The neuron pioneering the ventral nerve cord does not follow the common pathway of neurogenesis in the polychaete Malacoceros fuliginosus

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Research article

Keywords: Nervous system, Development, Evolution, Annelida, Pioneer neurons

Posted Date: January 3rd, 2020

DOI: https://doi.org/10.21203/rs.2.19981/v1

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Version of Record: A version of this preprint was published on September 14th, 2020. See the published version at https://doi.org/10.1186/s12862-020-01680-x.
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 early steps of nervous system development in the annelid *Malacoceros fuliginosus*.

**Results:** We find that the first pioneer neurons are already in place in the anterior and posterior pole when broad neurogenesis is just starting. They do not express serotonin or FMRFamide which are commonly used markers in studies on nervous system architecture. A single posterior neuron prefigures the main course of the ventral nerve cord and this mode is probably ancestral for majority of annelids. Notably, none of the studied sox and proneural genes, which are commonly involved in the generation of neurons, is expressed by this important neuron. The only transcription factor we found expressed is Brn3, which likely acts on a low hierarchical level.

**Conclusions:** We propose that the annelid ventral nerve cord pioneer neuron follows a highly divergent course of neurogenesis. The lack of Sox and proneural transcription factors, which are usually under control of patterning cell-extrinsic factors suggest a major influence of inherited cell-intrinsic properties on the development of this cell. Though cell-autonomous specification is generally an important pathway in the early development of spirally cleaving animals, its relevance for nervous system development is poorly understood. Our data suggest that closer investigation of the specification of pioneer neurons in animals featuring spiral cleavage will be highly informative to obtain a better understanding of how nervous systems form and evolve.

**Introduction**

The basic scheme of early neurogenesis, i.e. 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 displays 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 well conserved the main processes of nervous system patterning in bilaterians are 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], 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 scaffold of the later central nervous system. In this study, we aim to characterize the development of such pioneer neurons in the annelid Malacoceros fuliginosus. We found that those are already in place in very early developmental stages at a time, where neurogenesis and patterning of the vast majority of neurons is just starting. A single posterior neuron prefigures the main course of the ventral nerve cord. While differentiation markers confirm the neural identity of the cell it lacks expression of many neurogenic markers known from other organisms and observed throughout the developing nervous system in M. fuliginosus. Evidently, the pioneer follows a highly divergent course of neurogenesis. Accordingly, cells strongly impacting the later structure of the nervous system may not depend on the same patterning processes as other neurons do. We propose that closer and comparative investigation of CNS pioneer neurons probably will deepen the understanding of both the development and the evolution of nervous systems.

Methods

Malacoceros fuliginosus culture

Adult Malacoceros fuliginosus were collected from Pointe de Mousterlin, Fouesnant, France and were maintained in sediment containing sea water tanks at 18 °C. Individual males and females were picked, rinsed several times with filtered sea water and kept in separate bowls until they spawned. 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.

RNASeq and transcriptome assembly

Total RNA was extracted from cryofixed samples of various larval stages using the Agencourt RNAdvance 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 [28] followed by gene clustering using Corset [29].

**Gene cloning and probe generation**

Gene transcripts were retrieved from the assembled transcriptome by bidirectional blast. Specific primers were designed 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). Gene orthology was inferred by reciprocal blast against GenBank.

**In-situ hybridization**

Larvae 48 h and older were first relaxed with 1:1 MgCl$_2$-sea water 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 (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 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) double staining was according to the protocol described in Lauter et al. (2011).
Immunolabeling

Larvae 48 h and older were first relaxed with 1:1 MgCl$_2$-sea water 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) for 48 h at 4 ºC. 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 Scientifio) overnight at 4 ºC. 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 1% DABCO) at 4 ºC.

Microscopy and image processing

Samples were mounted in glycerol and imaged using 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

Anterior and posterior origin of the nervous system

The early development of the M. fuliginosus larva is highly synchronous and therefore allows for precise staging across batches. The nervous system development is relatively fast and with neglicent yolk content, detailed investigation is possible. For this purpose we performed antibody stainings against acetylated alpha-tubulin (a-tub) from post gastrulation stages (7 hpf) onwards where only the cilia of the prototroch and an apical tuft are visible.

Shortly later at 9 hpf, the first neurons appear at the anterior and posterior end of the larva and from 12 hpf first neurites start extending from the posterior along the route of the later developing ventral nerve cord. In the anterior we could see first neurites from 14 hpf onwards (Fig. 1, Fig. 3, Supplementary data 2). Studying stages in short time intervals allowed us to identify the 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 which starts to differentiate around 8 hpf with the accumulation of dense microtubules and acquires a distinct morphology by 9 hpf (Fig. 1J, Fig. 3, Supplementary data 1). This bifurcating cell projects two axons proceeding towards the prototroch while also acquiring a curved morphology with few sensory cilia extending outwards on the ventral side (Fig. 1K, inset). At 14 hpf, these axons extend already halfway towards the prototroch and reach the prototroch area around 18hpf (Fig. 1, Fig. 3). At around 16hpf, an additional neuron (without sensory cilia) and presumably a follower develops adjacent to the posterior pioneer and extends axons along the processes formed by the pioneer (Fig. 2L). More follower neurons
appear at 34 hpf, when a pair of weakly stained ciliated sensory cells are visible on either side of the posterior neuron and become more prominent at 36 hpf (Fig. 2M,M’).

In the anterior, the prototroch nerve starts to develop at 14 hpf from a pair of cells situated adjacent to the ventromedial prototroch cells. These cells have a triangular morphology and sensory cilia projecting externally. Interestingly, only one of these cells sends out processes extending on either side along the prototrochal cells (Fig. 1E, Fig. 3, Supplementary data 2). Few hours later the other cell which only has thin neurites projects onto the passing connectives from the adjacent cell. The surrounding prototroch cells seem to send extensions towards to the prototroch nerve (Supplementary data 4, 5 and 6). 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 the left side of the apical organ along with a descending axon (Fig. 3, Supplementary data 3) which becomes more prominent at 18 hpf (Fig. 2F). Around 19 hpf, this growing axon traverses contralaterally up to the prototroch region (on the right side) and extends posteriorly towards the anteriorly traveling neurite of the posterior pioneer neuron (Fig. 1F, Fig. 3, Supplementary data 4) and thereby closing the gap between anterior and posterior parts of the developing VNC. Meanwhile, at the same time, the growing axons from neurons pioneering the prototroch nerve travel posteriorly and join the developing VNC (Fig. 1G).

At 21 hpf more cells appear symmetrically around the apical organ and traverse contralaterally along the neurites established by the single ganglion cell which still persists (Fig. 1H, Fig. 3, Supplementary data 5). The crisscross of neurites originating alongside the apical organ creates a plexus which becomes the larval brain neuropil (Fig. 1I). By 24 hpf, the single ganglion cell becomes less prominent and is not easily identifiable as more and more differentiated cells start to innervate the brain neuropil (Fig. 1I, Fig. 3, Supplementary data 6). The prototroch nerve forming neurons, however, are still identifiable at 24 hpf which by 34 hpf become less conspicuous (Fig. 1I, Fig. 1D).

Around 24 hpf, a weakly stained commissure is arising in the anterior trunk region that connects the two VNC neurite bundles (Fig. 1C, Fig. 3, Supplementary data 6). The nervous system represented by the 34 hpf stage is of a typical polychaete trochophore composed of an apical tuft, prototroch, telotroch and neuronal elements such as a central cephalic neuropil, a prototroch nerve ring (semi-circular) and VNC. This basic neuronal architecture continues into the later larval stages and likely becomes part of the adult nervous system. In the anterior, numerous sensory cells develop throughout the head region with projections reaching the brain neuropil (Fig. 1D).

**Development of serotonergic and FMRFamidergic neurons**

For comparative purposes we studied immunoreactivity against 5HT and FMRFamide, which are common markers in studies on invertebrate neural development. While serotonin and FMRFamide is strongly expressed in the apical neuropil and in the trunk in 48 hpf stages (Fig. 2A,B), only few serotonergic and FMRFamidergic cells are present in younger stages. Anti-FMRFamide-LIR is detectable
first at 14 hpf as a single, ciliated flask shaped cell weakly labelled slightly left and dorsal from the apical tuft cell (Fig. 2C, Fig. 3, Supplementary data 2). This cell develops an axon which 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. 2E, Fig. 3, Supplementary data 6). At the same time a second pair of ciliated flask shaped cells with anti-FMRFamide-LIR also sending axons in the apical neuropil become visible ventrally from the apical tuft cell (Fig. 2F, Fig. 3, Supplementary data 6). The first anti-5HT-LIR shows up at 21 hpf stage 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. 2D, Fig. 3, Supplementary data 5). Up to 24 hpf stage neither anti-FMRFamide-LIR nor anti-5HT-LIR are 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 is immunoreactive against the used anti-FMRFamide and anti-5HT antibodies.

Progression of neurodevelopmental genes and domains of early differentiation

To determine the neurogenic components active during the early nervous system development we selected candidate genes corresponding early to late neurogenesis and studied their gene expression patterns. The earliest expressed of the investigated neuronal genes is Mfu-SoxB1, whose expression at 4 hpf is only confined to the animal pole, but soon expands to the whole neuroectoderm. At 8 hpf, the expression is not continuous along the neuroectoderm as a small gap appears in between the broad anterior and posterior expression domains (Fig. 4). In the subsequent stages, the expression profile is more segregated and gaps appear between domains in the anterior, middle and posterior regions of the larva (Fig. 4). By 18 hpf, Mfu-SoxB1 expression domain is mainly restricted to the anterior domains and this pattern continues in later stages. Although we identified another member of the SoxB group, Mfu-SoxB2 in our transcriptome, we could not detect any expression in the stages analyzed.

Mfu-SoxC appears around 4 hpf in 2–3 cells near the animal pole and by 8 hpf only a small anterior expression domain is present (Fig. 4). In general expression remains mainly confined to the anterior domains with some weak expression in the trunk region. At 14 hpf expression is much stronger in the anterior and middle regions and additionally a pair of cells with weak transient expression appears in the posterior.

We then characterized the expression of postmitotic markers and synaptic markers to identify newly born neurons. In general, conserved markers such as Prox1 and Elav define most if not all committed neuronal precursors. Mfu-Prox1 expression begins shortly after Mfu-SoxC expression. At 8 hpf, Mfu-Prox1 is expressed in distinct cells in the anterior and in few cells in the trunk region. By 14 hpf, expression has expanded until the posterior region where two pairs of cells are visible in the ventral region of the posterior (Fig. 4). In the next stages Mfu-Prox1 appears to be downregulated in the anterior domains and expands further along the trunk. In the posterior, however, the cell numbers do not expand and the expression remains persistent. The expression occurs in pairs of cells along the trunk and posterior and
its prolonged expression suggests the cells are post-mitotic and in the process of differentiation. In general, Mfu-Prox1 is expressed on the dorsal side in the anterior and trunk and in the posterior region its expression is more on the ventral side.

The onset of Mfu-Elav expression is much later at around 12 hpf and gradually gets stronger in the anterior near the apical organ and within a pair of cells in the posterior. By 18 hpf more cells in the brain region and cells around the trunk start expressing Mfu-Elav and at 24 hpf strong expression is observed in the anterior, trunk and posterior regions (Fig. 4).

The expression of synaptic genes begins simultaneously in the anterior and posterior domains at around 12 hpf (data not shown). Mfu-Syt1 expression was detected around 12 hpf in the posterior-most cell, with weak expression in the apical organ region and more lateral sensory cells. Mfu-Rab3 is expressed in a similar manner in the early stages. After 14 hpf, the expression of Mfu-Syt1 and Mfu-Rab3 rapidly expands in the brain region revealing the cells that contribute to the central neuropil (Fig. 4). In the posterior, however, Mfu-Rab3 is more broadly expressed than Mfu-Syt1 (Fig. 4). This pattern of expression continues until 24 hpf (Fig. 4) and by 48 hpf most of Mfu-Syt1 and Mfu-Rab3 staining is restricted to the anterior, oral and along the VNC (Additional file 2).

**Most neurons follow the common scheme of neurogenesis**

To determine whether the candidate genes that were analyzed do indeed correlate and overlap spatio-temporally, we performed double fluorescent in situ hybridizations. We first performed double labeling of Sox genes at certain developmental stages. At 14 hpf, as described earlier, Mfu-SoxB1 expression is found in broader domains in the anterior, trunk and posterior, whereas Mfu-SoxC is mostly confined to the anterior. We observed that several cells in the anterior neuroectoderm coexpress Mfu-SoxB1 and Mfu-SoxC (Fig. 5A-A”). Moreover, only Mfu-SoxC expression is observed underneath the region of the prospective brain, suggesting ingression of the cells from the neuroectoderm after downregulation of Mfu-SoxB1 (Fig. 5A”). In later stages (48 hpf), the expression pattern of Mfu-SoxB1 and Mfu-SoxC is more segregated and found mostly in the anterior and oral regions. In this stage, they are mostly expressed in proximity to each other in a mutually exclusive manner but in the few overlapping domains, only either one of the gene is strongly expressed (Additional file 1).

To investigate the next sequence of genes involved in neurogenesis, we performed double staining of Mfu-SoxC and Mfu-Prox1. As previously described, at 14 hpf, Prox1 is expressed in distinct domains in the anterior, middle and posterior. We detected a small number of cells coexpressing both genes in the anterior and middle (Fig. 5B-B”) whereas in the posterior we did not observe any coexpression at the stage analyzed. In addition, double labeling of Mfu-SoxB1 and Mfu-Prox1 revealed that the latter is exclusively expressed outside the Mfu-SoxB1 expressing domains which likely indicate regions of cellular differentiation (Additional file 1).

In continuation of the neuronal hierarchy we combined Mfu-Prox1 along with Mfu-Elav. At 24 hpf expression of both genes is broad and specifies neurons in the anterior, trunk and posterior domains. We
observed co-expression in all three domains (Fig. 5C-C”). Some cells persistently co-express Mfu-Prox1 and Mfu-Elav, particularly in a pair of posterior cells whereas most other cells in the anterior and trunk have transient coexpression.

A deviating course of neurogenesis in the posterior pioneer neuron

The first neuron to send out axonal processes is the bifurcating posterior ventral nerve cord pioneer neuron which starts differentiating around 8 hpf. At this stage, Mfu-SoxB1 is expressed throughout the neuroectoderm. Due to its distinct morphology, spatial organization and early onset, the posterior pioneer cell can be easily followed from 8 hpf stage onwards and correlated with gene expression profiles. By combining in situ hybridization along with immunohistochemical staining of tubulin, the differentiating posterior pioneer neuron was identified in the midst of proliferating Mfu-SoxB1 + cells (Fig. 6A). At this stage Mfu-SoxB1 is clearly absent from the posterior pioneer neuron and also does not show up at this position in later stages. Moreover, Mfu-SoxC remains mostly restricted to the anterior domains and does not extend to the posterior before 12–14 hpf and does not show up in the mid-posterior position. The broadly expressing Mfu-Prox1 and Mfu-Elav are present from 14hpf in the posterior, but only flank the posterior pioneer neuron (Fig. 6B,C). For complementing the dataset on proneural genes employed by the posterior pioneer neuron we screened also for expression of Mfu-Ascl1, Mfu-NeuroD, Mfu-Ngn and Mfu-Olig (Additional file 3). None of them are expressed in the posterior pioneer in the same stages as studied above. Their broad expression in other regions, however, suggests its function in most other neurons. The first neuronal gene we detected was the sensory neuronal marker, Mfu-Brn3 starting from 10 hpf (Fig. 6F-H) and the expression of which within this cell is maintained at least until 24 hpf. Mfu-Brn3 is also expressed in few cells in the anterior which likely become sensory neurons. At 14 hpf an additional pair of cells lying adjacent to the posterior neuron start to express Mfu-Brn3 and by 18 hpf many more cells throughout the larval body are revealed (Additional file 4). The posterior expression of Mfu-Syt1 and Mfu-Rab3 corresponds to the posterior pioneer cell and was validated either by counterstaining with acetylated tubulin (Fig. 6I,I’) or recognized by its distinct morphology. Since Mfu-Syt1 and Mfu-Rab3 could no longer be detected in the posterior after 48 hpf we could not track the development of the posterior pioneer in later stages.

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 central nervous system within annelids is widely accepted, it is still a matter of debate, how the annelid trunk nervous system relates to that of other bilaterians. Highly similar mediolateral patterning of nerve cord development between annelids and distantly related bilaterians suggest a general homology [19,20,31]. 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 [21–24,26,32–34], but not with focus on
the pioneer neurons prefiguring the main scaffold of the later nervous system. Data on the first steps in nervous system formation are thus highly interesting from both, a developmental and an evolutionary point of view.

**Neurogenesis in *Malacoceros fuliginosus* – variation of a common theme**

The basic processes of neurogenesis, i.e. specification of neurogenic areas, progression of progenitors, specification of precursors and differentiation of neurons in *M. fuliginosus* largely follows a conserved pattern observed in many organisms. The first of the studied proneural genes showing up in prospective neuroectoderm is an ortholog of SoxB, whose function in specification and proliferation of neural progenitors has been documented throughout the animal kingdom [35,36]. SoxC, known to promote cell-cycle exit follows little later [37]. Then, Prox1 and Elav, typical markers of postmitotic precursors [38–40] become expressed and finally markers for neural differentiation like Synaptotagmin and Rab3. The observed co-expression of succeeding markers in several cells suggests that this set of genes labels continuous lineages of developing cells. Other common neurogenic factors, such as Achaete-scute, NeuroD, Neurogenin and Olig are also broadly expressed in the neuroectoderm of *M. fuliginosus*. This set of genes is in a similar temporal order involved in neurogenesis in many animal groups [1,2,34,41].

A more detailed comparison to other representatives of annelids mainly reveals differences in the time course of neural development along the anterior-posterior axis. Compared to *Platynereis dumerilii* and *Capitella teleta*, neurogenesis progresses at more similar speed in the anterior, the trunk and the posterior in *M. fuliginosus*. Differentiation markers Synaptotagmin and Elav show up in the anterior region in *Platynereis dumerilii* and *M. fuliginosus* around 14 hpf and also onset of Prox expression may be similar, as it is already expressed in several cells in 12 hpf in *Platynereis dumerilii* [42]. However, in the trunk of *Platynereis dumerilii* 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 differentiation markers Elav and Synaptotagmin are reported from 32 hpf onwards in the trunk of *Platynereis dumerilii* [19], while they show up already at 14-18 hpf in the trunk of *M. fuliginosus*. In *Capitella teleta* SoxB, Prox and several bHLH proneural genes show up much later in the trunk and the posterior than in the anterior [22].

**The special case of the posterior pioneer neuron of the ventral nerve cord**

Owing to their easy identification and tracking, the function and features of pioneer neurons are best studied in arthropods, vertebrates and nematodes, and their role in formation of the axonal scaffold is established [4,43–45]. Several factors set them apart from follower and other neurons: from their precise positional cues to distinct molecular profiles and pathfinding abilities [46,47]. In particular, studies in *C. elegans* have shown that the positioning of pioneer neurons is different from other neurons as they considerably deviate from optimized neuronal wiring principle thereby suggesting strong underlying developmental constraint [48]. Moreover, different pioneers can be specified by different transcription factors and also have different developmental mechanisms [49,50].
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 yet been analyzed in detail. By correlating immunohistochemical data on nervous system differentiation with gene expression analysis we found that development of the first neuron showing up in the posterior deviates considerably from that of other neurons. This neuron probably takes in a very important role in the development of the nervous system of *M. fuliginosus*, since its axons pioneer the ventral nerve cord. This particular neuron starts differentiating very early evidenced by atub-LIR from 8 hpf and expression of the synaptic genes Synaptotagmin and Rab3, which are known to be involved in neurotransmitter release, from 12 hpf onwards. We have no evidence, but we also cannot rule out that the posterior pioneer neuron or its pre-differentiated form is expressing or maternally inheriting SoxB in very early stages, since we did not have a specific marker for this cell during the first 8 h of development. But from 8 hpf onwards, where the cell can be identified by its tubulin expression, SoxB cannot be detected within the cell. Further, our data suggests that this cell does not express SoxC, Achaete-scute, NeuroD, Neurogenin, Olig, Prox1 and Elav which are likely expressing in most if not all other neurons.

The detection of Mfu-Brn3 in the posterior neuron starting from 10 hpf corresponds to the stage where sensory cilia are developing. Brn3 has been identified in sensory neuron development in several bilaterian taxa suggesting an evolutionary conserved role [51–53], therefore it is likely that Mfu-Brn3 confers a sensory function for the posterior neuron. Functional studies in *C. elegans* and mice have shown the importance of Brn3 as a terminal selector in differentiation of neurons and maintenance of neuronal identity [54]. The continuous expression of Mfu-Brn3 in the posterior neuron and other cells suggests a similar role in *M. fuliginosus*.

Seemingly specification of neural fate of VNC pioneer depends on a highly deviant gene regulatory network devoid of a broad spectrum of genes involved in neurogenesis of other neurons. The only neural transcription factor we found expressed is Brn3, which likely acts on a low hierarchical level. Patterning processes highly relevant for the development of nervous system like Wnt and BMP signaling are known to drive neural fate determination by acting on the expression of Sox genes and other early neural determinants. Since the ventral nerve cord pioneer does not express those genes, it will be very interesting to find out, whether fate determination in this specific cell may depend less on cell-cell signaling, but rather on inherited cell-intrinsic properties. Such kind of cell-autonomous fate determination is generally an important mode of specification during development of spirally cleaving embryos of lophotrochozoans like annelids, molluscs and nemerteans and other Spiralia like flatworms [55–58]. mRNA segregation during several rounds of asymmetric cell divisions is supposed to be the main mechanism for passing on maternal factors [59–62] and probably is most relevant for specification of early differentiating cells, before regulative mechanisms mediated by extrinsic factors take over during later development of the embryos.

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 point 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 [42]. In difference, early differentiating neurons of the apical organ do not originate from bilateral symmetrical clones and do not express several transcription factors involved in head regionalization pointing towards cell-autonomous specification. In *Capitella teleta*, separation of the micromeres 1a-1d from the rest of the embryo in the 8-cell stage of *Capitella teleta* leads to head-only partial larva with cells expressing the neuronal marker Elav, seen as evidence for cell-autonomous specification of the respective neural fate [63]. Notably, the single ganglion cell in the future apical organ pioneering the anterior part of the circumoesophageal connective in *M. fuliginosus* also does not have a bilateral symmetrical counterpart, which might as well indicate cell-autonomous specification. But this remains speculative and needs further investigation. Our data, however, point for the first time towards possible cell autonomous specification of neural fate in the posterior end of a spiralian embryo, i.e. in the posterior pioneer neuron of the ventral nerve cord. This is not in contradiction with observations reported from *Capitella teleta* suggesting conditional specification of trunk neurons [63]. Here, no signs of trunk neurons were detected in partial larvae derived from the micromeres 1a-1d plus cell 2d, whose progeny in complete embryos gives rise to the trunk neuroectoderm. We suggest that while development of the vast majority of trunk neurons depends on extrinsic factors in annelids [19–21,24,31,63], a posterior ventral nerve cord pioneer which may be subject to cell-autonomous specification is secondarily missing in *Capitella teleta* (see discussion beyond).

**Position and role of pioneer neurons in Lophotrochozoa**

Insights into the role of pioneer neurons in early nervous system development of lophotrochozoans and other Spiralia rely mainly on immunohistochemical analysis of nervous system differentiation. Similar as in *M. fuliginosus* the first appearing neurons in many organisms were found in the anterior and/or the posterior region of the embryos. Observed variability in position, neurotransmitter and projection raised many questions on whether formation of the nervous system progresses in these animals 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 and 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 ventral nerve cord pioneer probably has been present in the last common ancestor of the two major polychaete subgroups, which are Errantia and Sedentaria [64]. A posterior bifurcating neuron highly similar to the cell we investigated in *Malacoceros fuliginosus* has been described from the errant polychaetes *Phyllodoce maculata* [65] and *Platynereis dumerilii* [66,67] and the sedentary polychaete *Pomatoceros lamarckii* [68]. 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 ventral nerve cord. These similarities strongly suggest homology. Thorough electron microscopy based characterization of neural circuitry in 3 days old
larvae of *Platyneris dumerilii* revealed that this cell beside its pioneering role also takes in a sensory-motor function by directly synapsing to multiciliated cells of the prototroch, which propel the larva through the water [69]. We could trace the axons of the posterior pioneer to the level of the prototroch making a similar function conceivable in *M. fuliginosus*. Neurotransmitter content of the posterior pioneer neuron seems to vary in polychaetes. While it is serotonergic in *Phyllodoce maculata* [65] and *Pomatoceros lamarckii* [68], it contains serotonin and FMRFamide in *Platyneris dumerilii* [67,69] and none of these transmitters in *M. fuliginosus*, even though the expression of Synaptotagmin and Rab3 suggest the capacity of transmitter release. Accordingly, presence of this cell may be overlooked, if studies on nervous system development rely mainly on stainings against few neurotransmitters like anti-FMRFamide-LIR and anti-5HT-LIR 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. A posterior ventral nerve cord pioneer is likely lost in *Capitella teleta*, where close examination of neural development provides no hints on presence of such a cell[70], 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, the nerves running posteriorly from the apical plexus associated with the apical organ is pioneered by neurites of a ganglion cell. This challenges the view that the CNS of annelid trochophores is generally prefigured only by peripheral sensory cells [65,67,71,72]. As in the case of the posterior pioneer neuron, these early anterior pioneer neurons of *M. fuliginosus* cannot be identified by studying anti-serotonin or anti-FMRFamide immunoreactivity. In accordance, cell-lineage data from *Platyneris dumerilii* show that several acetylcholinergic neurons appear much earlier in the anterior region than the first serotonergic neurons and that the former form already a considerable mesh of neurites 30 h after fertilization [42]. Data from *Capitella teleta* show that the first differentiating neurons in the apical region are not peripheral sensory cells, but form within the future brain [70].

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 groups of basally branching needs further investigation. The question in which direction CNS formation is progressing in annelids encompasses different aspects which are not necessarily linked to each other. For the differentiation of the vast majority of CNS neurons, existing data point towards an anterior-posterior progression. On the other side, the suggested ancestral presence of pioneers in both the anterior and the posterior means that scaffold formation of the CNS started from both sides. From which side 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 will arise that cell-autonomous specification drives formation of the first differentiating neurons in the anterior as well as in the posterior of annelid trochophores, scaffold formation of the whole annelid CNS may be under 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 few early developing cells in mollusks, nemerteans, and lophophorates. Obtaining deep data on pioneer specification, their 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.

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Availability of data and materials**

DNA sequences of Mfu-SoxB, 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.**

**Funding**

This work was supported by the University of Bergen and the Norwegian Research Foundation.

**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 analyzing data. SK and HH drafted, CH commented and SK and HH finalized the manuscript. All authors gave final approval for publication and agree to be held accountable for the work performed therein.

**Acknowledgments**

Not applicable.

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**Figures**
Figure 1

Anterior and posterior pioneer neurons create the main neuronal scaffold. (A-D) Nervous system development in Malacoceros fuliginosus as revealed by acetylated α-tubulin immunolabeling. (E-I) Pioneer neurons forming the main neuronal scaffold. (E) A pair of sensory cells (asterisk) visible clearly from 16 h gives rise to the prototroch nerve ring. The dotted circles show the adjacent prototroch cells. (F) A single ganglion cell (orange asterisk) with descending neurite is prominent from 18 h. (G-I) The
development of the anterior pioneers - ganglion cell (orange asterisk) and prototroch nerve sensory neurons (white asterisk). (H) Later developing neurons appear in pairs at around 21 h (red asterisk). (I) Additional neurites from peripheral sensory neurons (arrowheads) converge to form the central ganglia (F) Posterior pioneer neuron with dense tubulin at 9 h along with DAPI (blue). (G) Side view at 10 h showing ventral orientation of posterior pioneer (see inset). (H-I') follower neurons indicated by arrows. (H) A non-sensory follower neuron and a pair of sensory neurons (I, I'). Scale bars: 10 µm (except K: 20 µm). at: apical tuft, cec: circumesophageal connective, cg: central ganglia, prt: prototroch, sec: supraesophageal connective, tel: telotroch.

**Figure 2**

Expression of serotonin and FMFRamide during early nervous system development. (A) 5HT-like IR is detected in many neurons and neurites at 48h. (B) FMRFamide-like IR is detected in many neurons and few neurites at 48h. (C) First FMRFamide-like IR is detected at 14 h and restricted to one ciliated sensory cell located dorsally of the apical tuft cell. (D) First 5HT-like IR is detected at 21h 5HT-like IR and is restricted to one pair of ciliated sensory cells ventral to the forming apical plexus. (E, F) At 24h two pairs of ciliary sensory cells with FMRF 5HT-like IR are present in the apical region. Asterisks: nucleus of cells with FMRFamide-like or 5HT-like IR. Scale bars: A,B: 10 µm, D-F: 5 µm.
Figure 3

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

Progression of neurodevelopmental genes and domains of early differentiation. WISH images of neural genes in different stages of larval development. The arrowheads point to the gaps appearing in the expression of SoxB1. Scale bars: 50 µm.

Figure 5
Most neurons follow a general scheme of neurogenesis. (A-C'') Fluorescent dWISH, either full or substack of confocal images. (A) ISH of SoxB1 and SoxC. SoxB1 expression is only confined to the ectoderm indicated by dotted line. (A') Few SoxC expressing cells ingress (arrowheads) from the ectoderm. (B) ISH of SoxC and Prox1. (C) ISH of Elav and Prox1. Co-expressing cells are marked in orange asterisk. Scale bars: 20 µm.

**Figure 6**

The posterior pioneer neuron is a distinct Brn3 expressing sensory neuron. (A-A''') Confocal image of SoxB1 FISH combined with IHC of acetylated-tubulin. (B-E) Expression of post-mitotic genes, Prox1 and Elav and synaptic genes, Rab3 and Syt1 in the posterior. (F-H) FISH of Brn3. Images are substacks showing the expression in the posterior neuron. (I,I') Confocal image of Syt1 FISH combined with IHC of acetylated-tubulin. White arrowheads point to the posterior pioneer neuron. (J,J') Substack of Ngn along with IHC of acetylated-tubulin. Arrowheads point to the posterior pioneer neuron. Scale bars: 20 µm.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile2.tif
- Additionalfile4.tif
- Additionalfile3.tif
- Additionalfile1.tif