The development of early pioneer neurons in the annelid *Malacoceros fuliginosus*

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Abstract

Background Nervous system development is an interplay of many processes: the formation of individual neurons, which depends on whole-body and local patterning processes, and the coordinated growth of neurites and synapse formation. While knowledge of neural patterning in several animal groups is increasing, data on pioneer neurons that create the early axonal scaffold are scarce. Here we studied the first steps of nervous system development in the annelid Malacoceros fuliginosus. Results Here, we performed a dense expression profiling of a broad set of neural genes. We found that SoxB expression begins at 4 hours postfertilization, and shortly later, the neuronal progenitors can be identified at the anterior and the posterior pole by the transient and dynamic expression of proneural genes. At 9 hpf, the first neuronal cells start differentiating, and we provide a detailed description of axonal outgrowth of the pioneer neurons that create the primary neuronal scaffold. Tracing back the clonal origin of the ventral nerve cord pioneer neuron revealed that it is a descendant of the blastomere 2d (2d 221), which after 7 cleavages starts expressing Neurogenin, Achaete-Scute and NeuroD. Conclusions We propose that an anterior and posterior origin of the nervous system is ancestral in annelids. The specification of the relevant neurons starts very early and we suggest that closer examination of the first pioneer neurons will be valuable in better understanding of nervous system development in spirally cleaving animals, to determine the potential role of cell-intrinsic properties in neuronal specification and to resolve the evolution of nervous systems.

Introduction

The basic scheme of early neurogenesis, that is, the early steps in the development of individual neurons, depends in eumetazoans on a fairly conserved set of transcription factors, many of them belonging to the Sox and proneural bHLH gene families [1,2]. Though regulation and interdependency display variability between groups, these genes were in many organisms found to play important roles in neurogenesis: from providing neurogenic potential in the ectoderm of early embryos and controlling the step-wise transition of self-renewing multi-potential neuronal progenitors to more committed precursors which later differentiate into specific neurons or glia elements [3–6]. Where, when, and which neuronal types are formed depends on patterning processes, which are intimately linked to the above-described steps of neurogenesis and the subsequent process of differentiation, like broad anterior-posterior and dorso-ventral patterning of the ectoderm and more local patterning processes during subdomain development [7–11].

Yet nervous system architecture differs considerably in the different evolutionary lineages of bilaterian animals [12]. Many of them possess a centralized nervous system comprising of a central brain and longitudinal trunk nerve cords. But brain structure and the arrangement, number, branching, and organization of the nerve cords vary considerably between taxa, and some groups display only a low level of nervous system centralization and rather exhibit diffuse nerve nets. It is still intensely debated how conserved are the main processes of nervous system patterning in bilaterians and how nervous system centralization evolved, whether it is homologous or arose independently in different lineages [13–18]. In
This context, studies in the protostome group of lophotrochozoans received attention during the last years. While data on neurogenesis and nervous system patterning from some representatives suggest high similarity to vertebrates and arthropods [19–27], the nervous system architecture is quite diverse within the group and scenarios of common ancestry as well as independent evolution of nervous system centralization has been claimed [13,15].

One important aspect of nervous system development, which has hardly been addressed in these animals, is the specification and specific role of the pioneer neurons, which prefigure the primary scaffold of the central nervous system (CNS). Comparative immunohistochemical studies on early nervous system development in lophotrochozoans provide conflicting data on whether the nervous system forms from the anterior or the posterior pole or both [28,29]. This may partly result from the fact that only a small subset of neurons is stained by standard markers for certain neurotransmitters, but also from diversity, which is also obvious in the adult nervous system organization [12,30]. Within annelids, nervous system development is best characterized in *Platynereis dumerili*, representing the large subgroup of errant polychaetes and *Capitella teleta*, representing the sedentary subgroup. The data differ on the origin of neural structures (anterior and posterior in *Platynereis* and only anterior in *Capitella*), the patterning along the anterior-posterior, and the mediolateral axes [31,32]. We chose the sedentary polychaete *Malacoceros fuliginosus* as a study subject due to its experimental suitability and to find answers on the ancestral mode of early nervous development in annelids and beyond.

We describe for the first time in an annelid the very first steps of neurogenesis. We find that the pioneer neurons are already in place very early when neurogenesis and patterning of the vast majority of neurons are yet to start. Expression of proneural genes starts already at 5 h postfertilization and after only a few rounds of embryonic cleavages. The early expression is restricted to very few cells at the anterior and posterior pole. The expression in the early stages is very dynamic and punctuated before expanding in specific areas of neuronal proliferation. A single posterior neuron prefigures the main course of the ventral nerve cord (VNC), while in the anterior region, the central ganglia is initiated by a single apical neuron and a pair of sensory cells giving rise to the prototroch nerve ring. Due to its position, we could track the development of the posterior neuron in detail from early specification to the onset of differentiation and outgrowth of the neurites. From our data, an anterior and posterior origin of the nervous system is likely ancestral for the majority of annelids and possibly beyond. The early specification of pioneer neurons makes it interesting subjects for studies on neural specification, the role of cell-intrinsic and extrinsic factors on the development, and the evolutionary conservation of nervous system development.

**Methods**

**Malacoceros fuliginosus culture**

Adult *Malacoceros fuliginosus* were collected from Pointe de Mousterlin, Fouesnant, France, and were maintained in sediment containing seawater tanks at 18°C. Individual males and females were picked,
rinsed several times with filtered seawater, and kept in separate bowls until they spawned. The staging was started from the time gametes were combined in a fresh bowl. Bowls were kept at 18°C under 12:12 hr light-dark cycle, and water was replaced every day or every second day. Larvae were fed with microalga *Chaetoceros calcitrans* from 24 hpf onwards after each water change.

**RNA-Seq and transcriptome assembly**

Total RNA was extracted from cryopreserved samples of various larval stages using the Agencourt RNAAdvance Tissue Kit (Beckman Coulter). Library preparation and sequencing was performed by EMBL Genomics Core Facility (Heidelberg, Germany) using cation-based chemical fragmentation of RNA, Illumina Truseq RNA-Sample Preparation Kit and 1 lane of 100 bp paired end read sequencing on Illumina HiSeq 2000. Raw reads were trimmed, and error corrected with Cutadapt 1.2.1, the ErrorCorrectReads tool implemented in Allpaths-LG, and assembled with Trinity. A second assembly, including several steps for correction of sequence errors, chimerism, and elimination of redundancy, was generated using the DRAP pipeline [33] followed by gene clustering using Corset [34].

**Transcriptome screening and gene orthology**

The transcriptomic resources of *M. fuliginosus* were deeply screened by similarity searches using tblastn for genes of interest. For bHLH group A genes, we used all Acheate-Scute (ASC), Atonal, Neurogenin, NeuroD, and Oligo amino acid sequences from *Capitella teleta* and *Crassostrea gigas* discovered by # [35] as queries. We added the candidates which gave bHLH genes as hits by reciprocal blast against Genbank with MAFFT (option add) to the alignment of #[35] and computed gene trees with IQ-TREE 1.5.5 with the model calculated by the included ModelFinder, SH-like approximate likelihood ratio test (1000 replicates), ultrafast bootstrap (1000 replicates) and approximate Bayes test for estimating branch support. We set unsuccessful iterations to stop tree searching to 500 and perturbation 380 strength to 0.2. The same parameters were used for all other gene tree inferences as well. To get closer insights into the evolution of the Acheate-Scute (ASC) family we aligned (MAFFT) all sequences, which clustered in the respective group in the tree inferred above and used the conserved region of the alignment as query profile for HMMER searches against Metazoa RP15, Annelida, Mollusca, Platyhelminthes, and Brachiopoda Uniprot with a cutoff-evalue of 1e-15. To get a better sampling in Annelida we also screened the Genbank annelid protein and the TSA database of *Platynereis dumerilii* by blast and tblastn (e-value cutoff 1e-10) with all sequences from *Crassostrea gigas* and *Capitella teleta* which clustered in the bHLH gene tree in the ASC clade. For those ASC sequences from *Capitella teleta* and *Nematostella vectensis*, which matched sequences used by [21] and [36] we adopted the respective sequence annotations. We added all sequences from *M. fuliginosus*, which clustered in the ASC clade in the bHLH group A analysis above to the dataset. We removed duplicates and aligned the sequences with MAFFT (EINSI option) and kept only the conserved regions by manual curation. We removed sequences shorter than 60% of the alignment and calculated a tree with IQ-TREE with the same parameters as above. For POU genes, we screened the *M. fuliginosus* transcriptome resources with tblastn (e-value cutoff 1e-30) and all POU gene sequences from *Capitella teleta* uncovered by [37] as queries. We checked all hits for the presence of the
POU domain with SMART. For a first analysis we added the retrieved sequences to the POU gene sampling of [37], aligned them with MAFFT (EINSI option), and inferred a gene tree with IQ-TREE with the same parameters as above. For a second analysis, we removed three sequences with the longest branches. Further, we screened the Genbank protein database with all POU genes found in *M. fuliginosus* (blastp, e-value cutoff 1e-80). We kept only the longest isoforms, aligned the sequences with MAFFT (option EINS), and kept only the well-conserved regions containing the POU domain and the homeodomain. The gene tree was inferred as above. For Elav and CUGBP Elav-like, we generated a query profile by aligning (MAFFT EINSI) sequences from Mouse, *Drosophila*, *Tribolium*, and few Mollusk and annelid species and kept only conserved regions. With this profile, we conducted HMMER searches against RefSeq Metazoa (e-value cutoff 1e-100). We added few sequences with annotation nucleolysin TIA-1 and an e-value of 1E-39 as a potential outgroup. We removed highly similar sequences by clustering with 3 runs of h-cd-hit with identity cutoffs of 0.9, 0.7 and 0.6. For screening the *M. fuliginosus* transcriptome resources we used several Elav and CUGBP elav-like sequences as queries. The candidates retrieved were then used as queries against Genbank Lophotrochozoa (first 100 hits (only longest isoforms)). We removed duplicates from the data set, aligned it with MAFFT (EINSI option) and kept only the conserved regions by manual curation. We computed the gene with IQ-TREE as above. To identify prospero1 orthologs, we used the PFAM PF05044 profile alignment of the prospero domain as query profile for HMMER searches against RP15 (e-value cutoff 1E-10). From the resulting dataset we used the sequences from *Capitella teleta*, *Helobdella robusta*, *Crassostrea gigas* and *Mizuhopecten yessoensis* as queries for similarity searches with tblastn and blastp against the *M. fuliginosus* transcriptome and Genbank Lophotrochozoa proteins (e-value cutoff 1e-10 in both cases). We removed duplicates, kept only the longest isoforms, aligned the sequences with MAFFT (EINSI) and kept only well conserved regions by manual curation. The tree was inferred with IQ-TREE with the same parameters as above. To identify SoxB and SoxC orthologs, we used the alignment of conserved Sox gene domains from #*[38]*# and #*[39]*# as query profiles for HMMER searches against Uniprot Annelida, Mollusca, Brachiopoda and Platyhelminthes and Metazoa RP15 with e-value cutoff of 1e-30. We kept a copy of all sequences of *Homo sapiens*, *Mus musculus*, *Danio rerio*, *Strongylocentrotus purpuratus*, *Drosophila melanogaster*, *Anopheles gambia*, *Tribolium castaneum*, *Caenorhabditis elegans*, *Capitella teleta*, *Platynereis dumerilii*, *Crassostrea gigas*, *Lottia gigantea*, *Schmidtea mediterranea*, and *Nematostella vectensis* and clustered the sequences clustered with h-cd-hit (with identity cutoffs of 0.9, 0.6 and 0.3) to reduce the number of sequences. We then added the separately kept sequences and removed duplicates. We screened the transcriptome resources of *M. fuliginosus* by tblastn with all sequences retrieved above from *Capitella teleta* and *Crassostrea gigas* as queries and an e-value cutoff of 1e-30. We removed the mammalian sex determination SRY genes from the sampling as they are fairly diverged. For a first analysis we aligned the sequences with MAFFT (option LINSI), kept only the conserved residues in the the regions of the HMG box domain and calculated an unrooted tree with IQ-TREE (same parameters as above). For getting closer insights into SoxB evolution we used all sequences, which clustered in the above analysis with known SoxB sequences and few SoxC and SoxD sequences for a second analysis. We aligned the SoxB sequences again with MAFFT (option LINSI) and edited the alignment less restrictively. Especially downstream of the HMG box, we kept more residues. We added the SoxC and
SoxD sequenes to this alignment with MAFFT (option add) and calculated a tree with IQ-TREE (same parameters as above) and rooted it with SoxD. We checked the *M. fuliginosus* SoxB sequences for presence of the HMG domain with the SMART domain and InterproScan web servers.

**Gene cloning and probe generation**

Specific primers were designed for genes of interest retrieved from the transcriptome resources of *M. fuliginosus*, and fragments were amplified from either mixed stage or stage-specific cDNA. The PCR fragments were inserted into pGEM-T-easy vector (Promega) and cloned in Top10 *E. coli* cells (Invitrogen). DIG/FITC labeled RNA probes were generated by DIG RNA labeling mix (Roche) or using transcription reagents along with DIG-UTP/FITC-UTP (Roche) by SP6 or T7 polymerases (Roche).

**In-situ hybridization**

All animals were fixed in 4% PFA (in 1X PBS, 0.1% Tween20) for 2.5 hours. Larvae 48 h and older were first relaxed with 1:1 MgCl$_2$-seawater for 3-5 min before fixing them in 4% PFA (in 1X PBS, 0.1%TWEEN20) for 2.5 hours. Samples were stored at −20 °C in methanol until use. The larvae were rehydrated in series of 75, 50, and 25% methanol in PTW (1X PBS pH 7.4, 0.1% Tween20). Tissue was permeabilized by Proteinase K (100 ng/ml) treatment (30 s for 24 h larvae to 3 min for 5 d larvae). This was followed by two 5 min Glycine washes (2 mg/ml). The larvae were then acetylated with 1% triethanolamine (TEA) in PTW for 5 min and 0.5 µl/ml acetic acid in 1% TEA for 5 min. Two 5 min washes with PTW were done before fixing the larvae for 15 min in 4 % PFA (in 1X PBS-Tween20). The post-fixed samples were washed four times in PTW for 5 min each. The larvae were then equilibrated in hybridization solution (Hyb solution: 50% formamide, 5X SSC, 50 µg/ml heparin, 100 µg/ml salmon sperm DNA, 0.1% Tween20, 1% SDS and 5% dextran sulphate (Alfa Aesar J63690, MW 40,000) in sterile water) for 10 min.

Prehybridization was performed with Hyb solution at 65 °C for 2-4 h before hybridization with labeled RNA probes at a concentration of 1 ng/µl to 2.5 ng/µl for 48-60 h at 65 °C. After this, the samples were subjected to two post-hybridization washes of 5 min and 20 min with Hyb solution at 65 °C. The next washes with SSC were done as following – 2X SSC-Hyb solution in series of 25, 50, 75 and 100% 2X SSC each for 10 min at 65 °C, followed by two 30 min 0.05X SSC washes at 65 °C. The samples were then kept at RT for 10 min before washing in PTW-0.05X SSC in series of 25, 50, 75 and 100% PTW for 5 min each. Blocking was done before washing with Roche Blocking solution (in Maleic acid buffer pH 7.5) for 1 h and then incubated in anti-DIG-AP or anti-FITC-AP (Roche) Fab fragments (1:5000) overnight at 4 °C. After this, the larvae were washed six times in PTW for 1 h and then equilibrated first in Mg$_2$-free AP buffer (two 5 min washes), then in AP buffer (100 mM NaCl, 50 mM MgCl$_2$, 100 mM Tris (pH 9.5 for NBT/BCIP staining and pH 8.2 for Fast Blue/Fast Red staining), 0.1 % Tween20) (two 5 min washes). Probe detection was performed using NBT/BCIP (Roche) in AP buffer (pH 9.5) by adding 2.25 µl/ml NBT (from 100 mg/ml stock) and 3.5 µl/ml BCIP (from 50 mg/ml stock). Fast Blue (Sigma)/Fast Red (Roche) single or double staining was according to the protocol described in Lauter et al. (2011).

**Immunolabeling**
All animals were fixed in 4% PFA (in 1X PBS, 0.1% Tween20) for 30 min. Larvae 48 h and older were first relaxed with 1:1 MgCl₂-seawater for 3-5 min before fixing them in 4% PFA (in 1X PBS, 0.1% Tween20) for 30 min. After fixing, the samples were washed two times in PTW, followed by two washes in THT (0.1 M Tris pH 8.5, 0.1% Tween20). Blocking was in 5% sheep serum in THT for 1 h before incubating in primary antibodies (Monoclonal anti-acetylated α-tubulin, 1:300 Sigma product number T6793; Anti-5-HT, 1:500, Immunostar product number 20080; Anti-FMRFamide, 1:500, Immunostar product number 20091) for 48 h at 4 °C. The samples were then subjected to two 10 min washes in 1 M NaCl in THT followed by five 30 min washes in THT before incubating in secondary antibodies (Alexa Fluor 1:500, Thermo Fisher Scientific) overnight at 4 °C. Next, the samples were washed in THT, two 5 min washes followed by five 30 min washes. Specimens were stored in embedding medium (90% glycerol, 1x PBS, and 2% DABCO) at 4 °C.

**Embryology**

For studying embryology, we fixed larvae from a single batch starting from 30 mpf up to 6 hpf at every 10 min intervals. We used at least 100 larvae for each fixation (1 hr in 4% PFA). Next, the larvae were rinsed several times with PTW, stained with DAPI for 1 hr, and rinsed again with PTW. The stained specimens were then transferred to the mounting medium (Glycerol with 2% DABCO). We studied at least 5 embryos from each fixation and recorded two-channel (DIC and DAPI) Z stacks with a Zeiss Axio Imager Z2. In total, 165 embryos were studied.

**Microscopy and image processing**

Samples were mounted in glycerol and imaged using the Zeiss Examiner A1 microscope. Confocal imaging was done with a Leica SP5 microscope. Images were processed using ImageJ, Adobe Photoshop CS6, Imaris (Bitplane), and assembled using Adobe Illustrator CS6.

**Results**

The first pioneer neurons with long neurites appear at the anterior and the posterior pole of the larvae.

The early development of the *M. fuliginosus* larva is highly synchronous and therefore allows for precise staging across batches. Throughout the early development up to stages of 24 hpf, the larva is surrounded by a thick ornamented chorion of the egg (Fig. 1A-D, 1K). The chorion later becomes an integral part of the cuticle, which at 48 hpf already has a smooth surface (Fig. 1L). Cilia of the prototroch and the apical tuft penetrate the chorion from 7 hpf onwards. Pigmented eyes appear at around 14 hpf (Fig. 1B-D), and chaetae start penetrating the chorion around 24 hpf (Fig 1K) and form prominent bundles at 48 hpf (Fig. 1L). Immunohistochemistry shows that the prototroch and the telotroch are formed by bundles of cilia (Fig. 1E-J), which in later stages is less obvious by external examination (Fig. 1K, L). The prototroch band is not continuous on the dorsal body surface from the time of its emergence (Fig. 1E-L). The nervous system development is relatively fast, and due to a low yolk content of the larvae, a detailed investigation is possible by immunohistochemical stainings. For this purpose, we performed antibody stainings.
against acetylated alpha-tubulin (a-tub) from 7 hpf onwards, where only the cilia of the prototroch and an apical tuft are visible.

Studying stages in short time intervals allowed us to identify the first appearing pioneer neurons that send out the first neurites and initiate the formation of the early neuronal scaffold. The first neuron to send out axonal processes is a single posterior pioneer neuron (PPN), which starts to differentiate around 8 hpf with the accumulation of dense microtubules (data not shown) and acquires a distinct morphology by 9 hpf (Fig. 1E, 2E, Fig. 3, Supplementary Data 1). This bifurcating cell projects two axons proceeding towards the prototroch from 12 hpf onwards (Fig. 1F), which extends already one third to the prototroch at 14 hpf (Fig 1G, Fig. 3, Supplementary Data 2-4). During this phase, the PPN also acquires a curved morphology with few sensory cilia extending outwards on the ventral side (Fig. 2F, G). The axons of the PPN reach the prototroch area around 19 hpf (Supplementary Data 8, 9, Fig. 3). At around 16 hpf, a new neuron (without sensory cilia) and presumably a follower develops adjacent to the PPN (Fig. 2G). It extends axons along the processes formed by the PPN (Supplementary Data 5-7). More follower neurons appear at 34 hpf, when a pair of weakly stained ciliated sensory cells are visible on either side of the PPN and become more prominent at 36 hpf (Fig. 2H, H').

In the anterior end, the first neurons can be observed from 9 hpf underneath the apical tuft (Fig. 1E, Fig. 3, Supplementary Data 1), but we could not identify extending axons before 14 hpf. At this time, several cells lie underneath the apical tuft, but only one sends a short axon towards the prospective brain neuropil (Fig. 2, Supplementary Data 2-4). At the same time, the prototroch nerve starts to develop from a pair of cells situated adjacent to the ventromedial prototroch cells. We name these cells as the prototroch nerve forming neurons (PNNs). These cells have a triangular morphology and sensory cilia projecting externally. Interestingly, for a few hours, only one of these cells sends out processes extending on either side along the prototrochal cells (Fig. 2A, B, Fig. 3, Supplementary Data 5-7). From 19 hpf onwards, the other cell, which only has thin neurites projects onto the passing connectives from the adjacent cell (Fig. 2I, J, Fig. 3, Supplementary Data 8-12). On the other hand, the surrounding prototroch cells seem to send extensions towards the prototroch nerve (Supplementary Data 6-12). As the prototroch is discontinuous on the dorsal side, the prototroch nerve extends only until the dorsal-most prototroch cell without forming a complete ring.

At 16 hpf, a single ganglion cell appears on one side of the apical organ along with a descending axon (Fig. 3, Supplementary Data 5-7), which becomes more prominent at 18 hpf (Fig. 2C). We name this pioneer as the apical neuron 1 (AN1). Around 19 hpf, this growing axon from AN1 traverses contralaterally up to the prototroch region (on the other side). It extends posteriorly towards the anteriorly traveling neurite of the PPN (Fig. 2D, Fig. 3, Supplementary Data 8, 9) and thereby closing the gap between anterior and posterior parts of the developing VNC. Meanwhile, at the same time, the growing axons from PNNs travel posteriorly and join the developing VNC (Fig. 2I).

At 21 hpf, more cells appear symmetrically around the apical organ and traverse contralaterally along the neurites established by the AN1, which persists (Fig. 2J, Fig. 3, Supplementary Data 10-12). The
crisscross of neurites originating alongside the apical organ creates a plexus, which becomes the larval brain neuropil (Fig. 2J, Fig. 3). By 24 hpf, the AN1 becomes less prominent and is not easily identifiable as more and more differentiated cells start to innervate the brain neuropil (Fig. 2K, Fig. 3, Supplementary Data 13-16). The PNNs, however, are still identifiable at 24 hpf, which by 34 hpf becomes less conspicuous (Fig 2K, 1J).

Around 24 hpf, a weakly stained commissure is arising in the anterior trunk region that connects the two VNC neurite bundles (Fig. 1J, Fig. 3, Supplementary Data 13-16). The nervous system represented by the 34 hpf stage is of a typical annelid trochophore composed of an apical tuft, prototroch, telotroch, and neuronal elements such as central cephalic neuropil, a prototroch nerve ring (semi-circular) and VNC (Fig. 1J). This basic neuronal architecture continues into the later larval stages and likely becomes part of the adult nervous system. In the anterior end, numerous sensory cells develop throughout the head region, with projections reaching the brain neuropil (Fig. 1J).

### Development of serotonergic and FMRFamidergic neurons

For comparative purposes, we studied immunoreactivity against serotonin (5-HT) and FMRFamide, which are common markers in studies on invertebrate neural development. 5-HT and FMRF are important neurotransmitters in many animals [41,42]. While 5-HT and FMRFamide are strongly expressed in the apical neuropil and the trunk in 48 hpf stages (Fig. 2L, M), only a few serotonergic and FMRFamidergic cells are present in earlier stages. FMRFamide is detectable first at 14 hpf as a single, ciliated flask-shaped weakly labeled cell, which lies slightly left and dorsal from the apical tuft cell (Fig. 2N, Fig. 3, Supplementary Data 2, 3). This cell develops an axon that is later projecting into the area of the apical neuropile and is accompanied by a similar second cell on the right body side at 24 hpf (Fig. 2P, Fig. 3, Supplementary Data 14, 16). Already at 21 hpf, a second pair of FMRFamidergic ciliated flask-shaped cells also sending axons in the apical neuropil become visible ventrally from the apical tuft cell (Fig. 2Q, Fig. 3, Supplementary Data 11, 14, 16). 5-HT can be detected from 21 hpf onwards in a pair of cells ventral to the apical tuft cell, which likewise are ciliated and flask-shaped and send their axons into the forming apical neuropil (Fig. 2O, Fig. 3, Supplementary Data 10). Up to the 24 hpf stage, neither FMRFamide nor 5-HT is detectable in the mid or posterior body region. In all investigated stages, only a very small fraction of the identified neurons and none of the described neurons pioneering the ventral nerve cord, the prototroch, or the connection between the apical plexus and the VNC contains FMRFamide or 5-HT.

### The first neurons show synaptic activity likely from 12 hpf onwards

To get an idea when the developing neurons are entering differentiation and are getting functional on the molecular level, we screened the transcriptome resources of *M. fuliginosus* and public sequence
databases for orthologs to synaptotagmin-1 (Syt1), which is a conserved Ca\(^{2+}\) sensor for fast synaptic vesicle exocytosis in many neurons of metazoans, and ras-related protein 3 (Rab3), which regulates synaptic vesicle fusion. Two sequences were found, which after reciprocal blast against Genbank gave only Syt1 and Rab3 sequences as the first 100 hits, and we named them Mfu-Syt1 and Mfu-Rab3.

In order to find an ortholog for the RNA binding protein Elav1, which is a common marker for postmitotic neuronal precursor cells in many metazoans, we ran a maximum-likelihood tree of metazoan Elav and CUGBP Elav-like genes. As in many other lophotrochozoans, in *M. fuliginosus*, we found two CUGBP Elav-like sequences, and two Elav sequences, both of which have RRM RNA binding motifs. One sequence (Mfu-Elav2) groups with other lophotrochozoan sequences (Fig. S1) confirming the existence of a lophotrochozoan specific Elav2 gene [21,43]. The other one (Mfu-Elav1) clusters with high support with metazoan Elav1. Since the expression of Elav2 is not much studied and is not specific to neurons in *Capitella teleta* [21] and *Sepia officinalis* [43], we investigated only the expression of Mfu-Elav1.

In a similar manner, we ran an analysis of metazoan POU genes to identify the *M. fuliginosus* ortholog to POU4, which is an important regulator of terminal differentiation in many neurons of Metazoa. We found several POU genes in the transcriptomic resources of *M. fuliginosus*, all containing a POU-specific domain. Only one, Mfu-POU4, belongs to the well supported POU4 clade (Fig. S2). Likewise, most other lophotrochozoans have only one POU4 gene. If there are more, then they are the result of species-specific gene duplications.

*Mfu-Elav1* and *Mfu-Syt1* were first detected at 12hpf (Fig. 4A,4D). At this stage, *Mfu-Elav1* is only restricted to the anterior region close to the apical tuft, whereas *Mfu-Syt1* is expressed near the apical region, in a pair of bilateral cells and in the posterior region (Fig. 4A, 4D). While the expression of *Mfu-Syt1* in the bilateral cells corresponds to the developing eye photoreceptors (data not shown), the posterior region corresponds to the PPN, as shown by co-staining with ac-tubulin (Fig. 4I). Shortly later, at 14 hpf, *Mfu-Elav1* also starts expressing in the posterior region but not in the PPN. At the same stage, *Mfu-Rab3* starts expressing in few cells spanning the anterior, middle, and posterior regions in a similar pattern to *Mfu-Syt1*, nonetheless, in more cells (Fig 4G). From 18hpf onwards, several cells throughout the body express *Mfu-Elav1*, while *Mfu-Syt1* and *Mfu-Rab3* are mainly restricted to the anterior and posterior regions. The dense staining of *Mfu-Syt1* and *Mfu-Rab3* in the anterior region reflects the many neurons contributing to the central neuropil (Fig. 4F,4H). In the posterior region, *Mfu-Syt1* is only expressed in the PPN, whereas *Mfu-Rab3* is expressed in adjacent cells in addition to the PPN (Fig. 4H). By 48hpf, more neurons in the anterior, oral, and along the developing VNC express *Mfu-Syt1* and *Mfu-Rab3* (Sup. Fig. 8).

The terminal selector POU4 (*Mfu-POU4*) starts to express from 10hpf in two cells, one in the anterior region and one in the posterior region (Fig. 4J). The expression in the posterior region very likely corresponds to the PPN as determined by the position and morphology of the cell. At 14hpf, an additional pair of cells lying adjacent to the posterior neuron start to express *Mfu-POU4* (data not shown). In subsequent stages, the expression of *Mfu-POU4* expands mainly in the anterior peripheral cells (from
12hpf) and along the trunk (from 18hpf) (data not shown). The expression of *Mfu-POU4* in the PPN is maintained until the 24 hpf, after which it gets weaker.

**Evolution of *M. fuliginosus* Sox, Prospero and bHLH genes**

Sox, Prospero, and bHLH genes are important regulators of neurogenesis in many metazoans. Several genes of these groups duplicated and diversified during evolution, and many organisms have several copies of certain genes. To clarify the orthology relationships of the genes we found in the transcriptome resources of *M. fuliginosus*, we performed broad phylogenetic analyses with a focus on a good taxon sampling in Lophotrochozoa. Prospero1 (Prox1) genes are fairly distinct from other homeobox genes. Thus, we chose not to include any outgroup for tree inference. In our unrooted tree, the single Mfu-Prox1 groups with high support with other lophotrochozoan Prox1 genes (Fig. S3), and thus regarded as orthologs. We did not find more than one Prox1 in any lophotrochozoan.

bHLH genes are a large group of genes sharing the basic helix-loop-helix motif. Many genes of the bHLH subgroup A family: Achaete scute (ASC), Oligo, Beta3, Neurogenin, NeuroD, and Atonal play important roles in neural development. Based on a first analysis on the whole bHLH subgroup A, we obtained 6 Achaete scute (ASC), 3 Oligo, one each of Beta3, Atonal, Neurogenin and NeuroD sequence for *M. fuliginosus* (Fig. S4). For gene annotation, we adopted the terminology of #[35]#. The gene numbers in the specific families are similar as in other lophotrochozoans for which genomic data exist, and all families are well supported in the tree with the exception of NeuroD and Oligo, which are paraphyletic. Since Mfu-NeuroD clusters within this assemblage well-supported with NeuroD sequences from other lophotrochozoans, we regard it as an ortholog to those. Based on our tree, we could not resolve the 1:1 orthologs of the three *M. fuliginosus* Oligo sequences. Since not many comparative data exist on Oligo, we did not perform further analyses and named the sequences Mfu-OligoA, Mfu-OligoB, and Mfu-OligoC. We recorded clear expression patterns for Mfu-OligoA, Mfu-OligoB, Mfu-NeuroD, Mfu-neurogenin, and Mfu-ASCa1, Mfu-ASCa2, Mfu-ASCa3, and Mfu-ASCa4.

To get insights into the relationship of the *M. fuliginosus* ASC genes to those studied in other species, especially *Capitella teleta* and *Platynereis dumerilii*, we generated a further tree based on deeper sampling and a more ASC specific and longer alignment. It corroborates the three big metazoan clades ASCc, ASCb containing vertebrate ASC3-5, and ASCa containing arthropod ASC T1-T8 and vertebrate ASC1-2 (Fig. S5). *M. fuliginosus* has representatives of all ASC groups as do other lophotrochozoans. Four *M. fuliginosus* sequences belong to the clade ASCa. The evolution of this group is in lophotrochozoans seemingly more complex than hitherto anticipated, since many species have several gene copies, many of which are poorly studied. Mfu-ASCa1 and Mfu-ASCa2 are closely related to *Platynereis dumerilii* Achaete-scute 1 and to *Capitella teleta* Achaete scute 2 (Fig. S5), but also to two more transcripts from *Platynereis dumerilii*, which we received from the Genbank TSA database, for which no cellular expression data exist, but according to PdumBase [44] are highly expressed in larvae from early on. To give a final answer on clear orthology relationships amongst these 6 genes is not possible based on our
tree. It also remains unclear whether Mfu-ASCa3 has an ortholog in *Platynereis* and *Capitella*. Mfu-ASCa4 clusters with another transcript of *Platynereis dumerilii*, which according to PdumBase is highly expressed in early larvae.

SoxB and SoxC are important regulators of early neurogenesis. Still, the evolution and orthology relationships of SoxB genes in Lophotrochozoa and the relationship to vertebrate SoxB1 and Sox B2 is poorly understood [22,45,46]. This may be due to low taxon sampling in the respective phylogenetic analyses. Thus, we performed a broad taxon sampling, generated a first tree across most existing Sox genes, and found SoxC, SoxD, SoxE, SoxF, and at least a major part of SoxB well supported (Fig. S6). A second analysis focusing specifically on SoxB provides clear evidence that the SoxB1 group predates Bilateria and that Mfu-SoxB1 is orthologous to SoxB1 from *Capitella teleta*, *Platynereis dumerilii*, and *Crassostrea gigas* (Fig. S7). Mfu-SoxB2 likewise falls in a well-supported group, which contains the vertebrate SoxB2 representatives Sox-21 and Sox-14 and also *Capitella teleta* SoxB and *Platynereis dumerilii* SoxB, which we, accordingly, regard as SoxB2 orthologs. The basal branching of the SoxB group remains, however, elusive. We could not detect the expression of Mfu-SoxB2 in the stages analyzed, but we got clear expression patterns for *Mfu-SoxB1* and *Mfu-SoxC*.

**Expression of several neural developmental genes starts early at the anterior and posterior pole and is highly dynamic on the cellular level**

To determine the regions of neuronal specification during early development, we studied the expression of the aforementioned neural and bHLH class proneural genes. The bHLH class of proneural genes is known to be expressed in a transient manner in developing neurons [47]. Therefore, the expression of bHLH genes, but also SoxB1, SoxC, and Prox1, was investigated at 1-hour intervals starting from 2 hpf stage. We could not detect any expression of the genes studied at 2hpf and 3hpf. The first gene being expressed is Mfu-SoxB1, which at 4 hpf is only confined to few cells in the animal pole (Fig. 5). At 5 hpf, the expression of Mfu-SoxB1 extends throughout the animal pole cells while excluding the larger cells at the vegetal pole (Fig. 5). Shortly later, at 6 hpf stage, the expression of Mfu-SoxB1 spans throughout the body, and from 7 hpf stage, it is observed more prominently in the vegetal region. Around 12 hpf stage onwards, the expression of Mfu-SoxB1 gets more segregated as dedicated regions of proliferation and differentiation appear (Sup. Fig. 9). From 18 hpf, however, it remains mainly restricted to the anterior domain, and this pattern continues into later stages (Sup. Fig. 9).

*Mfu-SoxC* appears around 4 hpf in 2-3 cells near the animal pole. By 5 hpf stage, more cells start expressing *Mfu-SoxC* (Fig. 5). From 9 hpf stage onwards, some expression can be detected close to the posterior region, albeit transiently, but no expression was detected in the position of the PPN (Fig. 5). In general, expression of Mfu-SoxC remains mainly confined to the anterior and mid domains with expression in the trunk region starting later from 12 hpf stage (Fig. 5, Sup Fig. 9). The expression of Mfu-SoxC in later stages (24 and 48 hpf) is similar to that of Mfu-SoxB1, as both are mostly restricted to the anterior domain (Sup. Fig. 9).
Mfu-Prox1 is expressed from 6 hpf in few cells in the mid-region. In subsequent stages, the expression also continues to expand mainly in the anterior and mid domains. From 12 hpf, the expression of Mfu-Prox1 extends along the trunk and posterior regions and is mainly detected in pairs of cells (Sup. Fig. 9).

The expression of proneural genes Mfu-ASCa1 and Mfu-Ngn were detected from 5 hpf. While Mfu-ASCa1 is expressed in two cells in the anterior region, Mfu-Ngn was detected in both the anterior and posterior poles (Fig. 5). At 6 hpf Mfu-ASCa1 and now, Mfu-NeuroD also starts to express in the posterior region (Fig. 5). The posterior expression signal of all three genes corresponds to the position of PPN we observed by immunohistochemistry from 9 hpf onwards. Expression of both Mfu-ASCa1 and Mfu-Ngn in the posterior cell is more transient than Mfu-NeuroD, whose expression was observed from 6 hpf stage until around 8 hpf stage (Fig. 5). From 9 hpf stage onwards, none of the proneural genes were detected in the posterior cell, whereas expression domains expand in the anterior region (Fig. 5, Sup. Fig. 10).

Other Achaete scute genes – Mfu-ASCa2, Mfu-ASCa3, and Mfu-ASCa4 also begin expressing from 7-10 hpf stage onwards in an apparent non-overlapping manner (Sup. Fig. 10). The expression of Mfu-OligoA and Mfu-OligoB were detected from 9-10 hpf in a few cells in the anterior and along the developing trunk (Sup. Fig. 10). The Mfu-Oligo genes are expressed in restricted domains in comparison to other bHLH proneural genes. Around the stages 12-14 hpf, several proneural genes display an expanding pattern of expression (Sup Fig. 10). In summary, the expression of proneural genes is dynamic, and many display broad patterns in later stages of development (Sup. Fig. 10).

The first cell expressing proneural genes in the posterior region appears after 7 cleavages and is a descendant of the 2d blastomere

Having detected the expression of proneural genes from 5 hpf onwards at the position where the PPN differentiates, we were interested in tracing the clonal origin of the cell at the posterior pole. For this purpose, we fixed embryos from 30 minutes to 6 hours post-fertilization at every 10 minutes intervals. At least 100 specimens were used during each fixation. We then stained the nuclei with DAPI and recorded z-stacks of at least five larvae from each fixation (a total of 165 embryos). Since the pace of development differed to some extent between embryos, we ordered the image stacks up to the 64-cell stage not by time, but by the number of nuclei. We counted cleaving cells as two. At 6 hpf in the posterior pole, two small blastomeres are visible residing between two larger cells (Fig. 6S’, T). We traced this cell back through development as being a descendant of the D quadrant. Daughter cells were named according to the spindle orientation and the relative position of the cells to each other along the animal-vegetal axis. 30 min after fertilization, the zygote contains two polar bodies along with the male and female pronucleus (Fig. 1A, A’, Supplementary Data 17. 20 min later, the first cleavage generates a larger CD and a smaller AB cell (Fig. 6B, B’, B”C, C’, Supplementary Data 18, 19). The next cleavage gives rise to the 4-cell stage with a D-cell being considerably larger in size than the A, B, and C-cell (Fig. 6D, D’, D”’, E, E, Supplementary Data 20, 21). The spindle orientation of the first two cleavages is perpendicular to the animal-vegetal axis and tilts upwards. The D-cell is the first cell that enters the third cleavage around 1 h
50 min post-fertilization is dextral and gives rise to a smaller animal 1d and a larger vegetal 1D-cell (Fig. 6F, F', F", G, G', Supplementary Data 22,23). The cleavage is sinistral and gives rise to the cells 2D and 2d (Fig. 6H, H', H", Supplementary Data 24). Notably, in the 16-cell stage, the animal 2d cell is larger than the vegetal 2D cell (Fig. 6K, K', Supplementary Data 25). Cleavage 5 and 6 generate first the cell 2d\textsuperscript{2} on the right body side (Fig. 6L, L', L", 6M, M', Supplementary Data 26, 27), which then divides into 2d\textsuperscript{21} remaining on the right body side and 2d\textsuperscript{22} with a more central position. The 2d\textsuperscript{22} travels further posterior until it reaches the posterior-most region in the 64-cell stage, which is around 5 hpf (Fig. 6N, N', N", 6O, O', 6P, P', Supplementary Data 26-28). Cleavage 7, finally, generates the two small blastomeres 2d\textsuperscript{221}, which takes in the most posterior position in the embryo and 2d\textsuperscript{222}, which lies a bit more vegetal and anterior (Fig. 6R, R', R", 6S, S', Supplementary Data 29, 30). At 6 hpf, the cells 2d\textsuperscript{221} and 2d\textsuperscript{222} are flanked left and right by two cells with large nucleus (Fig. 6T, T'). These nuclei are also useful landmarks after fluorescence in-situ hybridization. Between the large nuclei, the \textit{Mfu-NeuroD} expression signal is surrounding a small nucleus, which corresponds to the position of 2d\textsuperscript{221} (Fig. 6U). Slightly anterior and vegetal is a second small nucleus that corresponds to the position of the cell 2d\textsuperscript{222} and is not surrounded by expression signal (Fig. 6U').

**Discussion**

Annelids, along with many other protostome invertebrates, share the presence of a brain and ventral longitudinal nerve cords. While homology of these parts of the CNS within annelids is widely accepted, it is still a matter of debate how the annelid trunk nervous system relates to that of other bilaterians. A highly similar mediolateral patterning of nerve cord development between annelids and distantly related bilaterians suggests a general homology [19,20,48]. The structural diversity of bilaterian nervous systems and molecular data on nervous system patterning from some groups were, however, also interpreted in favor of independent nerve cord evolution [15]. Molecular aspects of early neurogenesis have been studied in several species of Spiralia [21–24,26,49–51], but not on a cellular level and not with a focus on the pioneer neurons prefiguring the main scaffold of the later nervous system and on the dynamics of gene expression. Data on the first steps in nervous system formation are thus highly interesting from both a developmental and an evolutionary point of view.

**The general course of neurogenesis in \textit{Malacoceros fuliginosus} – a variation of a common theme**

The basic processes of neurogenesis, that is, specification of neurogenic areas, progression of progenitors, specification of precursors, and differentiation of neurons in \textit{M. fuliginosus} largely follows a conserved pattern observed in many organisms. SoxB1, whose function in specification and proliferation of neural progenitors has been documented throughout the animal kingdom [52,53] shows up very early in the prospective ectoderm and is expressed throughout the development of the nervous system in all of the investigated stages. SoxC, known to promote cell-cycle exit, follows a little later [54] is also amongst the early expressed genes. Then, several proneural genes of the bHLH families - \textit{Neurogenin, NeuroD, ASC}
and *Oligo* are broadly expressed, which are involved in neurogenesis in many organisms [1, 2, 51, 55]; and *Prox1* and *Elav*, typical markers of postmitotic precursors [56–58]; and finally markers for neural differentiation like *Synaptotagmin* and *Rab3*.

**The first steps in nervous system development in an annelid**

The specific value of our dataset is that, for the first time, we can describe the development of an annelid nervous system in short time intervals — from the onset of the expression of a broad set of neural genes to the first signs of differentiation and the outgrowth of the first neurites which form the scaffold of the later nervous system. Further, we reveal the clonal origin of one of the most important cells, the pioneer of the ventral nerve cord. From these data, we get insights into the dynamics of neural gene expression partly on the cellular level. We can also link the expression of neural genes to the specificities of spiral cleavage, and the data may serve as a sound basis for experimental studies on nervous system development and comparative and evolutionary studies across species and groups.

We do not have evidence that any of the studied genes are maternally expressed in the early embryos when neurogenic regions and the fate of prospective neurons are specified. At 2 hpf and 3 hpf, we did not get any positive signal on the expression of *Sox, Prox1*, and any proneural bHLH gene. *SoxB1* and *SoxC* expression start at the animal pole around 4 hpf, where according to the embryological data, the vegetal pole is not yet covered by a layer of smaller blastomeres. The extension of *SoxB* and *SoxC* towards the vegetal pole during the next 2-3 hours seems to correlate with epibolic coverage of the vegetal macromeres. Though, *SoxB* expression extends completely to the vegetal pole or the posterior end; we could not observe the expression of *SoxC* in these regions.

Before *SoxB1* expression extends towards the vegetal pole, expression of some proneural genes already starts in the PPN. We detected the first expression of few bHLH genes at around 5 hpf, wherein the embryos have completed the 6th cleavage, and most cells are soon entering the 7th cleavage. During the early stages, the expression is restricted to only very few cells in the anterior and the posterior region, and this pattern remains until the first neurons differentiate at around 9 hpf. The expression of proneural genes becomes broader when the first neurons already express differentiation markers and send out the first neurites. The axons of few cells create the main routes of the central nervous system. The axon of the AN1 travels on one body side down towards the prototroch and probably further towards the prospective course of the VNC. From the other side, the single bifurcating PPN sends axons upwards towards the prototroch region. The prototroch ring nerve is formed by two pioneers (PNNs) on the ventral side of the prototroch. Soon more cells are developing mainly in the anterior hemisphere, which mainly projects towards the prospective brain region. At the posterior end, another neuron differentiates, which, as a follower, sends axons along the axons of the VNC pioneer. Early expression of neural genes in the anterior region may partly result from the transcriptional activity of the AN1. Still, we were not able to localize the expression patterns clearly in this cell since other cells also start developing early. The paired
early expression signals of *Ngn* and *Prox1* in the middle of the body may likely be localized to the PNNs. Precise cellular localization of neural gene expression is possible in the PPN since we can observe it already at 9 hpf by immunohistochemistry and that we can see *NeuroD* expression up to 8 hpf in this specific position.

From the cellular localization pattern, we infer that the expression of some proneural genes is highly dynamic and differ in their regulation in different cells. While *Ngn* expression in the PPN is only at 5 hpf, it is likely to be expressed from 6-7 hpf in the PNNs, and 5-9 hpf in an anterior cell. ASCa1 is also expressed at one stage (6 hpf) in the PPN, while *NeuroD* remains expressed for 3 hours. The expression of many proneural genes is known to be transient and is downregulated prior to the differentiation phase [47]. In *M. fuliginosus*, the expression of *Ngn, ASCa1*, and *NeuroD* genes in the PPN is transient, lasting only for one to 3 hours, and ceases expressing before differentiation starts at around 9 hpf.

The inference of gene dependency or co-regulation of genes based on co-expression analyses is always speculative. The observed dynamics of gene expression provides an additional obstacle for this approach since the periods of overlapping expression in specific cells may be very brief. Nonetheless, it is worth noting that *Ngn* is the first proneural gene in the PPN, followed by *ASCa1* and *NeuroD*. Interestingly, *Ngn* expression in the posterior region is only at a time point when the 7th cleavage of the posterior pioneer precursor is not completed in all embryos of a batch. During vertebrate neurogenesis, *Ngn* is transiently expressed in proliferating precursors, which are then committed to a neural fate [59]. Similarly, expression of *Achaete-scute* and *Ngn* has been reported from proliferating neurons close to the ventral midline in later developmental stages in the annelid *Platynereis dumerili*[i][60].

Seemingly, in the prototroch pioneers, *Ngn* precedes the expression of *Prox1*, which is known to initiate the specification of neurons after they stopped cycling [57]. Although the molecular mechanisms by which proneural genes regulate neurogenesis and specify subtypes are being extensively studied, important questions remain as to what their main function is: general neurogenesis or subtype specification or both. Based on the short time intervals of our gene expression studies, we have the impression that many of the bHLH genes, especially the ASCa gene paralogs, are differentially expressed and probably involved in defining neural subtypes.

**Expression of Sox and bHLH genes in annelids**

A comparison of the general course of neurogenesis with other annelid representatives must take the orthology relationships of the genes investigated and the staging of the embryos into account. The gene expression patterns mainly reveal differences in the time course of neural development along the anterior-posterior axis. In contrast to *Platynereis dumerilii* and *Capitella teleta*, neurogenesis progresses at a more similar speed in the anterior, the trunk, and the posterior in *M. fuliginosus*. The differentiation markers *Synaptotagmin* and *Elav* show up in the anterior region in *Platynereis* and *M. fuliginosus* around 14 hpf, and also the onset of *Prox1* expression may be similar, as it is already expressed in several cells in 12 hpf.
in *Platynereis* [61]. However, in the trunk of *Platynereis*, SoxB is most broadly expressed around 24 hpf and stays active until 55 hpf [24], while we find broadest expression in the trunk of *M. fuliginosus* around 14 hpf and downregulation already around 18 hpf. The significance of the comparison is limited since the SoxB investigated in *Platynereis dumerilii* [24] clusters within SoxB2 in our analysis, and we obtained expression patterns only for SoxB1. Only for *Capitella* expression data of both SoxB1 and SoxB2 exist [22]. As they show overlapping expression, they may have a redundant function similar to arthropods rather than an antagonistic function as in vertebrates [22]. In the trunk, Elav and Synaptotagmin are reported from 32 hpf onwards in *Platynereis* [19], while they show up already at 14-18 hpf in the trunk of *M. fuliginosus*. In *Capitella*, SoxB, Prox, and several bHLH proneural genes show up much later in the trunk and the posterior than in the anterior [22].

The expression of several bHLH proneural genes is likewise not easy to compare between annelids, especially of Achaete-scute. According to our analysis, Mfu-ASCa1 (which shows up first and whose expression was detected in the PPN) and Mfu-ASCa2 are closely related to *Platynereis* Achaete-scute1 and Achaete-scute2 and Capitella Cte-Ash2. While Platynereis Achaete-scute2 is not yet an annotated sequence, Cte-Ash2, was shown to have a broad expression in several tissues and not further studied (Meyer & Seaver, 2009).

Both *Platynereis*, *Achaete-scute1*, and *Achaete-scute2* are known to be involved in nervous system development, but the description of their activity is only from later stages. In the study from [23,48] the earliest stage described is 24 hpf, during the stage where the VNC is populated with many neurons and in [27] during brain development at 48 hpf. Notably, during VNC development in *Platynereis*, *Achaete-scute1*, and Neurogenin are expressed in proliferating cells (Demilly et al., 2013). This may explain why these genes are expressed so early in *M. fuliginosus*. In *M. fuliginosus*, Neurogenin may be expressed before completion of the last cleavage of the PPN precursor. The other ASCa genes of *M. fuliginosus*, Mfu-ASCa3, and Mfu-ASCa4 are closely related to Cte-Ash1 and a transcript from *Platynereis*, which, according to PdumBase is highly expressed in early stages, but for which no cellular expression data exist. Cte-Ash1 is expressed early in the anterior region in *Capitella* during the formation of the brain and extends later also to the VNC and the posterior end (Meyer & Seaver, 2009; Sur et al., 2017). Mfu-ASCa3 and Mfu-ASC3 are also expressed relatively early in the anterior region, but not as early as Mfu-ASCa1 and also not in the PPN. Since the expression of Mu-ASCa4 starts around 8 hpf and from Mfu-ASCa3 around 10 hpf it is unlikely that they are involved in the early development of the first pioneers we observed in the anterior, midbody and posterior region. Neurogenin and NeuroD are also fairly early expressed in *Capitella* (Sur et al., 2017), and even a transient early expression is reported in the pygidium, but it remains unclear in what kind of cells and how they develop further.

Our gene expression data, due to the short time intervals, gives a first insight into the dynamic nature of proneural genes in annelids. And probably relevant for further studies on the function of the genes at the cellular level. Co-expression studies have to take into account that several of the genes are quickly up- and downregulated.
Position and role of pioneer neurons in Lophotrochozoa

On a comparative level, the role of pioneer neurons in lophotrochozoans and other Spiralia have been studied by immunohistochemical analysis of differentiated neurons. Similar to *M. fuliginosus*, the first appearing neurons in many organisms were found in the anterior and/or the posterior region of the embryos. The observed variability in position, neurotransmitter, and projection raised many questions, whether the formation of the nervous system in these animals progresses rather from anterior to posterior, vice versa, or from both sides and from central to the periphery or the other way round. A comparison of data is biased by the fact that many studies focus mainly on the small subset of serotonergic and FMRFamidergic neurons, while our data show that in *M. fuliginosus* the earliest differentiating pioneer neurons are devoid of these neurotransmitters. Moreover, many other neurons are already present before the first serotonergic and FMRFamidergic neurons are discernable. Nevertheless, evolutionary conservation of some patterns can be inferred. A posterior VNC pioneer probably has been present in the last common ancestor of the two major annelid subgroups - Errantia and Sedentaria [62]. A posterior bifurcating neuron highly similar to the cell we investigated in *M. fuliginosus* was described from the errant annelids, *Phyllodoce maculata* [63] and *Platynereis dumerilii* [31,64] and the sedentary annelid, *Pomatoceros lamarckii* [65]. In all four species, the respective cell is located on the very posterior tip of the developing larva, sends a bundle of cilia to the exterior, and two very long axons anteriorly along the future developing VNC. These similarities strongly suggest homology. Thorough electron microscopy-based characterization of neural circuitry in 3-day-old larvae of *Platynereis* revealed that this cell beside its pioneering role also takes in a sensory-motor function by directly synapsing to multiciliated cells of the prototroch [66]. We could trace the axons of the PPN to the level of the prototroch making a similar circuitry conceivable in *M. fuliginosus*. Neurotransmitter content of the PPN seems to vary in annelids. While it is serotonergic in *Phyllodoce maculata* [63] and *Pomatoceros lamarckii* [65], it contains serotonin and FMRFamide in *Platynereis dumerilii* [64,66] and none of these transmitters in *M. fuliginosus*, even though the expression of Synaptotagmin and Rab3 suggest the capacity of transmitter release. Accordingly, the presence of this cell may be overlooked, if studies on nervous system development rely mainly on stainings against few neurotransmitters like FMRFamide and 5HT and not on broad neuronal markers and also if time intervals between studied stages are too big to trace the outgrowth of the first neurites. The PPN is likely lost in *Capitella*, where a close examination of neural development provides no hints on the presence of such a cell [32], while this is difficult to judge for few other annelid trochophores, where less detailed data exist.

In the anterior region, we found two different kinds of pioneer neurons in *M. fuliginosus*. While the prototroch ring nerve is pioneered by axons of two peripheral sensory cells (PNNs), the nerves running posteriorly from the apical plexus associated with the apical organ is pioneered by neurites of a ganglion cell (AN1). This challenges the view that the CNS of annelid trochophores is generally prefigured only by peripheral sensory cells [29,63,64,67]. Like the PPN, the early anterior pioneer neurons of *M. fuliginosus* cannot be identified by studying serotonin or FMRFamide immunoreactivity. In accordance, cell-lineage
data from *Platynereis* show that several cholinergic neurons appear much earlier in the anterior region than the first serotonergic neurons and that the former already forms a considerable mesh of neurites at 30 hpf [61]. Data from *Capitella* show that the first differentiating neurons in the apical region are not peripheral sensory cells, but are from within the future brain [32].

In conclusion, it is likely that in the ancestor of errant and sedentary annelids, the first neurons pioneering the scaffold of the later CNS arose at the anterior as well as the posterior pole. While in the posterior only peripheral sensory cells take in this function, in the anterior, probably both peripheral sensory cells and central ganglion cells are involved. This may be valid for annelids in general, though the situation in the few basal branching groups needs further investigation. The question in which direction CNS formation in annelids is progressing encompasses different aspects that are not necessarily linked to each other. For the differentiation of the vast majority of CNS neurons, existing data points towards an anterior-posterior progression. On the other side, the suggested ancestral presence of pioneers in both the anterior and the posterior regions means that scaffold formation of the CNS started from both sides. From which region the first neurites extend faster in extant representatives is a matter of the exact onset of neurite outgrowth from the respective poles. This may be strongly affected by even small heterochronic changes in development underlying the observable variation between species.

If further evidence arises that cell-autonomous specification drives the formation of the first differentiating neurons in the anterior as well as in the posterior region of annelid trochophores, scaffold formation of the whole annelid CNS may be under the strong influence of neurons specified by maternally inherited factors. No molecular data exist from lophotrochozoans or Spiralia other than annelids on the neurogenesis and specification of early CNS pioneer neurons. Yet, a large number of immunohistochemical studies suggest that the first neurites prefiguring the main routes of the nervous system likewise develops from a few early developing cells in mollusks, nemerteans, and lophophorates. Obtaining deep data on pioneer neuron specification, cell-lineage, and role in axonal pathfinding in spiral cleaving animals may thus be highly informative for a better understanding of nervous system development and evolution.

**Cell lineage, spiral cleavage, and cell-autonomous specification**

In the large taxon of Lophotrochozoa and Spiralia, the molecular development of the neurons which pioneer the main routes of the developing nervous system has not been studied in detail. By correlating immunohistochemical and gene expression data, we could trace the development of the VNC establishing pioneer, PPN, from 5 hpf. By studying the embryology of *M. fuliginosus*, we could also reveal the clonal origin of the cell. The PPN is a derivate of the D quadrant of the spirally cleaving embryo. More specifically, it is the cell 2d^{221}, which can also be identified on the molecular level by transiently expressing *Neurogenin, NeuroD*, and *ASCa1*. In Spiralia, descendants of the first micromere quartet (1a-1d) are known to contribute anterior neurons and the brain [68–70]. A contribution of 2d descendants to
the nervous system has also been repeatedly reported, especially to the trunk nervous system. Either by labeling early blastomeres [69,71] or by arresting cleavage and studying tissue-specific markers. With the latter approach, it was shown that in *Platynereis dumerilii* 2d2, 2d12 and 2d112 and 2d1122 are expressing neural markers after days of arrested cleavage and that 2d2 and 2d12 take in a posterior position in the embryo [72]. Our data confirm the contribution of 2d descendants to the trunk nervous system. More specifically, 2d22 plays a central role in trunk nervous system formation from the very beginning. It will be interesting to investigate whether other cells that branched off earlier or other 2d descendants contribute to the formation of the VNC in *M. fuliginosus*. Interesting candidates are the descendants of the cell 2d222. We could not finally clarify whether the direct precursor 2d22 already starts expressing *Neurogenin* before it divides into 2d222 and 2d221.

In spirally cleaving animals like annelids, mollusks or flatworms, inherited cell-intrinsic properties are important for fate specification of early blastomeres [73–76], a process which often is called cell-autonomous specification. mRNA segregation during several rounds of asymmetric cell divisions is supposed to be the main mechanism for passing on maternal factors [77–80] and probably is most relevant for specification of early differentiating cells before regulative mechanisms mediated by extrinsic factors take over during later developmental stages.

The direct influence of cell-intrinsic properties on nervous system development in Spiralia, however, is poorly understood. Notably, most evidence has recently been provided from annelids, where data points towards cell-autonomous specification of neurons in the anterior region of the embryo. Correlation of 3D cell-lineage data of the trochophore episphere with gene expression data in *Platynereis dumerilii* revealed that many of the later appearing neurons with bilateral symmetry and similar features do not share corresponding lineages in left-right opposing quadrants suggesting a position related conditional specification [61]. In difference, early differentiating neurons of the apical organ do not originate from bilateral symmetrical clones. They do not express several transcription factors involved in head regionalization and therefore pointing towards cell-autonomous specification. In *Capitella*, separation of the micromeres 1a-1d from the rest of the embryo in the 8-cell stage leads to head-only partial larva with cells expressing the neuronal marker *Elav* and seen as evidence for cell-autonomous specification of the respective neural fate [81]. Notably, the AN1 in the future apical organ pioneering the anterior part of the circumesophageal connective in *M. fuliginosus* also does not have a bilateral symmetrical counterpart, which might as well indicate cell-autonomous specification. Nevertheless, this remains speculative and needs further investigation.

According to our study, the PPN (VNC pioneer neuron) may be an excellent study subject for future investigations on the mode of specification of early neurons and the involvement of cell-intrinsic factors. A known cell-lineage, the few numbers of cleavages, easy identification, and experimentally accessibility on the very posterior end may allow the application of a broad set of experimental tools. Respective studies, deep molecular characterization of the early pioneers, and molecular data on axon finding may deepen the understanding of nervous system development in Spiralia, and comparative studies may
undoubtedly be interesting in the context of the ongoing debate on the homology of nerve cords in Spiralia and Bilateria.

**Declarations**

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

DNA sequences of *Mfu-SoxB1, Mfu-SoxC, Mfu-Prox1, Mfu-Elav, Mfu-Syt1, Mfu-Rab3, Mfu-Ascl1, Mfu-Ngn, Mfu-Olig, Mfu-NeuroD, Mfu-Brn3* will be submitted to Genbank upon final submission of the manuscript. The supplementary data sets will be submitted to Dryad repository upon final submission of the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

SK carried out gene cloning, gene expression studies, immunohistochemistry and imaging. ST and CH also contributed to gene-expression studies, immunohistochemistry and imaging. HH did transcriptome assembly. SK and HH designed the study, analyzed and processed the data, and CH helped to analyze data. SK and HH drafted, CH commented, and SK and HH finalized the manuscript. All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

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**Figures**

![Diagram](image)

**Figure 1**

Development of early larval stages of Malacoceros fuliginosus. (A-D) Light microscopic images of larval development. (E-J) Nervous system development in Malacoceros fuliginosus as revealed by acetylated α-tubulin (ac-tubulin) immunolabeling (green). The primary ciliated structures such as apical tuft (at), prototroch (prt) and telotroch (tel) are also revealed. (K,L) SEM images showing the dorsal view of...
Malacoceros fuliginosus larva. The arrowheads (E-H) point to the developing posterior pioneer neuron (PPN) and bifurcating axons. Arrows (I) point to the first ventral nerve cord commissure. Scale bars: 20 µm. ch: chaetae.

Figure 2

Pioneer neurons forming the main neuronal scaffold. The schematic diagram indicates the larval regions shown in the figures (A-K). (A,B) A pair of sensory cells (white asterisk), visible clearly from 16 h gives rise to the prototroch nerve ring. The dotted circles show the adjacent prototroch cells. (C,D) An apical ganglion cell (AP1) (orange asterisk) with a descending neurite is prominent from 18 h. The orange arrowheads (D,I) indicate the converging axons. (E) Posterior pioneer neuron (PPN) with dense tubulin at 9 h along with DAPI (blue). (F) Side view at 10 hpf showing the orientation of the posterior pioneer (see inset). (G-H’) Follower neurons indicated by arrows. A single follower neuron (G) and a pair of sensory follower neurons (H,H’). (I-K) Development of the anterior neuronal scaffold. (J,K) Later appearing neuronal pairs (red asterisk) along with peripheral sensory neurons (arrowheads) converge to form the
central ganglia. (L-Q) IHC of serotonin (5-HT) and FMRFamide during early nervous system development. (L) 5-HT is detected in many neurons and neurites at 48h. (M) FMRFamide is detected in many neurons and few neurites at 48h. (N) First FMRFamide is detected at 14 h and restricted to one ciliated sensory cell located dorsally of the apical tuft cell. (O) First 5-HT is detected at 21hpf and is restricted to one pair of ciliated sensory cells ventral to the forming apical plexus. (P,Q) At 24 hpf, two pairs of ciliary sensory cells with FMRF and 5-HT are present in the apical region. Asterisks (N-Q): nucleus of cells with FMRFamide or 5-HT. Scale bars: 10 µm, (except F: 20 µm, N-Q: 5 µm). at: apical tuft, cec: circumesophageal connective, cg: central ganglia, prt: prototroch, sec: supraesophageal connective, tel: telotroch.

Figure 3

Schematic reconstruction of early nervous system development based on ac-tub, 5-HT and FMRFamide, and nuclear stainings.
Figure 4

Expression of differentiation genes during larval development. (A-H) WISH of neuronal genes Mfu-Elav1, Mfu-Syt1, Mfu-Rab3. (I) FISH of Mfu-Syt1 combined with IHC of acetylated-tubulin in the posterior region. (J-L) FISH of Mfu-POU4. Inset in (J) shows the expression of Mfu-POU4 in the anterior region. Arrowheads point to the posterior pioneer neuron, while arrows point to the neurons adjacent to it. Scale bars: 20 µm
Figure 5

Expression of neural and proneural genes during 4-9 hpf stages of larval development. Scale bars: 20 µm
Figure 6

Clonal lineage of the cell 2d222 in the embryo of M. fuliginosus. (A-T’) Images of fixed, DAPI stained specimens from first to 7th cleavage. The images are labeled either by their stage (1c-64c, 5h, 6h) or by the cleavage (cl1-cl7). The positional labels animal and vegetal do not refer to the absolute position in the embryo, but the relative position of image pairs of the same stage or cleavage. Arrows indicate the spindle direction. (U-U) FISH with probe against Mfu-NeuroD at 6h stage, posterior part of the embryo. (Yellow asterisks) corresponding landmarks between image pairs, (black asterisks) spindle pole in focus plane, (pb) polar body, (pn) pronucleus. Scale bars: 20 µm.

Supplementary Files

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- FigS3.pdf
