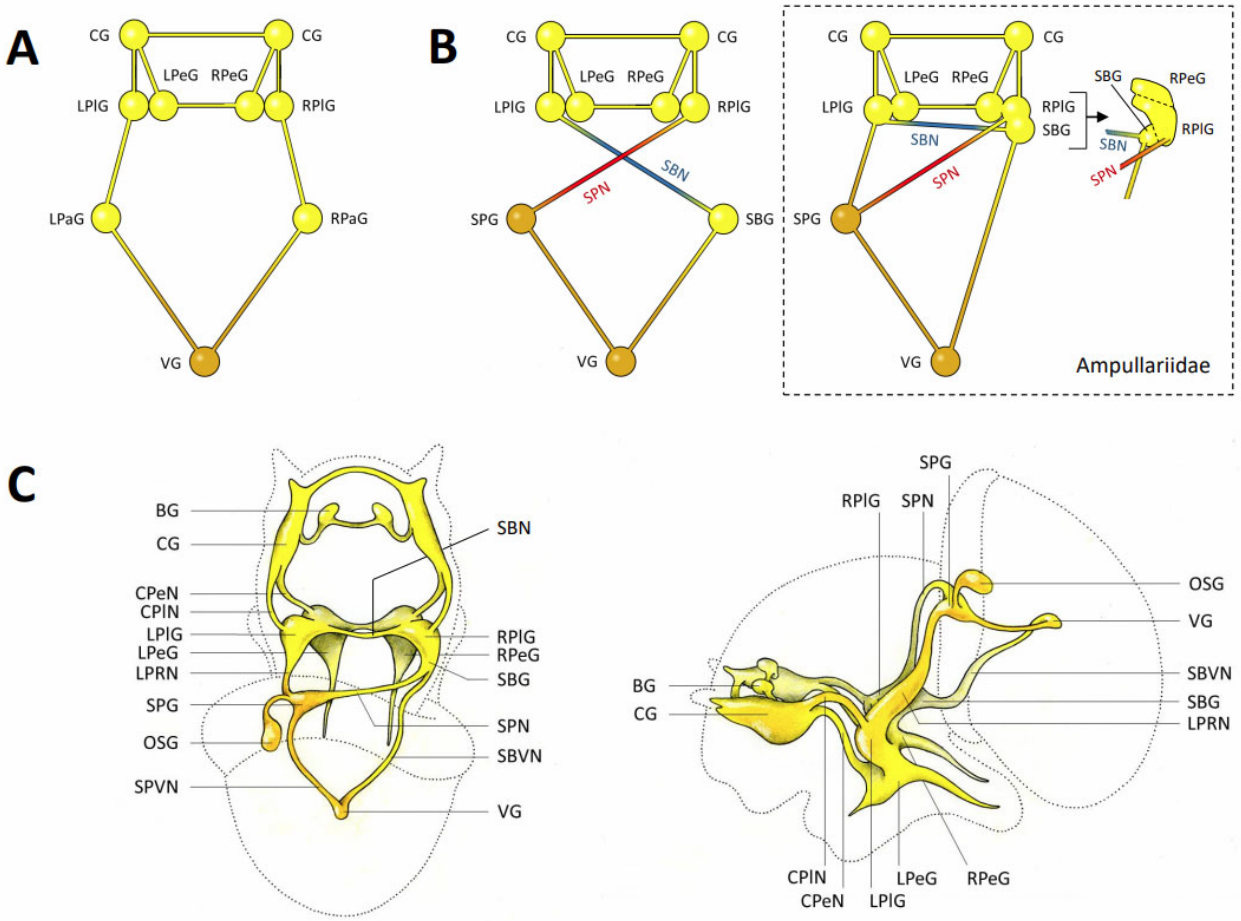

Fig. 1. Schematic drawings of the organisation of the gastropod nervous system. Portions held in orange point to the observer. (A) Situation proposed for the ancestral, non-torted pre-gastropod mollusc, dorsal view. The pleurovisceral nerve cords connecting the pleural ganglia and the visceral ganglion (VG) symmetrically 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. The originally left parietal ganglion, which now lies on the right side of the body, is now called the subintestinal ganglion (SBG) because of its more ventral position. The originally right parietal ganglion, now lying more dorsally on the right side of the body, is re-named the supraintestinal ganglion (SPG). Right (in dashed box): The situation in ampullariids, such as *Marisa cornuarietis*: As the subintestinal ganglion (SBG) has fused with the right pleural ganglion (RPlG) (and, to some extent, also with the right pedal ganglion (RPeG)), 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. The rightmost image detail displays the situation in the ampullariid snail *Afropomus balanoideus* (redrawn after Figure 12 in [39]) in which the crossing of the SBN and the supraintestinal nerve (SPN, red) is even more camouflaged by the posterior extension of the right pleural ganglion within the fused ganglionic mass. Nevertheless, the transverse course of both the supraintestinal and the subintestinal nerves 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 [21]. Left: Dorsal view, corresponding to the scheme displayed in B, left side. Right: Lateral view from the left. Labels: BG: buccal ganglion; CG: cerebral ganglion; CPeN: cerebro-pedal connective; CPlN: cerebro-pleural connective; LPaG: left parietal ganglion; LPeG: left pedal ganglion; LPlG: left pleural ganglion; LPRN: 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.

gastropod has never been provided. In general, such a generalising concept across all basal gastropods seems quite unlikely, as some neritimorph and caenogastropod families with streptoneury (e.g., Neritidae, Hydrobiidae, Aciculidae, Pomatiidae) do not show any externally visible signs of ontogenetic torsion. Thus, doubts about the strict association of ontogenetic torsion and streptoneury have increasingly emerged over the past two decades.

The present study deals with the development of the nerve system in the caenogastropod apple snail, *Marisa cornuarietis* (Linnaeus, 1758). Its normal development has been examined in great detail by Demian & Yousif [21]. As outlined in that study and also for another ampullariid [22], neurogenic placodes for the parietal, osphradial, and visceral ganglia are formed by thickened portions of mantle epithelium at discrete positions of the embryonic visceral sac which later invaginate and/or delaminate inward to form these ganglia [7, 23]. Rudiments of the cerebral, pedal, pleural and parietal ganglia arise at a rather early stage, the so-called stage V (nomenclature according to [21]). In the next stage VI, pedal and pleural ganglia fuse to form a pleuro-pedal ganglionic mass and the two parietal ganglia are still symmetrical. At stage VII ontogenetic torsion, the 180° horizontal rotation of the visceropallium relative to the cephalopodium, starts. Concurrently, the right parietal ganglion crosses to the left over the gut and persists as the supraintestinal ganglion. The left parietal ganglion shifts to the right and forward and becomes the subintestinal ganglion. Under normal conditions, the rotation of the visceropallium, the hallmark feature of ontogenetic torsion, is completed after 100 hours [24]. According to Demian & Yousif [21], the two nerves that typically mirror streptoneury because of their crossing, the supraintestinal and the subintestinal nerve, are first visible after full completion of the ontogenetic torsion, namely in stages VIII and IX, respectively.

Through previous work on the effects of platinum ions on rapidly outgrowing tissues [25, 26, 27] we were able to block the counter-clockwise horizontal rotation of the visceropallium around the dorsoventral body axis during development and thus ontogenetic torsion in *M. cornuarietis*, and to replace torsion by a 90° leftward tilt of the visceral sac around the longitudinal body axis (Fig. 2). This fundamental, induced change in the movement of the visceropallium during ontogeny opened, for the first time, a path to experimentally tackle the falsifiability of the long-lasting doctrine on the required association between streptoneury and horizontal ontogenetic torsion which is still widespread in textbooks. We chemically prevented the TGF-β cytokine-dependent [28] differential outgrowth of the mantle and thus the counter-clockwise horizontal rotation of the visceropallium by Pt^{2+} treatment of embryos which results in individuals with a posterior ctenidium. An additional effect of this treatment is that these individuals lack both a mantle cavity and an external shell [25, 27] (Figs. 3, 4A). Instead of torsion, these non-torted individuals show a slight, approximately 90° leftward tilting of the visceral sac around the longitudinal axis [26], most likely because the weight of the internal shell growing on the left side mechanically pulls this side of the body down. Even though this platinum-induced leftward tilting around the longitudinal body axis is in a completely different direction of movement than the ontogenetic torsion (which is 180° around the dorsoventral axis) it also results in displacement of pallial structures and organs associated with the visceral sac epithelium – however, according to the tilting direction, in a different way than usual. This affects the osphradium, the renopericardial complex, including the heart, and the shell field. The positions of the embryonic anlagen of these pallial structures, their movement during platinum-induced tilting of the visceral sac and the positions of these organs in the fully developed transformed snail are visualised in Fig. 3. Except for this 90° tilting of the visceral sac epithelium there are no other signs of displacement which should have been expected if conventional ontogenetic torsion could not have been blocked chemically. Only the U-shaped form of the intestinal tract and the anterior position of the anus give the impression that conventional ontogenetic torsion also took place under Pt^{2+} exposure. However, it must be taken into account that even under normal conditions the anus is formed for the first time as

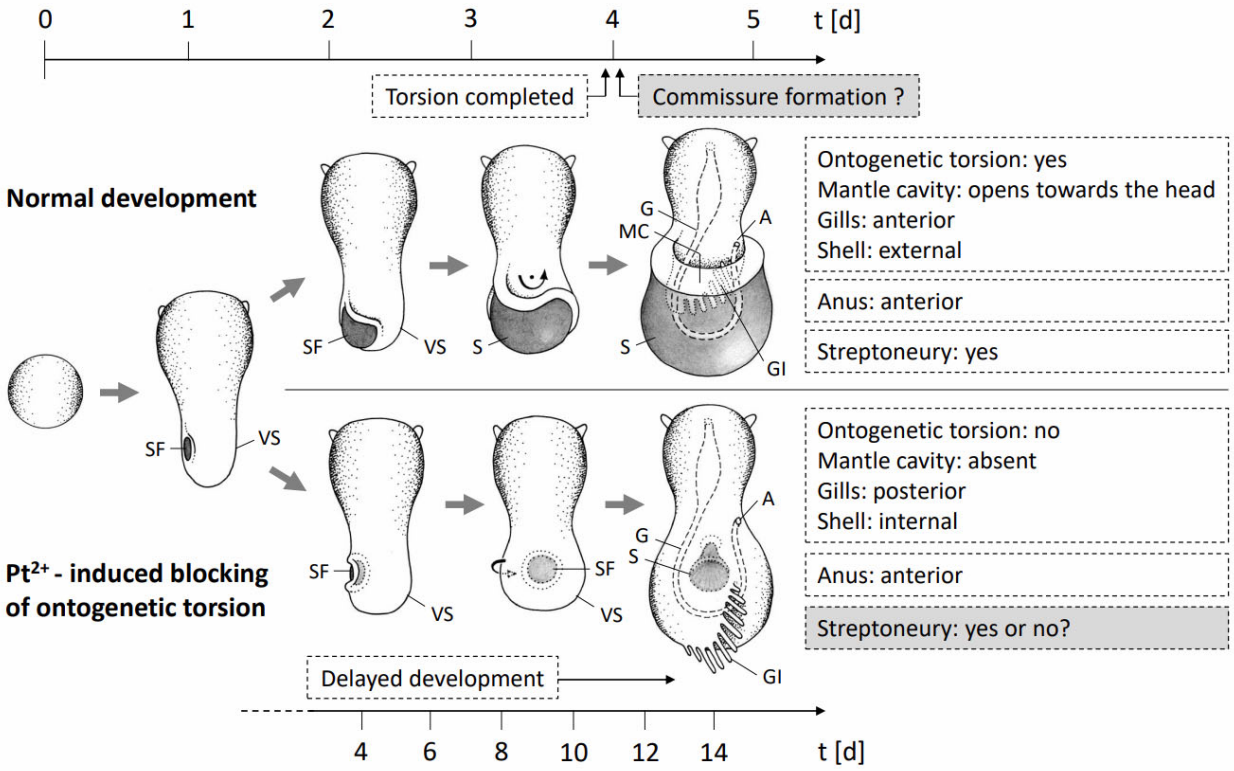

Fig. 2. Course of development. Development and morphological differences between a normally developed, torted (upper row) and a Pt^{2+}-modified, non-torted individual in which ontogenetic torsion was experimentally blocked (lower row). Time scales (top and bottom) differ due to delayed development of Pt^{2+}-treated individuals. The first developmental stage in which the shell field is formed does not differ between the two courses of development. Under normal conditions, ontogenetic torsion, symbolized by the curved arrow, is completed 4 days post fertilization. When ontogenetic torsion is blocked by treatment with Pt^{2+}, the visceral sac tilts to the left and shifts the shell field in a downward rightward movement to the centre of the ventral side. The internal shell is later formed by invagination of the shell-forming epithelium. Shell in grey, external shell: dark grey, internal shell: light grey. Dashed boxes list the key features of individuals undergoing the different courses of development. Shaded boxes: Features in question addressed in this study: commissure formation in torted individuals by immunolabeling and CLSM, formation of streptoneury in a non-torted individual by serial section-based 3D-reconstruction of the nervous system. Labels: A: anus; G: gut; GI: gill; MC: mantle cavity; S: shell; SF: shell field; VS: visceral sac.

a new perforation in the ectodermal layer at stage IX, i.e. quite late in individual development at a post-torsional stage, and that no trace of a proctodeal ectodermal invagination exists [29]. It can therefore be assumed that the direction of rectum growth and the position of the anus are guided by processes other than torsion.

We visualized the nerve system of such a non-torted individual after completed embryonic development by serial sectioning and 3D reconstruction. Since it has not yet been possible to image the nervous system of such small snails by micro-computed tomography, we had to take the methodologically complex approach with serial sections and thus limit ourselves to a single specimen, following the strategy of Kubilius *et al.* [30] who also described the 3D-microanatomy of the nervous system of a mm-sized snail by means of serial sections of a single individual. Furthermore, we immunolabelled the nervous system of a normally developed torted individual to check 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.

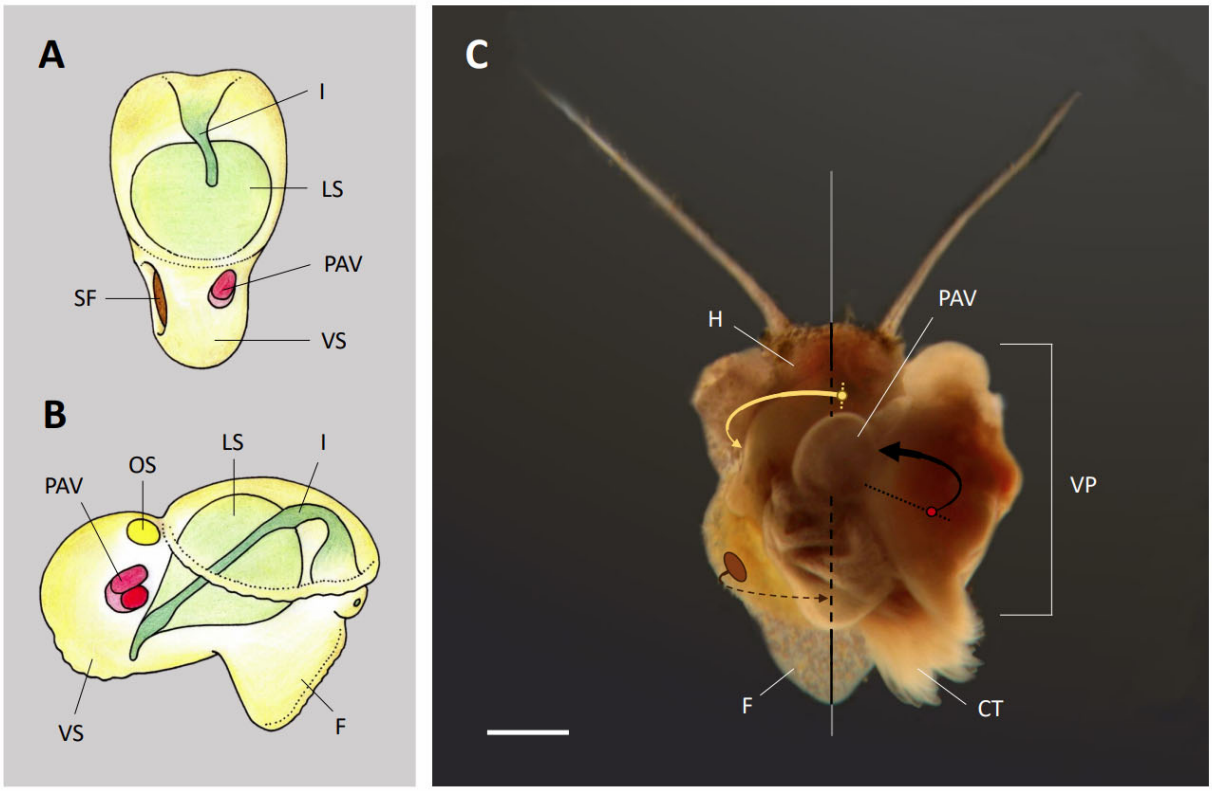

Fig. 3. Visceral sac tilting in transformed snails. Positions of the anlagen of pallial structures, their movement during platinum-induced tilting of the visceral sac and the positions of these organs in fully developed *Marisa cornuarietis*. Together with the blocking of conventional ontogenetic torsion, a leftward tilting movement of the visceral sac occurs during treatment with Pt^{2+}, resulting in a displacement of organs. (A) and (B) Developmental state VI according to [24] and redrawn in a simplified way from the same source, normal development before start of ontogenetic torsion, dorsal view (A) and (B) lateral view from the right. (C) Two-months-old Pt^{2+}-transformed individual, dorsal view. Dots and arrows indicate the original position of the anlagen for the pericardial complex (pericard, auricle, ventricle; in red), the osphradium (in yellow) and the shell field (in brown) as described in [24]. Arrows indicate their tilting around the longitudinal body axis with an angle of approximately 90°. The ultimate positions of pericard and heart (median on the dorsal side), osphradium (lateral on the left) and [internal] shell (median on the ventral side) are indicated by the respective arrowheads. Labels: CT: ctenidium; F: foot; H: head; I: intestine; LS: larval stomach; OS: osphradium; PAV: pericardial complex (pericard/auricle/ventricle); SF: shell field; VP: visceropallium; VS: visceral sac. Scale bar: 1 mm.

Since *M. cornuarietis* has so far been the only species in which horizontal ontogenetic torsion could be reliably blocked experimentally, we were bound to this species with our approach, even though the classical anatomy of the nervous system, including streptoneury, is secondarily modified here (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 obscuring 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 body side, 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 Yousif [21] (Fig. 1C), which provided an excellent basis for structural comparison

with the 3D reconstruction of the non-torted individual's nervous system that was conducted in this study.

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 $PtCl_2$ using the protocols of Osterauer *et al.* [25] and Marschner *et al.* [27]. The rearing conditions of the lab stock culture were the same as in their work [25, 27, 31]. The embryonic development and organogenesis of *M. cornuarietis* has been described in detail in a series of papers [21, 24, 29]. The nomenclature used in these 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) or polyclonal rabbit-anti FMRFamide (ImmunoStar, Hudson, WI, USA; 1:250)) 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)) which was diluted 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 in PBS. Samples were cleared at room temperature for 7 weeks in Sca*le*B4 [32], which was also used as the 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.sc, [33]). The overview image shown in Fig. 4B (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 Fig. 4B (right).

After optimizing the technique with numerous samples, the sample size of this approach amounted to four individuals, both for serotonin and FMRFamide.

Blocking 180° horizontal ontogenetic torsion

Egg clutches of *M. cornuarietis* were scraped off the aquarium wall with a razorblade and extracted from the clutch with pipette tips [25, 34]. Treatment with $PtCl_2$ was executed according to Osterauer *et al.* [25]. In the 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. $PtCl_2$ concentrations (400 µg/l) were higher than in [25], as it was the aim to obtain non-torted individuals as reliably as possible. One individual that was randomly selected for serial sectioning was approximately 1 mm long after 14 days post-fertilization (dpf). It should be noted that $PtCl_2$, apart from having the desired effect of interfering with torsion also slows down growth [25].

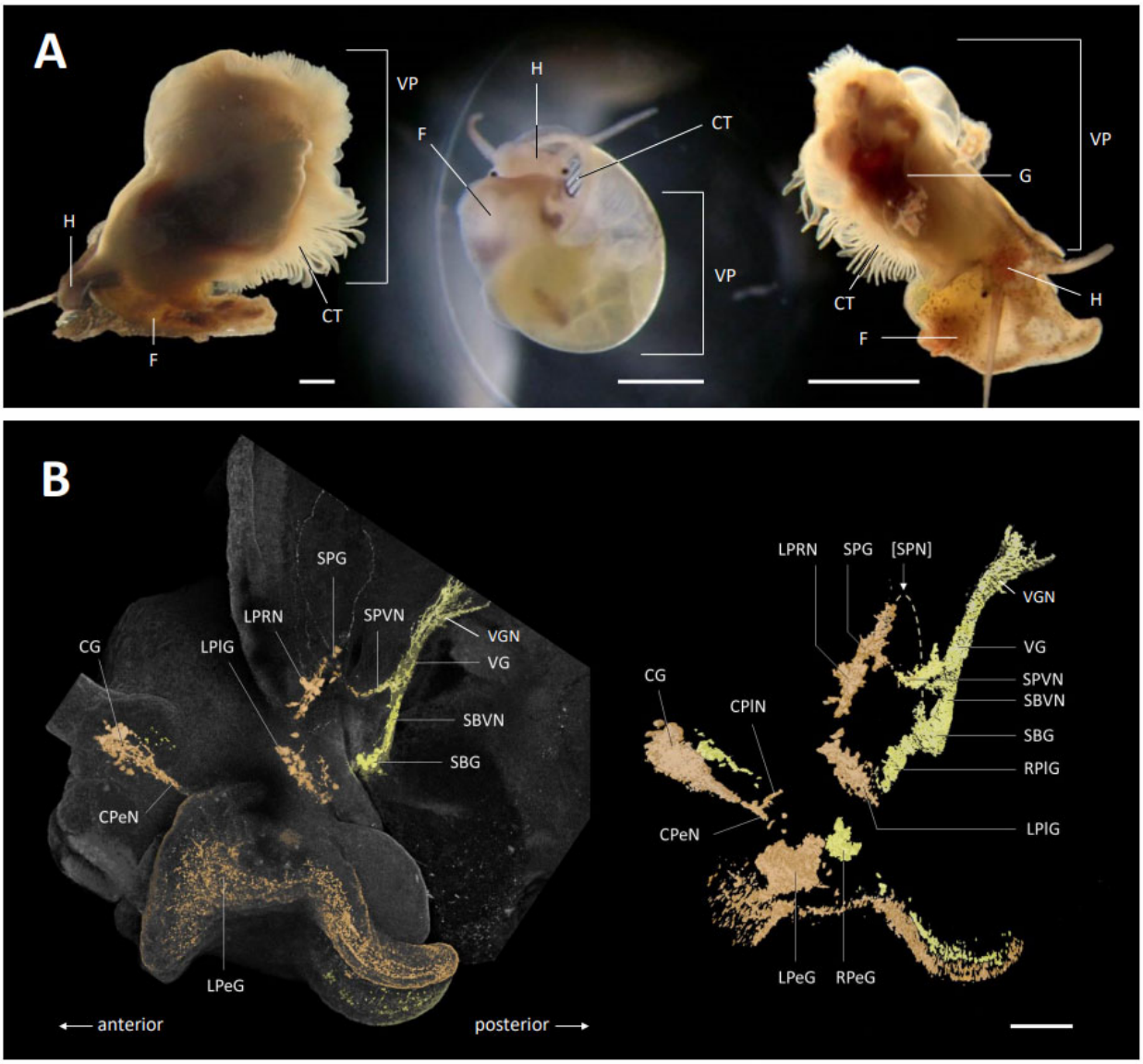

Fig. 4. Habitus and organization of the nervous system of *Marisa cornuarietis*. (A) Habitus. Two-month-old non-torted individual (left), Torted, non-transformed individual prior to hatch, 10 dpf (middle) and hatched non-torted individual, 14 dpf (as analysed in this study, right). Labels: CT: ctenidium (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. cornuarietis* individual, torsion completed at 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). Location of ganglia corresponds to the situation displayed in Fig. 1B, dashed box. The gaps between the ganglia imply that the 'adult' nervous system is not yet fully formed. Labels: CG: cerebral ganglion; CPeN: cerebro-pedal connective; CPlN: cerebro-pleural connective; LPeG: left pedal ganglion; LPlG: left pleural ganglion; LPRN: left zygosis; RPeG: right pedal ganglion; RPlG: right pleural ganglion; SBG: subintestinal ganglion; SBVN: subintestinal-visceral connective; SPG: supraintestinal ganglion; SPN: supraintestinal nerve; SPVN: supraintestinal-visceral connective; VG: visceral ganglion; VGN: visceral ganglion nerve. Scale bar: 500 µm.

Histological fixation and embedding

After 14 dpf, the individual used in this experiment was close to the maximal size that would still allow 3D reconstruction by serial sectioning. The sample was fixed in 2% glutardialdehyde 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 (OsO_4) 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 [35]. After washing the sample first in PBS, then in H_2O, 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 $PtCl_2$ treatment [27]. 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 hours.

Series sectioning and digitalization

Serial sections of 600 nm thickness were cut with a Diatome Histo Jumbo 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 [33] using TrakEM2 [36] first by a rigid alignment followed by a second alignment using the 'Elastic Stack Alignment' plugin [37]. 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 [21], 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. Generated surface meshes were exported to Meshlab [38] for mesh simplification (Screened poisson surface reconstruction) and smoothing (Laplacian smooth filter). The parameter settings of both filters were adapted accordingly to the specific meshes (nervous system, alimentary tract, retina and vitreous body of the eyes) to preserve their volumes, thin threads and delicate protrusions. Visualization of the final meshes for the image plates was done in Amira 6.0.

For the evaluation of possible effects on the anatomy of the nervous system and other organs caused by blocking ontogenetic torsion, we used the work of Demian & Yousif [21, 24, 29] as a control.

RESULTS

Individuals in which ontogenetic torsion has been blocked by Pt^{2+} are morphologically very different from normally developed snails. The suppression of horizontal torsion is caused by the suppression of the outgrowth of the left body side to the right, which leads to the situation that all organs whose development or displacement is linked to this outgrowth do not develop (e.g. the mantle cavity) or remain elsewhere (e.g. the gill and the shell) (Fig. 2). However, the course of the intestinal tract is not altered by the prevention of ontogenetic torsion, i.e. the anus is directed anteriorly.

Untreated snails with torted visceropallium

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 the individuals four days post fertilization (dpf) which had completed torsion (Fig. 4B). At this developmental stage,

several connectives and commissures were either incomplete or only partially visualized. Particularly the labelling of the supraintestinal nerve revealed immunoreactivity just for 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. Also FMRFamide-like immunolabelling revealed the presence of the pleuro-pedal ganglionic mass along with the staining of peripheral innervation of the foot and parts of the digestive tract but with much lower intensity than in serotonin-like labelling (not shown). Likewise, anti-FMRFamide staining did not indicate that the formation of the supraintestinal nerve in four-day-old individuals immediately after completion of torsion has taken place yet. The absence of immunological evidence of the connectives crucial for streptoneury by either method for a total of eight individuals that had completed the rotation of the visceropallium suggests that the nerve cords are not yet fully developed when the torsion is ontogenetically accomplished, i.e. streptoneury in the adult snail does not seem to be mechanically caused by twisting of nerve cords during ontogenetic torsion, but seems to develop independently.

Pt^{2+}-treated snails lacking a horizontally torted visceropallium

The 3D reconstruction of the nervous system, together with the intestinal tract in a Pt^{2+}-treated non-torted individual 14 days post fertilization (Fig. 5) largely mirrors the neuroanatomy described by Demian & Yousif [21] for non-manipulated, 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 (LPlG and RPlG) adjacent to the pedal (LPeG and RPeG) rather than to the cerebral ganglia (CG). Paired cerebral, pedal and pleural ganglia are arranged in a circumenteric ring. Distant from this ring, there 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 just as in an untreated torted individuum and is indicative for the type of streptoneury typical of ampullariids. 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. 6A-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 untreated torted ampullariids. The essential criteria for a typical streptoneuric nervous system of the Ampullariidae as described by Demian & Yousif [21] and Berthold [39], in particular the position, orientation and direction of the subintestinal and supraintestinal nerves were thus clearly fulfilled by the Pt^{2+}-treated non-torted individual examined here. In addition to the reconstruction of Demian & Yousif [21], we observed a rather prominent visceral ganglion nerve, both by immunolabelling in the torted individual and by 3D-reconstruction in the individual without 180° rotated visceropallium, which leads from the visceral ganglion in posterior direction upwards.

Despite the overall similarities between conventionally torted and experimentally non-torted individuals, minor modifications in the neuronal anatomy of the individual with blocked torsion and induced leftward tilting occurred: the positions of the supraintestinal and subintestinal ganglia were at approximately the same level in the body, and the osphradial ganglion (OSG) was shifted to the ventral (Fig. 5C-D). Both modifications very reasonably result from the visible, approximately 90° leftward movement of the visceral sac around the longitudinal axis in artificially created non-torted individuals, whereby the ganglia were obviously displaced as well (Fig. 6C).

DISCUSSION

The aim of our work presented here is neither a detailed description of the embryonic development of Pt^{2+}-transformed individuals of *Marisa cornuarietis* in the style of Demian & Yousif [21, 24] nor a temporal tracing of nerve growth by immunological

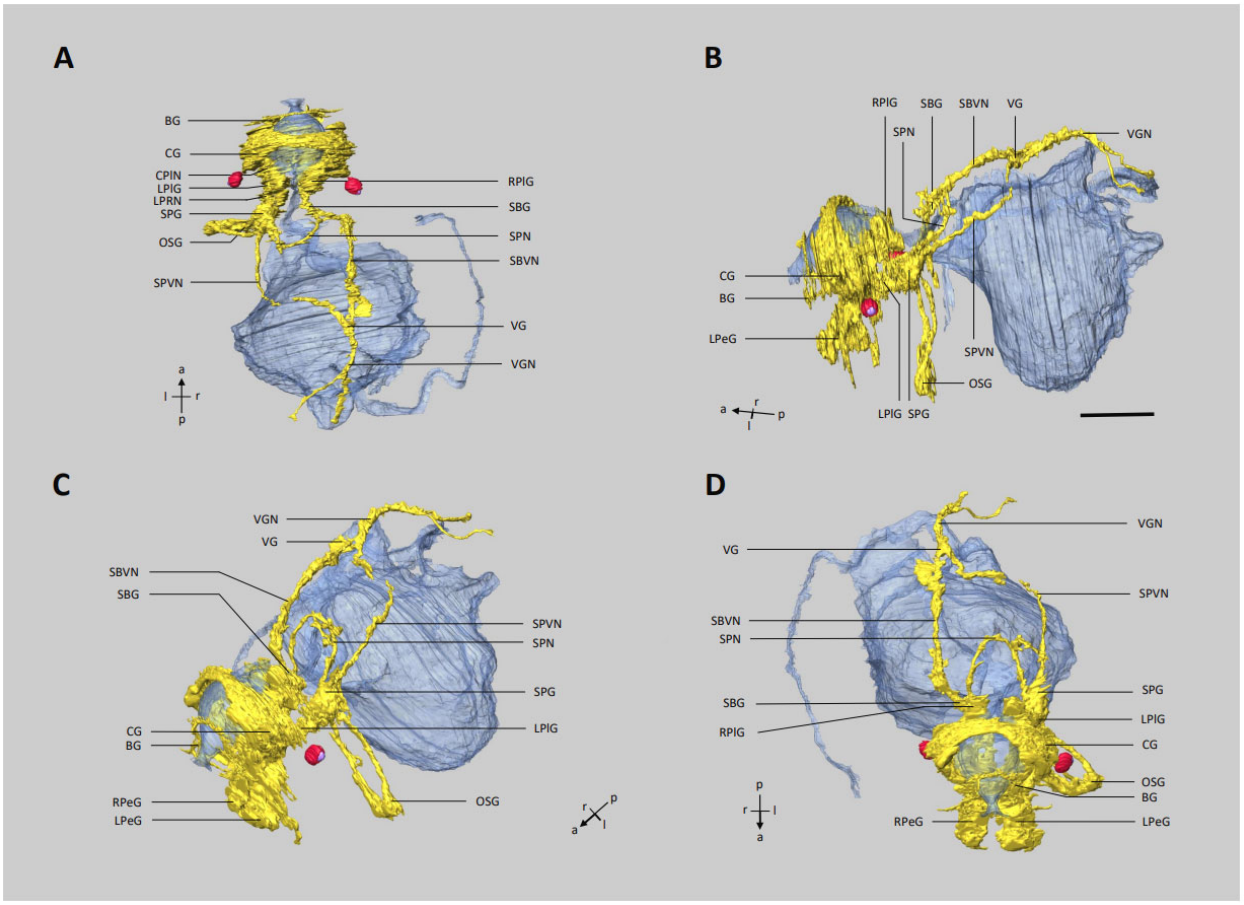

Fig. 5. The nervous system in the transformed snail. Three-dimensional reconstructions of the nervous system of a Pt^{2+}-treated non-torted individual of *M. cornuarietis*, 14 dpf, based on serial sections. This individual exhibits streptoneury as indicated by the transverse course of the supraintestinal nerve (SPN) across the longitudinal body axis. Nervous system in yellow, intestinal tract including hepatopancreas in blue, and eyes in red and purple. Orientation markers: a: anterior; l: left; p: posterior; r: right. (A) Dorsal view. (B) Lateral view from the left. (C) View obliquely from the left above. (D) View obliquely from the front above. Labels: BG: buccal ganglion; CG: cerebral ganglion; CPeN: cerebro-pedal connective; CPlN: cerebro-pleural connective; LPaG: left parietal ganglion; LPeG: left pedal ganglion; LPlG: left pleural ganglion; LPRN: left zygosis; OSG: osphradial ganglion; RPaG: right parietal ganglion; RPeG: right pedal ganglion; RPlG: right pleural ganglion; SBG: subintestinal ganglion; SBN: subintestinal nerve; SBVN: subintestinal-visceral connective; SPG: supraintestinal ganglion; SPN: supraintestinal nerve; SPVN: supraintestinal-visceral connective; VG: visceral ganglion; VGN: visceral ganglion nerve. Scale bar: 200 µm.

labelling. Nor is the formulation of a new alternative theory on the origin and displacement of ganglia explicitly our concern. The central statements of our work are merely that (1) we provide experimental evidence for the currently more often expressed view that streptoneury, the enigmatic twisted condition of the nervous system found in gastropods, can result even when classical 180° counter-clockwise ontogenetic torsion around the dorsoventral axis is blocked and (2) that this is also plausible, since even in normal individual development the pleurovisceral connectives are formed after torsion has been completed and thus do not need to be mechanically twisted – the latter has been implied by theoretical considerations on how to come from a non-yet torted, non streptoneurous pre-gastropod to a torted, streptoneurous gastropod on one hand, and mechanical models that are often

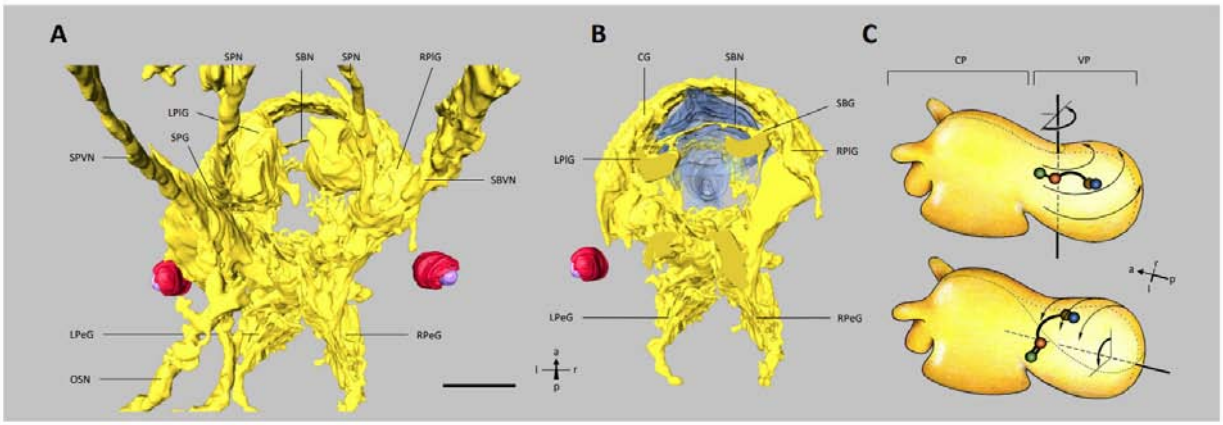

Fig. 6. Posterior view of the nervous system in the transformed snail and axes of rotation. Orientation markers: a: anterior; l: left; p: posterior; r: right. (A) Non-torted individual, 14 dpf, posterior view of the anterior part of the nervous system. Intestinal tract electronically removed. (B) Identical 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. Labels: CG: cerebral ganglion; LPeG: left pedal ganglion; LPlG: left pleural ganglion; OSN: osphradial nerve; RPeG: right pedal ganglion; RPlG: right pleural ganglion; SBG: subintestinal ganglion; SBN: subintestinal nerve; SBVN: subintestinal-visceral connective; SPG: supraintestinal ganglion; SPN: supraintestinal nerve; SPVN: supraintestinal-visceral connective. Scale bar: 100 µm. (C) Rotation axes and direction of rotation of the visceropallium (VP) *versus* the cephalopodium (CP) in normal, torted *M. cornuarietis* individuals (180° horizontal rotation, top) and in non-torted individuals (90° vertical leftward tilting, bottom). For clarity, solely those ganglia that were displaced the most by the 90° rotation and their connecting nerves are marked: subintestinal ganglion (blue), right pleural ganglion (brown), supraintestinal ganglion (orange) and osphradial ganglion (green).

used in textbooks to visualise supposed relationships in a misleading way on the other hand. Despite the existing limitations of our study, these two statements can be undeniably derived from our work and are discussed in the following, although generalising implications for the torsion of gastropods are not possible, because torsion, in comparison between different gastropod taxa, has been designated as a loosely constrained developmental process with multiple pathways to the more constrained end result of the body plan [40], and because our results refer to one species only and are based on a singular lab observation.

Potential heterochrony of torsion and formation of the nervous system

Although one cannot necessarily assume that anti-serotonin and anti-FMRFamide labelling stains all neurons of the nervous system, our immunolabeling CLSM studies suggest that the pleurovisceral cords have not yet fully formed when ontogenetic torsion takes place. This corresponds to previous failures to visualize fully established pleurovisceral nerve cords in pre-torsion stages of snail larvae which pass directly into the formation of the adult nervous system. These are not likely to be attributed to inadequate staining techniques, but rather to the absence of them in these stages, as serotonin has been detected in all ganglia and in the vast majority of nerves in those gastropod species studied to date [17]. The species *Marisa cornuarietis* was already investigated decades ago by Demian & Yousif [21], who found that the cerebral, intestinal, pleural and pedal ganglia arise and develop simultaneously and separately by delamination from the ectoderm at an early embryonic stage. However, according to these authors, the visceral ganglion develops later by delamination from the right side of the visceral sac. Already this study has proposed a temporally distant secondary formation of commissures and connectives developing as ‘extensions’ from the

periphery of the ganglia. Similarly, in *Haliotis kamtchatkana*, the first two neurites of a solitary neuron that was formed prior to the ontogenetic torsion and appeared to delineate the trajectory of the future pleurovisceral nerve cords did not cross over during torsion [20]. Nevertheless, also in this study, a full crossing of the pleurovisceral connectives occurred secondarily at later stages in ontogeny when the torsion of the visceral mass was already accomplished. In the basommatophoran *Lymnaea stagnalis*, the pathways of embryonic neurites seem to exhibit streptoneury and, later, also detorsion (a feature typical for pulmonate snails), but these structures did not appear to join the ganglia of the future adult nerve system, ceased to express immunoreactivity and disappeared after hatching [18, 19]. Hence, a whole series of studies suggest that the central and peripheral neurons that comprise the adult nervous system appear at later stages in ontogeny, and many neurons are lost during the transition from the larval to the juvenile stage and are replaced by new ones [41, 42]. Also, during the direct development of *M. cornuarietis*, there was no histological evidence in the past that the nerval cords, which are crucial for streptoneury, are already formed before ontogenetic torsion [21]. We therefore conclude that, at least in the species investigated so far, the establishment of streptoneury in the post-embryonic nervous system is a secondary process that is, at most, triggered by the early neurons´ pioneer pathways and their signals which may act as guidance for the neuronal outgrowth of the developing adult nervous system [17].

No mechanical coupling of 180° horizontal torsion and streptoneury

Based on the data from our study we cannot judge – and refrain to speculate – on any uncoupling of ganglia from the position of their respective placodes and independent migration. We are convinced that, in non-transformed snails, the displacement of pallial structures, including neurogenic placodes, is due to the interaction of torsion and morphogenetic movement of the mantle epithelium, and we have no reason to claim that this principle should not apply in the same way to individuals whose visceropallium morphogenesis has been experimentally altered. But, in our opinion, later fusion processes of ganglia and the formation of nerve cords between ganglia by neurite outgrowth from neuronal cell bodies that reside in these ganglia are very likely controlled by torsion-independent processes, so that streptoneuric condition is possible even if ontogenetic torsion is blocked.

Our observations show that it is possible that streptoneury can develop without preceding horizontal rotation of the visceropallium around the dorsoventral body axis. This finding is in very good accordance with the observation that the U-shaped course of the intestinal tract and the anterior position of the anus – both criteria usually attributed to ontogenetic torsion – remain independent of the rotation of the visceropallium in all Pt^{2+}-treated individuals of *M. cornuarietis* [25].

The development of streptoneury, at least in our singular observation, was independent of a horizontal rotation of the visceral sac and, therefore, eventually also independent of whether or not ganglia had exchanged their places on the left and right sides of the body during ontogeny. Plausibly, any movement of the visceropallium may mechanically relocate structures. This is confirmed by our study as the observable 90° leftward tilting of the visceropallium in the non-torted individual [26], which most likely is due to the increasing weight of the growing internal shell that pulls down the left side of the body, displaced pericard/heart, osphradium and shell field (Fig. 3) and went also along with a leftward shift of the position of at least four posterior ganglia (Fig. 6C). On the one hand, the osphradial ganglion that develops by proliferation directly below the osphradium was shifted ventrally (Figs. 5C, 6C) and, on the other hand, the arrangement of the supra- and subintestinal ganglia (which are considered homologous to the parietal ganglia, the latter fused with the right pleural ganglion; cf. Fig. 1A-B) relative to one another was tilted in the same direction and came to lie at almost the same height (Fig. 5D). Even though torsion likely is not a uniform process [15, 25] and thus may result in gradual interspecific variation in the degree to which different organs are displaced [15], 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 in gastropods. Although we cannot judge whether the experimental prevention of ontogenetic torsion also prevented the parietal ganglia from exchanging places, the key criterion of streptoneury, i.e. the transverse crossing of the midline axis by the supra- and subintestinal nerves (as shown in Figs. 1B: right, 1C), was nevertheless established also in the Pt^{2+}-treated snail which did not undergo conventional torsion. Our findings suggest that, in torted *Marisa* snails, the pleurovisceral connectives, which are probably formed only after ontogenetic torsion, grow crosswise towards the opposite ganglia across the central axis. The same obviously occurs when ontogenetic torsion has been artificially blocked: also in this case, the pleurovisceral connectives connect the opposite ganglia in a crosswise manner. This suggests that, independent of a horizontal 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.

It has long been known that larval structures of the nervous system of gastropods largely disappear during individual development and are replaced by other structures that characterise the adult nervous system (reviewed in [17]). It is not until the mid-larval stage that the first structures appear that persist into adulthood. Thus, the final formation of neurons within the ganglia occurs long after their formation, and the outgrowing axons of these neurons determine the orientation of the commissures and connectives connecting the ganglia in the adult nervous system [43]. However, the overwhelming majority of these studies were carried out on aquatic gastropods with true veliger larvae, and hence it is unclear to what extent these results can be transferred to species with a derived direct development, such as *M. cornuarietis*. Thus, in principle, it is also possible that the concept of the causal link between ontogenetic torsion and streptoneury formulated for gastropods with original larval forms (and even questioned in these species) does not apply in the same way to *M. cornuarietis* and that the strongly derived individual development does not necessarily support a role for ontogenetic (mechanical) torsion as the most important factor in this process.

In fact, our findings argue against streptoneury being ontogenetically caused by mechanical coupling to torsion of the visceral mass in *M. cornuarietis*. This supports views stated in the past that suggest a formation of the adult nervous system by the spatially determined 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 [10]. On the basis that a mechanical twisting of connectives and the formation of crossed pleurovisceral nerve cords within the developing visceropallium have never been confirmed experimentally, Haszprunar [10] has already 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* [44] and the pulmonate *Lymnaea stagnalis* [18, 45, 46] 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 [47]. We know that asymmetric expression of genes connected to 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 [5, 48, 49]. 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 was already established in these ancient organisms. Although the results of our study cannot shed light on this, they suggest that directional growth of nerve fibers across the midline axis can be 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 [50, 51], 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 [52]. Thus, as implied 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.

CONCLUSIONS

The present experimental evidence for the potential independence of streptoneury and the horizontal rotation of the visceropallium in the apple snail *Marisa cornuarietis* refutes, despite restricted to a singular case, the universality of the assumption still widely used in textbooks that these two criteria are necessarily based on each other, at least from the view of ontogeny. The fact that the formation of streptoneury in molluscs is, in individual cases, also possible without conventional torsion does not necessarily refute the generally accepted phylogenetic view of the mechanisms behind this distinctive feature of the Gastropoda flatly. However, it needs to be discussed whether either the developmental programme of some present-day gastropods does not necessarily reflect the phylogenetic origin of streptoneury in gastropods or whether the phylogenetic 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.

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AUTHOR CONTRIBUTIONS

Conceptualization: Heinz-R. Köhler
Data curation: J. Anton Morath, Stefan Fischer, Simon Schwarz
Investigation: J. Anton Morath, Stefan Fischer, Simon Schwarz
Supervision: Heinz-R. Köhler, Oliver Betz, Rita Triebskorn
Writing – original draft: J. Anton Morath
Writing – review and editing: Heinz-R. Köhler, Oliver Betz, Stefan Fischer

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interests.

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