



MASTERARBEIT | MASTER'S THESIS

Titel | Title

Characterization of neurogenic cell types in *Pycnogonum litorale* (Strøm, 1762)
via gene expression studies

verfasst von | submitted by

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angestrebter akademischer Grad | in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien | Vienna, 2025

Studienkennzahl lt. Studienblatt |
Degree programme code as it appears on the
student record sheet:

UA 066 831

Studienrichtung lt. Studienblatt | Degree
programme as it appears on the student
record sheet:

Masterstudium Zoologie

Betreut von | Supervisor:

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Acknowledgements

This research work was supported by the grant RR2025 of the “Association Haus des Meeres Wissenschaft und Forschung” to Paul Arthur Höller.

I would like to sincerely thank my supervisor Dr. Georg Brenneis, not only for introducing me to the truly wonderful and fascinating group of Pycnogonida, but also for guiding me through my thesis project and all the associated challenges.

Further, I would like to express my gratitude to the members and entire staff of the Department of Evolutionary Biology for their help and advice during my studies as well as for providing a generally pleasant working environment. Not least of all I thank the heads of the department Univ.-Prof. DDr. Andreas Wanninger and Univ.-Prof. Mag. Dr. Mihaela Pavličev for providing the access to the facility and resources needed to pursue my thesis.

Special thanks go to:

Antonia Grausgruber MSc and Gudrun Schwarze MSc for their advice and company as well as for their welcoming and kind attitude.

Mag. Marion Wanninger for instructing me the Aqua Lab and caring for the animal culture together with Nadja Hattinger.

My fellow master's students and friends Ines Müller BSc, Maximillien Jadin BSc and Filip “Fudi” Šetena BSc for standing by me, keeping me company and making the experience even more enjoyable.

Last but not least, I thank my brother, Felix Otto Höller, my sister, Gina Lara Regina Höller as well as my mother Sonja Matuš and her partner Manfred Bogad for putting up with me and supporting me throughout the years.

Abstract

Sea spiders (Pycnogonida) are a fascinating, yet understudied group of marine chelicerates that form the sister group of all remaining extant taxa (Euchelicerata). Notably, nervous system development in pycnogonids has been reported to rely on large neural stem cells (NSCs) with high mitotic activity and a morphologically asymmetric mode of division. The latter results in their self-renewal and the formation of a second, intermediate neural progenitor cell (INP), which in turn divides symmetrically. This mode of neurogenesis differs significantly from that found in euchelicerates and myriapods, while showing several similarities with neuroblasts and ganglion mother cells, the well-characterized neurogenic cell types driving neurogenesis in Pancrustacea (Crustacea + Hexapoda). Until now, however, the gene regulatory networks governing sea spider neurogenesis and neural differentiation have not been studied, impeding validation of the morphology-based distinction of pycnogonid cell types and further comparisons with pancrustacean neuroblasts and ganglion mother cells on the molecular level. In order to redress this knowledge gap, this study employed HCR-FISH to study the expression patterns of a suite of key genes with conserved roles in neural cell specification, maintenance and differentiation during the development of the sea spider *Pycnogonum litorale*. The results of this yielded first insight into the transcriptional profiles of the cell types involved in sea spider neurogenesis, revealing a general congruence between previous morphological cell type classifications and gene expression characteristics. Beyond this, it is shown that the first neural precursor cells are specified in segmentally stereotypic positions in the neuroectoderm, similar to those of pancrustaceans. This work contributes to the understanding of neurogenic cell types in sea spiders and allows several parallels to be drawn to the neurogenic processes of pancrustaceans, revealing potential insights into the ancestral state of neurogenesis in arthropods altogether.

Zusammenfassung

Asselspinnen (Pycnogonida) sind rein marine Vertreter der Chelicerata, welche die Schwestergruppe aller anderen Cheliceraten (Euchelicerata) bilden. Die Entwicklung des Nervensystems der Pycnogoniden erfolgt mittels großen neuralen Stammzellen (neural stem cells; NSCs) mit hoher mitotischer Aktivität und morphologisch asymmetrischer Zellteilung. Letzteres führt einerseits zu ihrer Selbsterhaltung durch die Entstehung einer weiteren neuralen Stammzelle und andererseits zur Bildung einer „intermediate neural progenitor“ (INP) Zelle, die sich ihrerseits symmetrisch teilt. Dieser Modus der Neurogenese unterscheidet sich deutlich von dem der Eucheliceraten und Myriapoden, während er mehrere Ähnlichkeiten mit Neuroblasten und Ganglien-Mutterzellen, den bereits gut charakterisierten neurogenen Zelltypen, welche die Neurogenese bei Pancrustaceen (Crustacea + Hexapoda) steuern, aufweist. Da bislang die genregulatorischen Netzwerke, welche die Neurogenese und neurale Differenzierung bei Asselspinnen steuern, noch nicht untersucht wurden, konnten die auf der Morphologie basierenden Beschreibungen nicht validiert werden. Weiters konnten keine Vergleiche mit den Neuroblasten und Ganglien-Mutterzellen auf molekularer Ebene durchgeführt werden. Um diese Wissenslücke zu schließen, wurde in dieser Studie HCR-FISH eingesetzt, um bei der Asselspinne *Pycnogonum litorale* die Expressionsmuster einer Reihe von Genen zu untersuchen, die eine konservierte Rolle bei der Spezifizierung, Erhaltung und Differenzierung von Nervenzellen und deren Vorläufern spielen. Die Ergebnisse lieferten erste Einblicke in die Transkriptionsprofile der Zelltypen, die an der Neurogenese von Pycnogoniden beteiligt sind, und zeigten eine allgemeine Übereinstimmung zwischen früheren morphologischen Zelltypklassifizierungen und Genexpressionsmerkmalen. Darüber hinaus wird gezeigt, dass die ersten neuralen Vorläuferzellen in segmental stereotypen Positionen im Neuroektoderm spezifiziert werden, ähnlich wie bei den Pancrustacea. Diese Studie trägt zum Verständnis der neurogenen Zelltypen bei und ermöglicht es, mehrere Parallelen zu den neurogenen Prozessen von Pancrustaceen zu ziehen, was potenzielle Einblicke in den ursprünglichen Zustand der Neurogenese bei Arthropoden allgemein offenbart.

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Introduction

The central nervous system (CNS) of arthropods and its development

Arthropods are one of the most successful groups in the animal kingdom, have conquered virtually every habitat and make up 80% of extant metazoan diversity, with more than a million species described to date [1]. This incredible species richness is the result of a long evolutionary history which has led to an equally astonishing diversity of body plans present in extinct and extant lineages alike [2]. Nevertheless, features such as the possession of an exoskeleton, jointed limbs and the segmentation of the body are fundamental similarities shared by all arthropods [3]. Another shared feature among arthropods is their segmented central nervous system (CNS), which can generally be divided up into a brain situated at the antero-dorsal end of the animal, followed by a variable, taxon-specific number of ventral ganglia, also known as the ventral nerve cord (VNC) [3]. These ganglia are composed of two bilaterally symmetrical, often fused hemiganglia present in each segment [3] and like the rest of the nervous system are made up mainly of neurons and glial cells [4]. During early development the CNS of all arthropods is formed by homologous segmental units of developing neural tissue (neuromeres) that differentiate into the ganglia of adults [3, 5-9]. Surprisingly, however, the cellular mechanisms underlying neuromere formation differ between the four major arthropod groups (Chelicerata, Myriapoda, crustaceans and Hexapoda) [10]. This has been best studied in hexapods, particularly *Drosophila*, in which the neuroepithelium generates a stereotypic set of neural stem cells called neuroblasts (NBs), per neuromere. Each NB is specified from a proneural cluster, which is a group of neuroepithelial cells expressing genes of the *achaete-scute* complex. These genes encode transcription factors that convey ectodermal cells the potential to adopt neural fate [11]. Members of the *achaete-scute* complex have previously been shown to be required for NB commitment, showing some degree of functional redundancy in this respect [11]. From each proneural cluster, a single NB delaminates and moves into the interior of the embryo, where it then divides repeatedly in asymmetric fashion. Each NB division results in one daughter cell that inherits NB identity and a daughter cell of a different type, the so-called ganglion mother cell (GMC). The GMCs in turn divide typically only once and symmetrically, producing two post-mitotic neurons/glial cells [10]. Notably, NBs are also present in various crustacean taxa [10, 12, 13], the closest relatives of Hexapoda with which they form the taxon Pancrustacea [14]. The crustacean NBs also divide asymmetrically, producing GMCs that --as they are being budded off by the NBs-- form columns of cells reaching into the interior of the embryo. The GMCs then also divide once, resulting usually in a pair of post-mitotic neurons/glial cells. One significant difference to hexapods is that

crustacean NBs remain part of the apical epithelium, rather than delaminating into the embryo [10]. Despite this difference, cell lineage and gene expression data strongly support the homology of hexapod and crustacean NBs [15], making them a shared and, likely apomorphic trait of Pancrustacea [16].

In contrast to pancrustaceans, early neurogenesis in myriapods and most chelicerates lacks specialised NBs and GMCs. Instead, groups of neural precursor cells arise in stereotypic domains of the neuroepithelium that also express the proneural genes of the *achaete-scute* family [17]. These groups form local cell immigration sites that are maintained throughout neurogenesis, but may later coalesce into larger neuroepithelial invaginations that detach from the apical surface [17-19]. Deviating from the pancrustacean NBs and GMCs, the immigrating neural precursors are usually already post-mitotic as they immigrate, i.e., most of them do not divide further and instead differentiate directly into neurons or glial cells [10].

Genetic control of neurogenesis

Other than the proneural genes of the *achaete-scute* family, which are expressed in the clusters giving rise to the NBs in pancrustaceans as well as in the neuroepithelial regions of future cell immigration sites in most chelicerates and myriapods [10, 17], a conserved cassette of other genes are known to be involved in arthropod neurogenesis.

1) *SoxNeuro* (*SoxN*) is a transcription factor gene of the *SoxB* group containing the high-mobility group (HMG) box, a DNA-binding domain, which is highly conserved across the lineages of the Panarthropoda [20, 21]. In hexapods, this gene has been shown to act both in parallel and up-stream of other proneural genes. Among others, it contributes to NB selection and delamination by playing a substantial role in the process of lateral inhibition, which mediates the selection of individual NBs from the proneural clusters [22, 23]. Similarly, *SoxN* is expressed broadly in the neuroepithelium and NBs of crustaceans, therefore likely providing these epithelial cells the ability to adopt neural fate [24]. Despite their differing modes of neurogenesis, myriapods and most chelicerates show similar patterns of broad *SoxN* expression across the complete ventral neuroectoderm, including --but not restricted to-- embryonic neurogenesis [25, 26]. A similar function of the myriapod and chelicerate orthologs to the pancrustacean *SoxN* has been suggested based on their expression in domains prefiguring the future sites of neural precursor formation and immigration [26].

2) Another pan-neural gene is *deadpan* (*dpn*), a member of the Hairy and Enhancer of Split (Hes) family of genes. As its characteristic basic helix-loop-helix (bHLH) domain suggests, *dpn* is involved in transcription regulation [27, 28]. It represents a NB-specific marker expressed in

all NBs and only very transiently in newly formed GMCs [29, 30], is involved in both cell-cycle regulation and specification of NB fate [31], and has previously been shown to be necessary and sufficient for maintaining NB self-renewal [28].

3) The two genes *Prospero* (*pros*) and *Brain tumor* (*brat*) are known to cooperate in the exit of the NB daughter cell from the cell cycle and specifying the fate of the resulting GMCs in hexapods [32]. During NB division the product of both of these genes is segregated asymmetrically only into the forming GMCs via interaction with the scaffolding protein Miranda [29, 32, 33]. Although partially redundant in their function, both genes are required to specify GMC fate [32]. *pros* is a homeodomain transcription factor that simultaneously represses stem cell fate and cell cycle genes while simultaneously activating terminal cell differentiation genes. It therefore acts as a binary switch between neural stem cell fate and neural differentiation [31]. Accordingly, *pros* is mainly expressed in NBs, GMCs and only occasionally and transiently in newly formed neurons [33, 34]. *brat* on the other hand is a conserved member of tumor suppressor genes that inhibit NB self-renewal [29, 32]. Its gene product acts as a translation repressor that binds the mRNA of NB markers such as *dpn* and thereby facilitates their degradation [30]. Loss of function of *brat* has previously been shown to result in overproliferation of neural tissue due to GMCs reverting to NB identity [32]. Similar to *pros*, *brat* is also expressed in a subset of hexapod NBs as well as GMCs and maturing neurons [29, 30, 32].

In crustaceans *pros* has been likewise reported to be expressed in several NBs in the ventral neuroepithelium, with especially strong expression as these cells undergo asymmetric cell division [12, 24]. Additional expression domains are present in the subapical layers of the neuroepithelium containing the GMCs, whereas expression does not persist in post-mitotic neurons [24]. Interestingly, the expression of *pros* in NBs has been shown to co-localize with expression domains of both *SoxN* and *achaete-scute* homologs (*ASH*) in crustaceans [12, 24]. As in hexapods, *brat* expression domains in crustaceans show notable overlap to *pros*. They partially attain a subapical position followed by strong ubiquitous expression in both apical and subapical cell layers, in line with the expression of *brat* in NBs, GMCs and early neuronal cells [24].

In most members of the Chelicerata and myriapods the gene *pros* has been found to be expressed in the invaginating neural precursor cells from their formation up to their differentiation into neurons, with no evidence for asymmetric segregation of the *pros* protein [35, 36]. Based on these expression patterns it has been suggested that *pros* plays a role in the differentiation process of neural precursors and possibly the termination of cell proliferation of spiders [36].

4) Finally, the gene *embryonic lethal abnormal vision (elav)* is ubiquitously expressed across the nervous system of pancrustaceans, being required for its development and maintenance [24, 37]. Importantly, however, the expression is restricted to differentiating and mature neurons, while remaining absent from NBs and GMCs, making *elav* orthologs ideal neuron markers in the arthropods studied so far [37].

Sea spiders and their deviating mode of neurogenesis

Pycnogonida (sea spiders) is a group of bizarre arthropods with a cosmopolitan distribution in the world's oceans [38]. Despite previous debate on their phylogenetic position, sea spiders are nowadays widely accepted as a member of the Chelicerata, forming the sister group of all remaining extant chelicerates (termed Euchelicerata) [14, 39, 40].

In contrast to euchelicerates, most sea spiders show a pronounced indirect development, which plausibly resembles the plesiomorphic condition of the pycnogonid lineage and potentially of chelicerates in general [41]. After the hatching of a minute protonymphon larva, pycnogonids undergo an anamorphic postembryonic development [42], characterised by a series of molting stages (instars) [41] featuring the successive formation of new body segments at the posterior pole until the adult body plan is complete [42, 43]. During this developmental phase both the walking legs and the corresponding ventral segmental ganglia are successively formed (e.g., [44]). As in other arthropods, the CNS of adult pycnogonids can be divided up into the anterodorsal brain and the ventral nerve cord (VNC). The VNC is composed of the composite subesophageal ganglion (SEG) including the palpal and ovigeral neuromeres and usually four walking leg ganglia (WLG) [45]. Interestingly, neurogenesis in pycnogonids shares similarities with pancrustaceans as well as with chelicerates/myriapods [9]. It features an initial phase, during which primarily post-mitotic neural precursors immigrate from the neuroepithelium. This is later followed by the formation of a central invagination in each hemisegment [9], first described by T.H. Morgan as “ventral organs” (VOs) [46]. These invaginations are apically lined by large neural stem cells (NSCs), which show high mitotic activity, as well as a morphologically asymmetric mode of division, similar to the pan crustacean NBs [9]. Further, so-called intermediate neural progenitors (INPs) have been described in pycnogonids, which are smaller than the apical NSCs and form a subapical layer where they undergo morphologically symmetrical division(s) [9]. Due to these characteristics it was proposed that the INPs are the daughter cells of the NSCs and may possibly correspond to the GMCs of pancrustaceans [9]. Based on these morphological descriptions alone, however, the question

whether the two pycnogonid cell types should be considered homologous to the ones of pancrustaceans or instead a convergent evolution remains currently unanswered [9].

Thesis aims: Characterizing neurogenic cell types in pycnogonids via gene expression studies

So far, gene expression studies addressing pycnogonid neurogenesis are completely lacking. Such data, however, are essential to make more informed comparisons with other arthropod groups, in which expression pattern analyses of key neurogenic genes enabled molecular characterisation of the involved cell types [11, 12, 17, 24]. Comparable data for pycnogonids would therefore not only provide first insights into the transcriptional profiles of their NSCs and INPs but also allow their evaluation compared to the pancrustacean NBs and GMCs versus the myriapod/euchelicerate neural precursors. Given the early divergence of sea spiders at the base of the chelicerate tree of life, the molecular characterisation of their cell types may contribute to the reconstruction of the ancestral state of Chelicerata, with the potential to impact current hypotheses on the evolution of neurogenesis within arthropods.

For these reasons, this study aims to gain a better understanding of sea spider neurogenesis by examining the expression patterns of the candidate genes/gene families discussed above, known to be involved in neurogenic processes in other arthropod groups. This will allow more informed conclusions to be drawn about the potential homology of the aforementioned pancrustacean and pycnogonid cell types as well as the ancestral mode of neurogenesis in arthropods altogether.

Choice of study organism

To address these knowledge gaps on sea spider neurogenesis, this study investigates neurogenic gene expression during the postlarval development of the pycnogonid *Pycnogonum litorale* (Strøm, 1762), a member of the Pycnogonidae. This representative was chosen, as it is one of the few species whose complete life cycle has previously been described and because it can be kept together with its prey in a closed life cycle, making it well-suited for long-term laboratory husbandry [47-49]. Unlike most other pycnogonids, the adult VNC of *P. litorale* consists of only four ganglia since the SEG includes the neuromere of the first walking leg segment (WLN1) [44]. *P. litorale* hatches as a minute protonymphon larva (instar I) whose VNC only consists of the SEG (already including WLN1) and the VOs of the prospective WLG2. During the subsequent anamorphic developmental phase with its five postlarval stages (instars II-VI) the segmental ganglia of the VNC, including two posterior ganglia (PG) that later fuse with the last WLG, are successively formed [44]. Accordingly, the VNC displays a pronounced anterior-

posterior developmental gradient, providing access to all stages of nervous system development [44]. Taking advantage of this, this study leverages the recently established method of hybridisation chain reaction fluorescent in-situ hybridisation (HCR-FISH) in *P. littorale* [50, 51] combined with nuclear staining and immunolabeling to elucidate the gene expression profiles of neurogenic cell types in *P. littorale*.

Material & Methods

Animal husbandry

Lab-reared adult and juvenile specimens of *P. littorale* were kept in aquaria operated with recirculating artificial sea water (ASW; 32 ‰ salinity, 15°C). Groups of both males and females were placed into cages together with their anthozoan prey *Metridium senile* (Linnaeus, 1761) and the males were regularly checked for egg batches, which were carefully removed from the ovigers using needles and fine forceps. The eggs were then transferred into aerated plastic containers filled with ASW until the protonymphon larvae started hatching. At this point the egg batches were divided into smaller pieces that were attached to colonies of the hydrozoan *Clava multicornis* (Forsskal, 1775) - the preferred prey of the postlarval instars - growing on small plastic plates. The subsequent development of the animals was monitored via regular checking under a stereomicroscope and several individuals of each developmental stage (instars I-VI; Alexeeva & Tamberg 2020) collected for fixation as they occurred. However, this study focuses predominantly on instars II, III and IV since these are sufficient to address the research questions while also being easier to document with high resolution microscopy than later stages due to their smaller size.

Specimen fixation and pre-staining treatment

The collected specimens were briefly relaxed in freshly carbonated ASW, prepared using a SodaStream sparkling water maker and subsequently fixed in 4% PFA/ASW (20% paraformaldehyde [Sigma-Aldrich; 158127] diluted 1:5 in ASW) for approximately 1.5 hours at room temperature (RT). The samples were then washed for at least 30 minutes in phosphate-buffered saline (PBS, 1.86 mM NaH₂PO₄, 8.41 mM Na₂H₂PO₄, 175 mM NaCl; pH 7.4) with several changes of the buffer and either used immediately for fluorescent histochemical staining and immunolabeling or alternatively dehydrated in an ascending methanol series on ice and stored in absolute methanol at -20°C until further use.

Prior to fluorescent histochemistry and immunolabeling, the larval legs as well as walking legs (if present) were partially cut off using surgical micro-scissors under a stereomicroscope.

Additionally, all samples were briefly sonicated in an ultrasonication bath in order to increase the permeability of the cuticle and tissue, followed by permeabilisation in PBTx (PBS + 1.5% DMSO [dimethyl sulfoxide] + 0.5% Triton-X) for at least one hour at RT.

For hybridisation chain reaction fluorescent in-situ hybridisation (HCR-FISH), the methanol-stored samples were first gradually rehydrated and then washed several times in PBST (PBS + 0.1% Tween20) on ice, followed by the removal of the larval/walking legs and several brief pulses in the bath sonicator. The samples were then additionally permeabilised for 30-60 minutes in detergent solution (1% SDS [sodium dodecyl sulfate], 0.5% Tween, 50 mM Tris-HCl [pH 7.5], 1 mM EDTA [ethylenediaminetetraacetic acid; pH 8.0], 150 mM NaCl).

Fluorescent histochemistry and immunolabeling

F-actin labeling using Alexa Fluor[®]488-phalloidin (1:50 in PBTx; Thermo Fisher Scientific; A12379) was performed overnight at 4°C, after which the staining quality was checked under a Nikon SMZ25 epifluorescence stereomicroscope and, if needed, the incubation time extended up to 48 h.

Additionally, the structural protein acetylated α -tubulin was stained via immunolabeling. After two 30-minute blocking steps in 5% normal goat serum (Thermo Fisher Scientific; PCN5000) + PBTx, the monoclonal mouse primary antibody (Sigma-Aldrich; T6793) diluted in PBTx (1:300) was added and the samples left to incubate for 24-48 h on the shaker at RT. The incubation was followed by at least 2 h of rinsing in PBTx at RT with several changes of the buffer and two more 30-minute blocking steps, after which the Alexa Fluor[®]568-coupled polyclonal secondary goat antibody (Thermo Fisher Scientific; A11004) was added and left to incubate for another 24-48 h. The samples were then washed several times in PBTx followed by additional washing steps in PBS.

These two methods have previously been shown to reliably visualize the cell shapes and sizes in the neuroepithelium and the ventral organs [9, 52, 53] as well as the tubulin-rich neurites of the ganglia. In some of the samples both phalloidin and tubulin-immunolabeling were combined.

For nuclear staining either DAPI (4',6-diamidino-2-phenylindole; Sigma-Aldrich; D9542) or SYBR[®]Green (Thermo Fisher Scientific; S7563) was applied to the samples, which were left to incubate for at least 1 h at RT and if needed overnight at 4°C.

The samples were then transferred into Vectashield[®] Mounting Medium (Vector Laboratories, Inc; H-1000) and subsequently mounted on microscope slides, applying small pieces of plasticine to the corners of the coverslip to prevent the sample from being squashed. The

samples were either mounted directly in Vectashield® or, if needed, first cleared and then mounted in RapiClear® (SunJin Lab; RC149001).

Identification of target gene sequences in *P. litorale*

Published amino acid sequences of the target genes from other arthropod species (see Appendix 1) were downloaded from the NCBI database and used as queries for local BLAST searches in Geneious Prime (version 2024.0.7; Insightful Science, LLC) against a draft genome as well as transcriptomic resources of *P. litorale* (Papadopoulos et al. 2024) to identify the sequences of the putative orthologs. To validate the gene identity of the resulting sequences they were reciprocally blasted against the NCBI databases.

The sequences were then used to design a set of probe pairs for each gene, using a modified version of the Easy-HCR in situ probe generator [https://github.com/rwnull/insitu_probe_generator; 54, 55] adapted by N. Papadopoulos (University of Vienna, Dept. Evolutionary Biology). When designing the probe sets it was made sure that as many genes as possible had a different amplifier sequence so that they could be multiplexed. However, since a total of seven genes were investigated, but hairpin stocks (Molecular Instruments, Los Angeles, USA) with only five different amplifier sequences (B1-5) were available for the study, not all of the genes were multiplexed. To overcome this limitation for *Plit_SoxNeuro*, a second probe set with a different amplifier was ordered, enabling combination with any of the other genes. The probe sequences (including amplifier sequences) were then submitted to IDT (Integrated DNA Technologies, Coralville, USA) for synthesis of DNA oligoPools. Each of the gene-specific DNA oligoPools was reconstituted in molecular grade water from the probe stock solution (1µM stock) and stored at -20°C (see Appendix 2).

Deviating from this procedure, the probe set of the gene *Plit_Elav* and one of the two probe sets for *Plit_SoxNeuro* were designed and synthesised by Molecular Instruments (Los Angeles, USA; 1µM stock solution) after direct submission of the gene sequence (see Appendix 3).

Hybridisation chain reaction fluorescent in-situ hybridisation (HCR-FISH)

For HCR-FISH a protocol largely based on Choi et al. [56], Bruce et al. [57] and Schwarzkopf et al. [58] but slightly adapted after personal communication with Emily Setton (The Whitney Laboratory for Marine Bioscience, University of Florida, USA) and Carsten Wolff (Marine Biological Laboratory, University of Chicago, USA) was followed (see Appendix 4). DAPI was used as a nuclear counterstain.

The target genes were run both in separate HCRs as well as multiplexed in different pairs in order to determine whether the two probes co-distribute with one another (see Appendix 5). A negative control was conducted in which gene-specific probes were omitted and two sets of different hairpins (with different fluorochromes) were used, which resulted in a strong diffuse labeling in the lumen of the midgut. This points to the midgut or rather its contents acting as a hairpin trap leading to unspecific signal.

All HCR-FISH samples were cleared and mounted in 75% 2,2'-Thiodiethanol (TDE) + PBS. To prevent the samples from collapsing, they were gradually cleared in 65% TDE/PBS for 15 minutes, followed by another 15 minutes in 75% TDE/PBS.

Confocal laser-scanning microscopy (CLSM), data analysis and data presentation

A Leica DM6000 microscope coupled with a Leica TCS SP5 unit was used to create image stacks, with the 40x glycerol immersion objective generally being used for overview scans (depending on the size of the instar), while details of the central nervous system were taken with the 63x glycerol immersion objective. The z-increment between successive scanning planes ranged from 0.25µm to 0.7µm, depending on the intended z-resolution as well as the maximal resolution of the objective. A combination of different laser lines was used corresponding to the excitation spectra of the different fluorochromes (UV laser - 405 nm → DAPI; argon laser - 488 nm → Alexa Fluor® 488, Sytox™ Green, SYBR™ Green, cuticular autofluorescence; diode pumped solid state laser - 561 nm → Alexa Fluor® 594; helium-neon laser - 633 nm → Alexa Fluor® 647). When possible, the argon laser was used to capture just the autofluorescence of the cuticle in a separate channel.

The resulting CLSM data were analysed and documented using the 3D reconstruction software package 'Imaris' (version 7.3.1; Oxford Instruments). In the 'Display Adjustment' window the signal of the different channels was adjusted (background signal reduced and weak signal boosted) to achieve a clearer visualisation. Maximum intensity projection (MIP) 3D renderings were generated using the 'Surpass mode' and 'Clipping planes' applied to remove obstructing regions (such as the cuticle) and gain a better view of the underlying structures. Additionally, the 'Section mode' was used to create virtual sections (horizontal, sagittal and transverse simultaneously) through the scanned specimens, enabling more detailed analysis of the gene expression patterns. As some areas of interest such as certain cell divisions could not be clearly depicted in these views a "Ortho slicer" was applied allowing virtual sections to be created across any combination of axes.

Additional analysis and documentation was done using the software package ‘Amira’ (version 2020.2; Thermo Fisher Scientific). Here, the ‘Threshold’ tool in the segmentation editor was applied to label the separately recorded channel of the cuticle which could thereafter be used to mask cuticular signal in the other channels, using the ‘Arithmetic’ function. The resulting data were used to create 3D volume renderings (maximum intensity projections), while clipping planes were used in a similar fashion as in ‘Imaris’.

All figures/plates were compiled in Adobe Illustrator (version 25.3.1; Adobe Inc.). In images in ventral view, the anterior end is always oriented towards the top of the image, while in lateral views and virtual sagittal sections anterior is towards the right and ventral towards the top, unless stated otherwise. Global brightness and contrast was adjusted in plates as needed using Adobe Photoshop (version 26.7.0; Adobe Inc.).

Results

Identified target gene sequences

The BLAST searches against the genomic and transcriptomic resources yielded several hits with varying degrees of similarity for each of the target gene queries. Out of these hits the one with the greatest sequence similarity was selected for the genes *SoxNeuro*, *Brain tumor*, *Prospero* and *Embryonic lethal abnormal vision*. To further support gene identity of these selected candidate sequences, the presence of highly conserved, gene-specific domains was checked for and confirmed. As expected, the *Plit_Pros* sequence contains the unique ‘homeo-prospero domain’ (HPD) and the one of *Plit_SoxN* the ‘high mobility group’ (HMG) domain characteristic of the Sox gene family. In the *Plit_Brat* sequence, a typical ‘NHL repeat’ domain is found and *Plit_Elav*, contains two different ‘RNA recognition motif’ domains belonging to the ‘ELAV_HUD_SF’ superfamily.

Two different paralogs of the *achaete-scute-complex* (*Plit_ASH1* & *Plit_ASH2*) were identified, featuring the conserved ‘basic helix-loop-helix’ (bHLH) DNA-binding domain characteristic of this transcription factor family. Only for the target gene *Deadpan* no unequivocal candidate sequence was found, but the HES gene family-specific bHLH and ‘Hairy_orange’ domains of the sequence with the lowest E value showed the highest correspondences to the *Deadpan* orthologs of insects (*Drosophila melanogaster*, *Tribolium castaneum*) and was therefore selected for this study and referred to as *Plit_Dpn-like*.

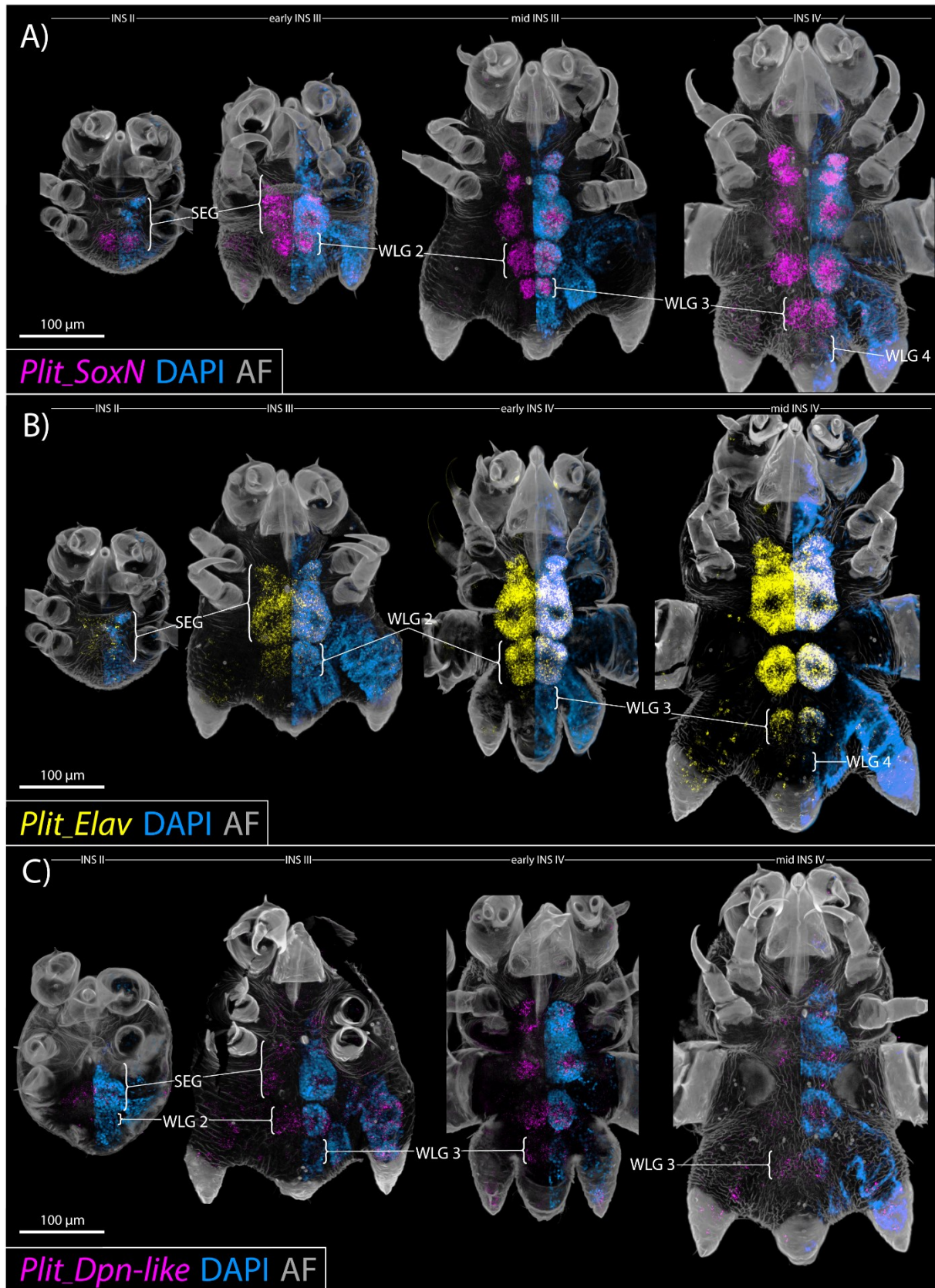


Figure 1. Overview of the general expression patterns of neurogenic genes in postlarval instars of *P. litorale*. 3D renderings (MIP) of instars II-IV in ventral view. Gene expression patterns of *Plit_SoxN* (A; magenta), *Plit_Elav* (B; yellow) and *Plit_Dpn-like* (C; magenta) respectively with nuclear counterstain via DAPI (blue) and autofluorescent signal of the cuticle (gray). DAPI signal shown only in one half of the body to reveal weaker HCR-FISH signal. Note more posterior expression domains of *Plit_SoxN* as compared to *Plit_Elav* in corresponding stages. *Abbreviations:* SEG – subesophageal ganglion, WLG – walking leg ganglion.

General gene expression patterns in the developing VNC of *Pycnogonum litorale*

Plit_SoxN

In the VNC of instar II the expression of *Plit_SoxN* is restricted to the SEG, in the anterior half being limited to the VO regions of the palpal and ovigeral neuromeres (Fig. 1A, 2A). The strongest expression however can be found in the posteriormost part of the SEG, which corresponds to the neuromere of the first walking legs (Fig. 2A). In early instar II, walking leg neuromere 1 is not yet detached from the apical neuroepithelium and *Plit_SoxN* expression is evenly distributed along its entire apico-basal extension. By late instar II, walking leg neuromere 1 has fully separated from the apical neuroepithelium and expression has concentrated in the ventral half of the ganglion, including the corresponding VOs. At this stage, two small, apical expression territories can be seen posterior to the SEG, marking the primordial anlage of WLG2 (Fig. 1A, 2B).

The spatio-temporal expression dynamics observed during instar II progress in corresponding fashion as additional ganglion anlagen develop posteriorly. Accordingly, in early instar III, *Plit_SoxN* is prominently expressed in the SEG, but predominantly concentrated around the three VOs, with strongest signal in the cells directly lining these central invaginations (Fig. 2C). Posterior to the SEG intense signal is found in the entirety of the growing WLG2 anlage, which has not yet detached from the apical neuroepithelium. Posterior to WLG2 only weak, scattered signal remains which for the most part is limited to the neuroepithelium (Fig. 2C). In late instar III, the expression of *Plit_SoxN* has further concentrated around the VOs of the SEG and WLG2 which has by now increased in size and detached from the neuroepithelium (Fig. 1A, 2D). Posterior to this the small, apical anlage of WLG3 is now visible in the neuroepithelium showing strong signal (Fig. 2D).

As the WLG3 anlage detaches from the neuroepithelium during instar IV, *Plit_SoxN* expression increasingly starts to concentrate in the apical half of the ganglion. Simultaneously two stripe-shaped expression domains become apparent in the posterior neuroepithelium (Fig. 1A, 2E). Strong expression is also present in the anlagen of the first and second posterior ganglia which emerge in instars V and VI, respectively. Even in these later stages *Plit_SoxN* continues to be expressed in the VOs of the more anterior ganglia, but at lower levels than in the less developed ganglia (Fig. 2F).

Plit_Elav

In the SEG of early instar II, the expression of *Plit_Elav* is prominent in the palpal and ovigeral neuromeres, while the neuromere corresponding to the first walking leg only shows comparatively little expression basally, at its antero-dorsal margin (Fig. 1B, 3A). Although the expression domains of the more anterior portions of the SEG are spread out more broadly across this structure, they are noticeably concentrated around the dorsal side of the ganglion, in line with an expression of *Plit_Elav* in further advanced neuronal cells. When the SEG starts to detach from the apical neuroepithelium in late instar II, its posterior part has increased in size and the expression of *Plit_Elav* becomes stronger and more widespread but remains mostly confined to the dorsal half of the ganglion (Fig. 3B). The newly emerged anlage of WLG2 contains only single *Plit_Elav*-positive cells with relatively weak expression laterally on the dorsal margin of the ganglion (Fig. 3B).

In instar III, the expression domains in SEG and WLG2 continue to expand further ventrally, but do not reach to the regions surrounding the VOs (Fig. 3C). As in the previous instar, the newly formed ganglion anlage (WLG3) almost completely lacks expression, except for single cells at its dorsal margin (Fig. 3C).

The same characteristic patterns - the expansion of *Plit_Elav* expression toward the ventral side in the more developed ganglia and the presence of single cells expressing *Plit_Elav* in the early ganglion anlagen at the posterior pole of the animal - continue in the last three remaining postlarval instars (Fig. 1B). Notably, gene expression can also be seen at the antero-dorsal end of the first posterior ganglion, which emerges in these later stages (Fig. 3D). At this point, *Plit_Elav* is expressed almost throughout the entirety of the more developed ganglia, often including even cells belonging to the shrinking VOs (Fig. 3D).

Plit_Dpn-like

Out of all the genes studied *Plit_Dpn-like* yielded the weakest signal by far (Fig. 1C, 4A), rendering its description more challenging. In instar II the expression pattern is reminiscent of that of *Plit_SoxN*. The expression domains concentrate around the VOs of the SEG, however the signal is more diffuse and, in some cases, appears to reach further dorsally into the ganglion (Fig. 4B). The anlage of WLG2, if present, shows weak *Plit_Dpn-like* signal as well, but unlike *Plit_SoxN* it is not present throughout the entire structure, but rather restricted to cells in the ventral half (Fig. 4B).

In instar III the expression domains in WLG2 have expanded, still reminiscent of the expression of *Plit_SoxN* (Fig. 1C, 4C). The anlage of WLG3 shows very restricted, weak signal located in

the ventral-most cells of the *Plit_SoxN* expression territories (Fig. 4C). The same is true for the VNC of instars IV, in which the diffuse expression domains continue to be located in the ventral-most portion of the ganglia constituting the apical cell layer of the VOs (Fig. 4D). Notably, in this later stage the signal in the VO cells of the more developed anterior ganglia is not noticeably weaker (as opposed to *Plit_SoxN*).

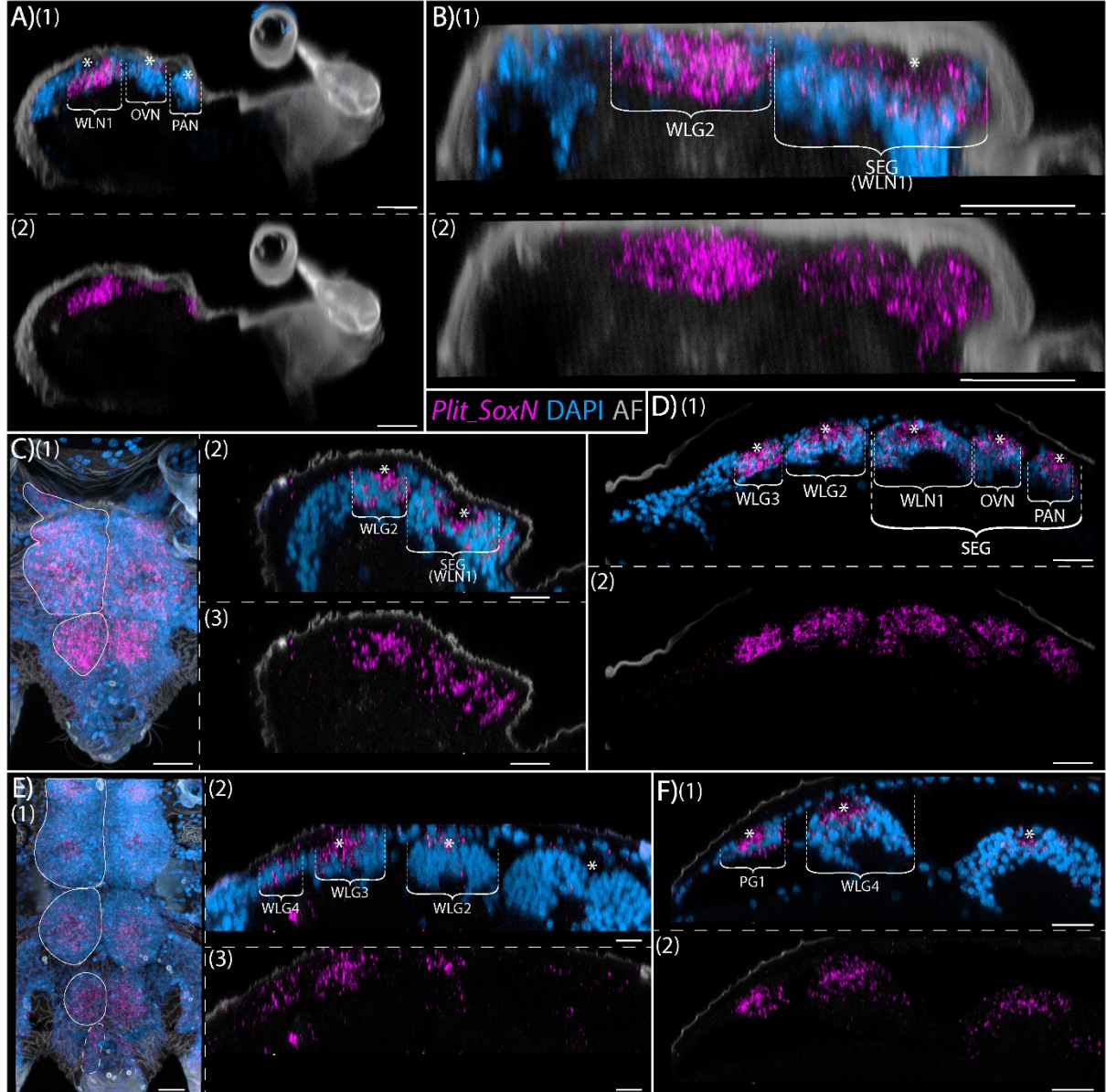


Figure 2. Expression of the proneural gene *Plit_SoxN* in the developing VNC of *P. littorale*. Virtual extended sections across the sagittal plane (A-F) and additional 3D renderings (MIP; C1 & E1) of several postlarval instars. *Plit_SoxN* gene expression (magenta) with nuclear counterstain via DAPI (blue) and autofluorescent signal of the cuticle (gray). All virtual sagittal sections with anterior pointing towards the right. 3D renderings in ventral view with anterior pointing towards the top. Asterisks indicate the positions of the VOs central cavity. **A:** Early instar II. Note the absence of the WLG2 anlage and *Plit_SoxN* expression posterior of the SEG (1) with and (2) without nuclear staining. **B:** Late instar II (1) with and (2) without nuclear staining. **C:** Early instar III. (1) 3D rendering with the outline indicating the position of the SEG and WLG2. (2) with and (3) without nuclear staining. **D:** Late instar III. (1) with and (2) without nuclear staining. **E:** Instar IV. Dashed outline in (1) indicates the shape of the forming ganglion anlage (2) with and (3) without nuclear staining. **F:** Instar V. (1) with and (2) without nuclear

staining. Scalebars = 20µm. *Abbreviations:* OVN – ovigeral neuromere, PAN – palpal neuromere, SEG – subesophageal ganglion, WLG – walking leg ganglion, WLN – walking leg neuromere.

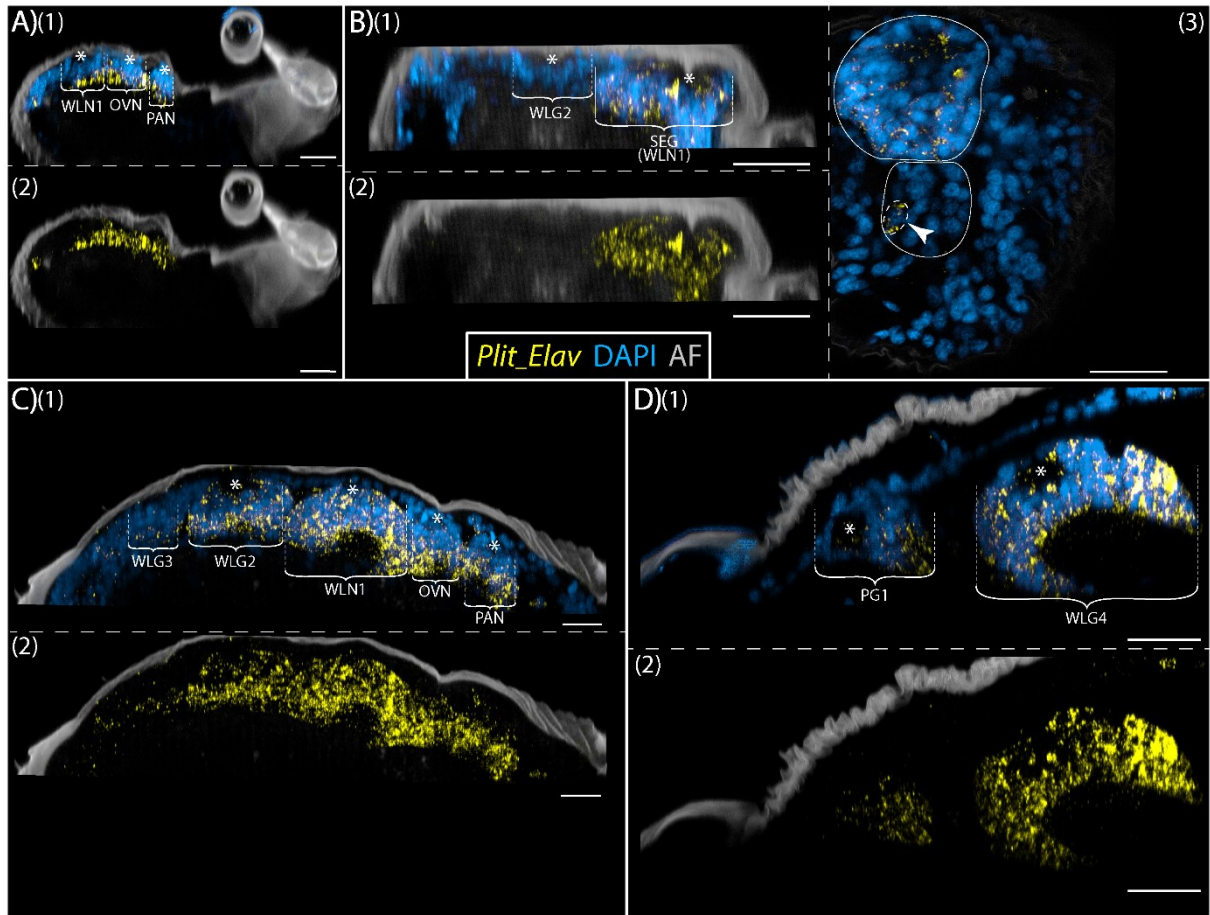


Figure 3. Expression of the neuronal marker *Plit_Elav* in the developing VNC of *P. littorale*. Gene expression patterns of *Plit_Elav* (yellow) combined with nuclear counterstain via DAPI (blue) and autofluorescent signal of the cuticle (gray). Virtual extended sections across the sagittal plane (**A-D**; (1) with, (2) without nuclear staining) and an additional 3D rendering (MIP; **B3**). Virtual sections in lateral view with anterior pointing towards the right. 3D renderings in ventral view with anterior pointing towards the top. **A:** Early instar II. **B:** Late instar II. Note that this is a slightly oblique section, thus the right side shows a more apical portion of the VNC than the left side. Note the virtual lack of expression in the WLG2 anlage. The outlines in **B3** indicate the shape of the SEG and WLG2 anlage. The arrowhead in **B3** highlights a single cell with expression **C:** Instar III. **D:** Instar VI- Note the expression at the antero-dorsal margin of PG1. Asterisks indicate the positions of the VOs central cavity. Scalebars = 20µm. *Abbreviations:* OVN – ovigeral neuromere, PAN – palpal neuromere, PG – posterior ganglion, SEG – subesophageal ganglion, WLG – walking leg ganglion, WLN – walking leg neuromere.

Plit_Brat

In instar II *Plit_Brat* is expressed both in the SEG and the WLG2 anlage (Fig. 5A, 6A). In the former, expression in the palpal and ovigeral neuromeres is located in the regions of the apical VOs, but also extends further basally into the ganglion, almost reaching its dorsal margin. Posteriorly in the SEG, the WL1 neuromere shows much stronger signal, expressed broadly throughout most of the structure (Fig. 6A). The signal is strongest in the cells of the dorsal ganglion half and gradually decreases ventrally, generally not reaching the apical layers of the

VOs. A similar dorsoventral gradient is present in the early WLG2 anlage of late instar II, with dorsal cells showing more elevated expression (Fig. 6B).

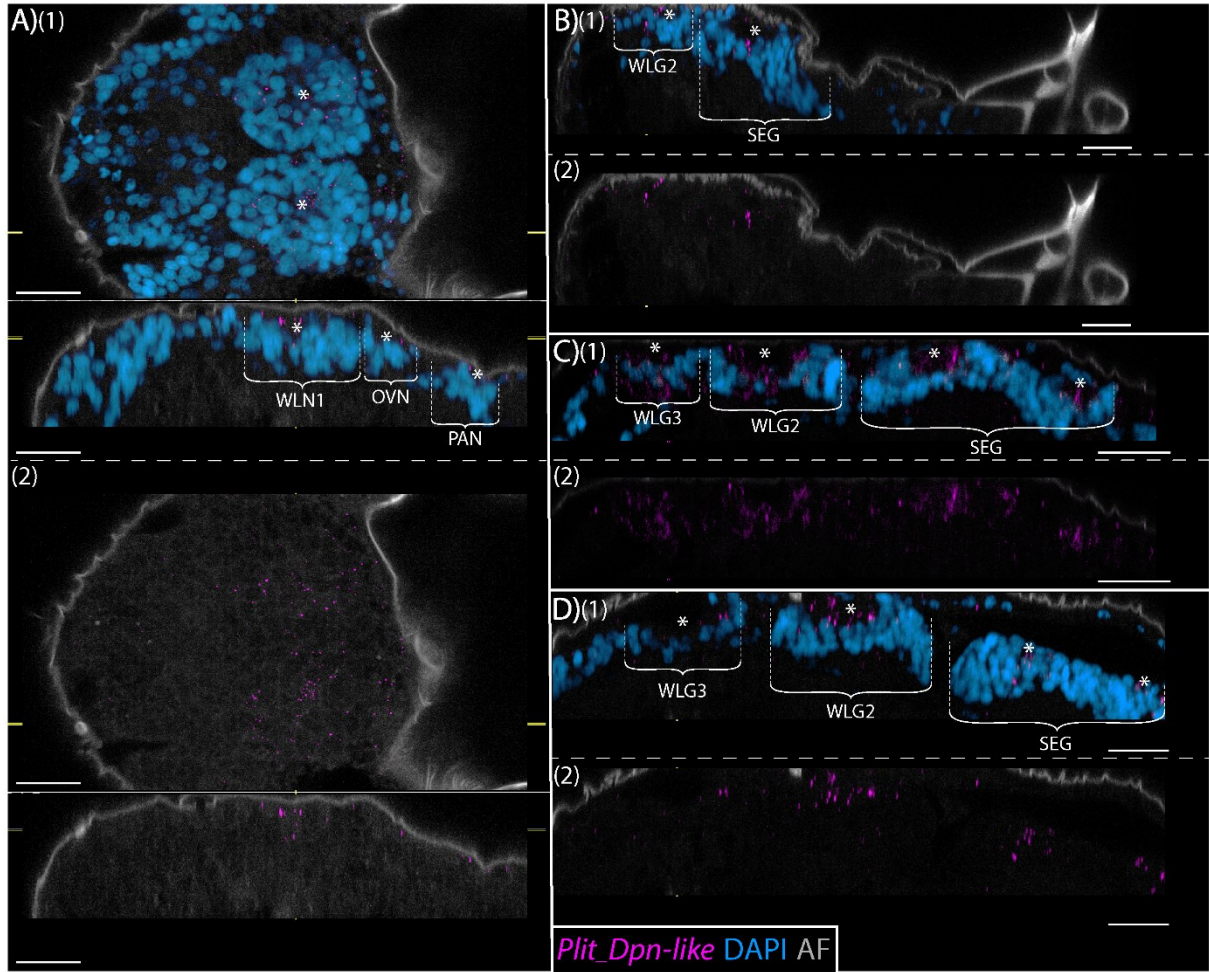


Figure 4. Expression of the Hes-family member *Plit_Dpn-like* in the developing VNC of *P. litorale*. Gene expression patterns of *Plit_Dpn-like* (magenta) combined with nuclear counterstain via DAPI (blue) and autofluorescent signal of the cuticle (gray). Virtual extended sections (MIP) across the horizontal (top) and sagittal (bottom) planes in A and across only the sagittal plane in B-C. (1) with, (2) without nuclear staining. Anterior oriented towards the right. Asterisks indicate the positions VOs central cavity. Note the generally weak signal. **A:** early instar II. **B:** late instar II. **C:** instar III. **D:** instar IV. Scalebars = 20µm. *Abbreviations:* OVN – ovigeral neuromere, PAN – palpal neuromere, SEG – subesophageal ganglion, WLG – walking leg ganglion, WLN – walking leg neuromere.

Instar III shows similar expression patterns (Fig. 6C, D). The signal of palpal and ovigeral neuromeres localises to the cells of the VOs and dorsally thereof, and the expression domains of the WL1 neuromere encompassing almost the entire structure (Fig. 6C). The dorsoventral gradient has become less pronounced, since the expression in the ventral half is stronger. The weakest signal is found in cells in or directly adjacent to the VOs (Fig. 6C). In the dorsal half of the SEG the strongest expression occurs in cells surrounding the neuropil, which is recognizable as the nucleus-free structure located dorsally in more developed ganglia (Fig. 6C). WLG2 also shows strong expression throughout the ganglion with a decrease in signal intensity

towards the VOs (Fig. 6D). In the newly formed WLG3 anlage only very faint expression is detectable, with the exception of a lateral cluster of cells anterodorsally located in the ganglion which shows significantly stronger signal (data not shown).

By instar IV the expression territory in the SEG has concentrated centrally in the hemiganglia, directly ventral to and bordering the respective neuropils (Fig. 5A, 6E). Only weak to no signal is present in more peripheral regions of the prospective soma cortex (Fig. 5A, 6E). In WLG2 a corresponding condensation starts to be noticeable, but it is not yet as pronounced as in the SEG (Fig. 6E). By contrast, WLG3 displays quite broad expression with a dorsoventral gradient, similar to the WLG2 in the preceding instar (Fig. 6E).

These patterns repeat themselves in the three most posterior ganglia of instars V and VI, respectively (Fig. 6F). In the more anteriorly located ganglia (SEG and WLG2), however, the aforementioned central condensation of expression domains is no longer visible. Instead, only scattered individual cells strongly express *Plit_Brat*, in addition to low signal throughout the entire ganglion (Fig. 6G). For the most part, these single cells are located ventral to the significantly size-decreased VOs. Only few of them are situated further basally around the neuropil.

Plit_Pros

The expression pattern of *Plit_Pros* is quite similar to that of *Plit_Brat* (Fig. 5). In instar II the expression domains in the SEG are clustered around the VOs of the palpal and ovigeral neuromeres, while the gene is almost ubiquitously expressed in the posterior part corresponding to WLN1. However, there is no anteroventral gradient visible in the latter region (Fig. 7A). In the WLG2 anlage only one to two individual cells with strong *Plit_Pros* expression are discernible at the anterior margin of each hemiganglion, while the rest of the structure lacks any expression (Fig. 7A). In the SEG of instar III, the territories of strong expression have concentrated concentrically around the VOs, resulting in decreased expression towards the midline of the body (Fig. 5B, 7B). Throughout WLG2 the signal is generally still ubiquitously present, whereas in the anlage of WLG3 only three *Plit_Pros*-positive cells in triangular configuration are visible (Fig. 7B). During the following instars, the expression domains of the more developed ganglia continue concentrating into an ellipsoid shape located between the respective VOs and neuropils, with additional domains clustered anteriorly around the neuropils (Fig. 5B, 7C, D). By instar VI the signal in these further developed ganglia becomes weaker and more diffuse, with only some cells in the ganglionic soma cortex showing elevated levels of expression, similar to the expression dynamic observed for *Plit_Brat* (data not shown).

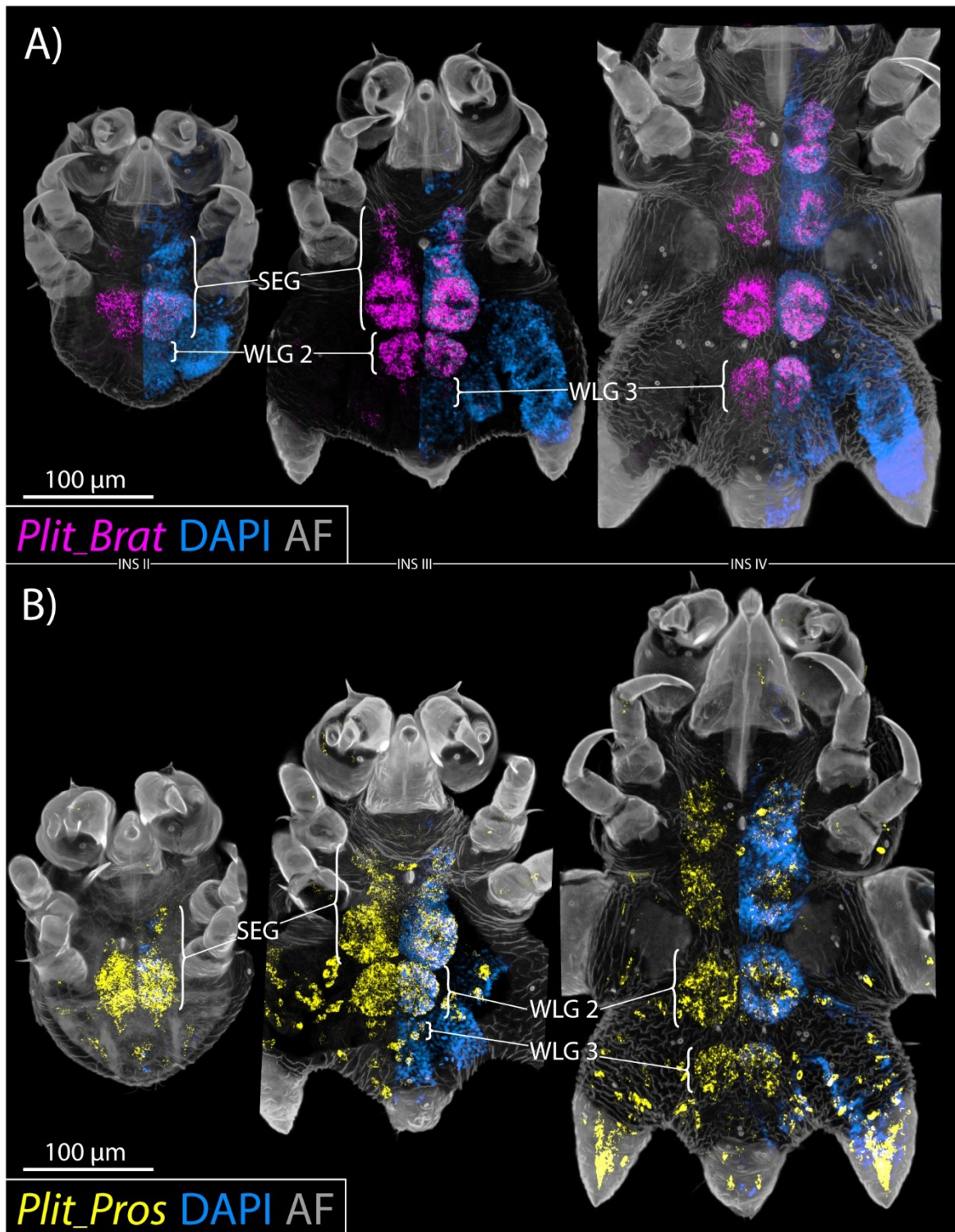


Figure 5. Overview of the general expression patterns of *Plit_Brat* and *Plit_Pros* in postlarval instars of *P. littorale*. 3D renderings (MIP) of instars II-IV in ventral view. Gene expression patterns of *Plit_Brat* (A; magenta) and *Plit_Pros* (B; yellow) with nuclear counterstain via DAPI (blue) and autofluorescent signal of the cuticle (gray). DAPI signal shown only in one half of the body to reveal weaker HCR-FISH signal. Note the central concentration of expression domains in more developed ganglia of instars IV. *Abbreviations:* SEG – subesophageal ganglion, WLG – walking leg ganglion.

Plit_ASH1

Compared to the previous genes the expression of *Plit_ASH1* is rather weak and therefore more difficult to interpret (Fig. 8A, 9). The expression domains are quite comparable to those of *Plit_SoxN*. In instar II, the expression domains cluster around the VOs of the SEG, but also reach further dorsally into the subapical cell layers (Fig. 9A). In the WLG2 anlage the expression pattern is similar, with the exception of individual cells showing slightly higher levels of expression (data not shown).

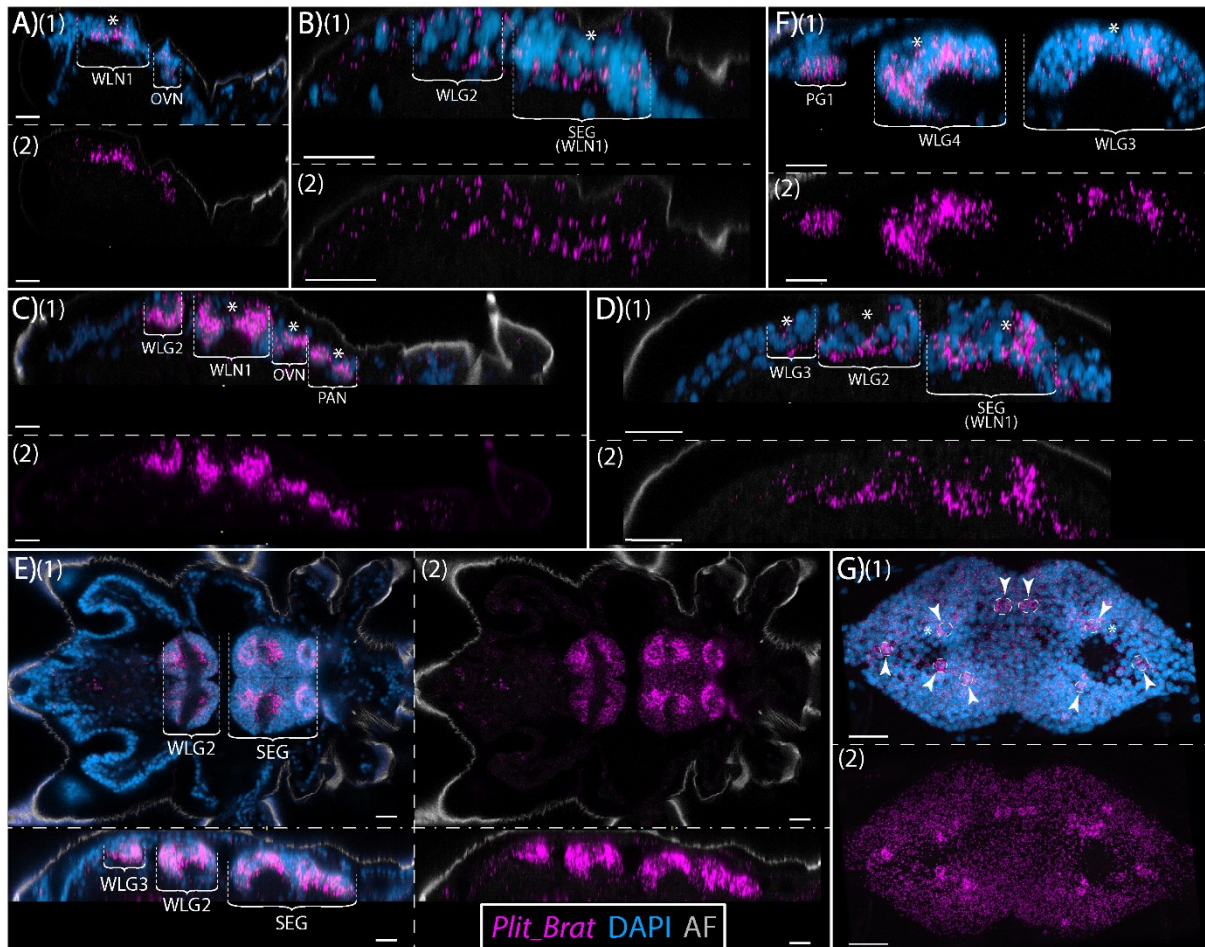


Figure 6. Expression of the neuronal differentiation marker *Plit_Brat* in the developing VNC. Gene expression patterns of *Plit_Brat* (magenta) combined with nuclear counterstain via DAPI (blue) and autofluorescent signal of the cuticle (gray). Virtual extended sections across the horizontal (top) and sagittal (bottom) planes in **E** and across only the sagittal plane in **A-D** and **F**. (1) with, (2) without nuclear staining. Anterior in virtual sections oriented towards the right, in 3D-rendering towards the top. **A:** early instar II. **B:** late instar II. **C:** early instar III. **D:** late instar III. **E:** instar IV. Note the central concentration of expression in the SEG. **F:** detail of posterior region of instar VI. **G:** 3D rendering (MIP) of WLG2 in instar VI. Arrowheads and dashed-lines highlight the bilaterally symmetrical arrangement of *Plit_Brat*-positive cells. Asterisks indicate the positions of the VOs central cavity. Scalebars = 20µm. Abbreviations: OVN – ovigeral neuromere, PAN – palpal neuromere, SEG – subesophageal ganglion, WLG – walking leg ganglion, WLN – walking leg neuromere.

Similar patterns are found in instar III, where the expression domains of the SEG are restricted more or less to the VOs, while in WLG2 the signal is spread further across the ganglion (Fig.

8A, 9B). In the anlage of WLG3 a pattern of three cells in triangular formation with strong expression is visible, corresponding to that seen in *Plit_Pros* (Fig. 10).

In instar IV this three-cell pattern is no longer discernible in WLG3, which at this point has increased in size and shows expression throughout the entire structure (Fig. 8A). The expression domains of WLG2 start concentrating around the VOs, similar to the SEG making them comparable to the expression of *Plit_SoxN* in corresponding stages (Fig. 8A). The anlage of WLG4 also shows elevated expression in individual cells, however unlike in previous stages no clear pattern is recognizable, in part due to this ganglion anlage having a more stripe-shaped elongated appearance (data not shown).

This pattern of single cells with elevated expression emerging in the posteriormost ganglion anlage, followed by broad expression throughout the entire developing ganglion and subsequent concentration around the respective ventral organs is maintained in the following stages and includes the posterior ganglia (Fig. 9C).

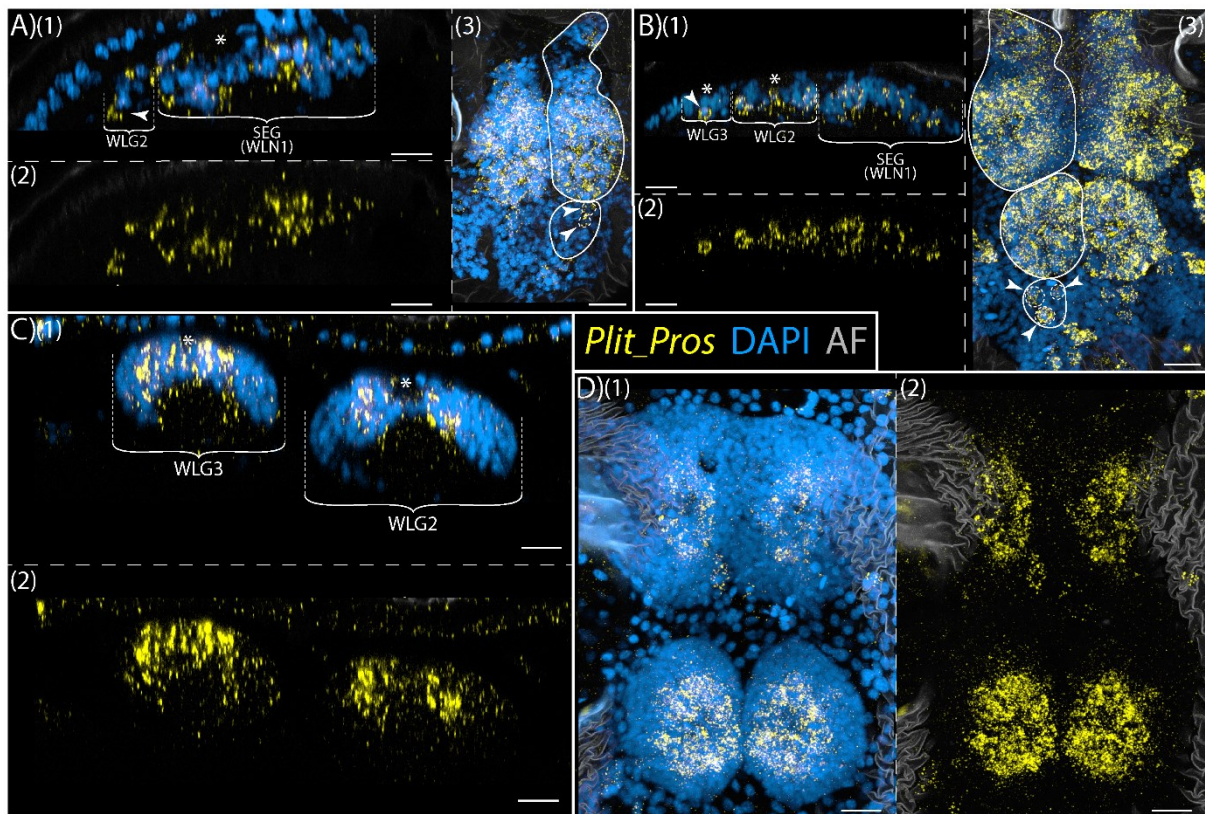


Figure 7. Expression of the neural differentiation marker *Plit_Pros* in the developing VNC. Gene expression patterns of *Plit_Pros* (yellow) combined with nuclear counterstain via DAPI (blue) and autofluorescent signal of the cuticle (gray). Virtual extended sections across the sagittal plane (A-C) and 3D-renderings (MIP; A3, B3, D). (1) with, (2) without nuclear staining. Anterior in virtual sections oriented towards the right, in 3D-rendering towards the top. **A:** instar II. **B:** instar III. **C-D:** instar V. The outlines indicate the shape of ganglia. Arrowheads highlight single cells that express *Plit_Pros*. Asterisks indicate the positions of the VOs central cavity. Scalebars = 15µm. *Abbreviations:* SEG – subesophageal ganglion, WLG – walking leg ganglion, WLN – walking leg neuromere.

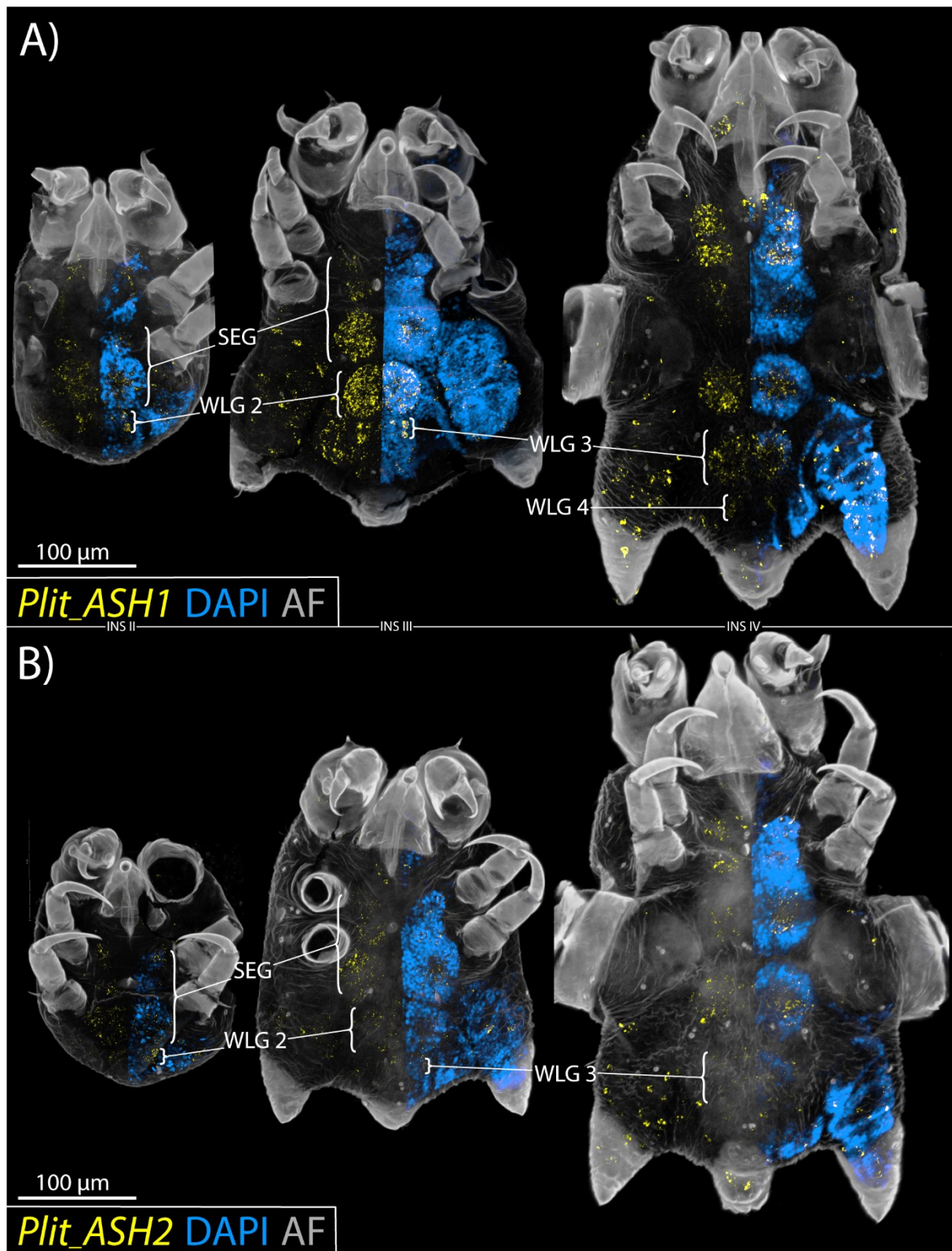


Figure 8. Overview of the general expression patterns of the two *Plit_ASH* genes in postlarval instars of *P. litorale*. 3D renderings (MIP) of instars II-IV in ventral view. Gene expression patterns of *Plit_ASH1* (A; yellow) and *Plit_ASH2* (B; yellow) with nuclear counterstain via DAPI (blue) and autofluorescent signal of the cuticle (gray). DAPI signal shown only in one half of the body to reveal weaker HCR-FISH signal. *Abbreviations:* SEG – subesophageal ganglion, WLG – walking leg ganglion

Plit_ASH2

The expression patterns of the two *achaete-scute* homologs are strikingly similar if not identical. Like *Plit_ASH1*, *Plit_ASH2* also generally shows very weak, which is in the SEG of instars II somewhat concentrated around the VOs (Fig. 8). The primordial WLG2 anlage likewise exhibits the triangular formation of strongly expressing cells (Fig. 11A). In later stages this pattern expands first into ubiquitous expression across the entire ganglion anlage and subsequently starts to concentrate around the VOs (Fig. 11B, C). The posteriormost ganglion anlage usually shows from one to all three of the cells forming the triangular pattern, possibly dependent on their degree of development.

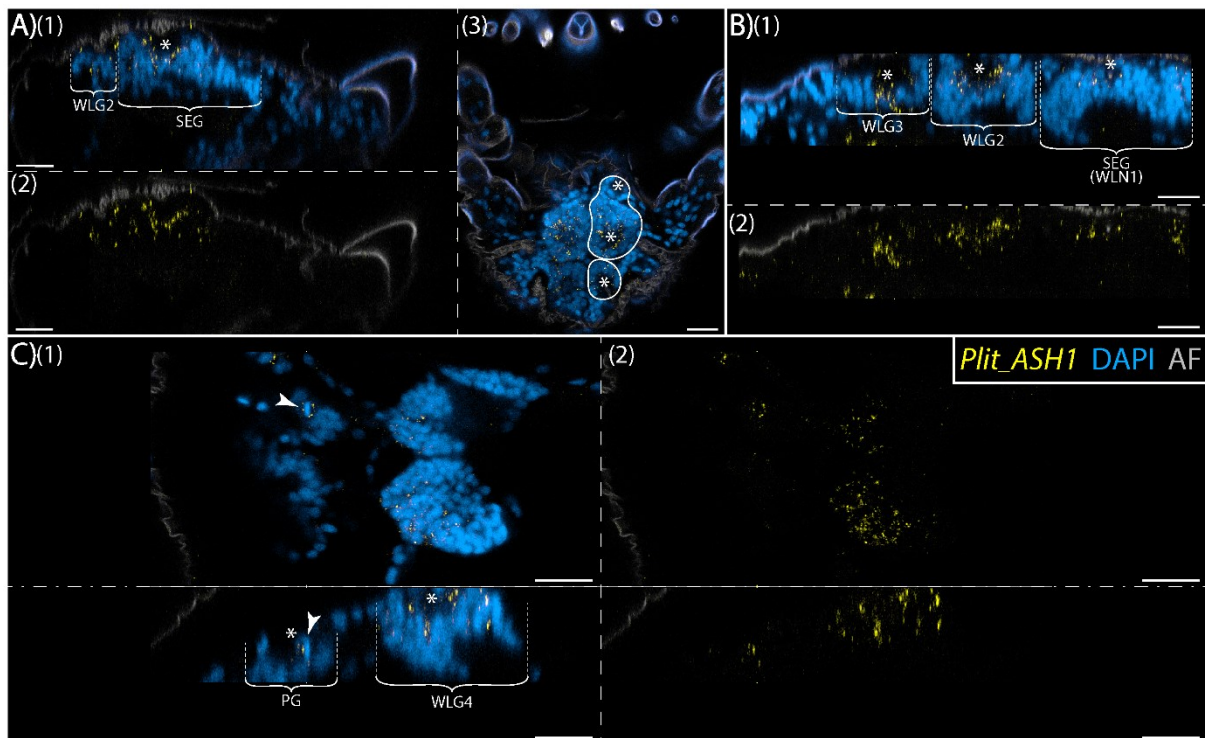


Figure 9. Expression of *Plit_ASH1* in the developing VNC. Gene expression patterns of *Plit_ASH1* (yellow) combined with nuclear counterstain via DAPI (blue) and autofluorescent signal of the cuticle (gray). Virtual extended sections across the horizontal (top) and sagittal (bottom) planes in C and across only the sagittal plane in A and B. (1) with, (2) without nuclear staining. Anterior in virtual sections is oriented towards the right. **A:** instar II. (3) showing an additional horizontal section (anterior oriented towards the top). **B:** instar IV. **C:** instar V. The outlines indicate the shape of the SEG and WLG2 anlage. Arrowheads highlighting single cells expressing *Plit_ASH1*. Asterisks indicate the positions of the VOs central cavity. Scalebars = 20µm. Abbreviations: PG – posterior ganglion, SEG – subesophageal ganglion, WLG – walking leg ganglion, WLN – walking leg neuromere.

Co-expression of neurogenic genes in the developing VNC

Since selected pairs of genes were multiplexed in the HCR-FISH reactions, it is possible to make additional statements about the co-expression of these genes.

The expression domains of *Plit_SoxN* and the putative *deadpan* homolog overlap in the region of the VOs, but the expression domains of *Plit_Dpn-like* were observed to variably extend further dorsally than those of *Plit_SoxN* (Fig. 12A).

By contrast, *Plit_ASH1* is more or less exclusively expressed in *Plit_SoxN*-positive regions (Fig. 12B). However, especially in single cells that strongly express *Plit_ASH1* the expression level of *Plit_SoxN* is noticeably reduced yet still clearly present (Fig. 12C).

Plit_Pros also shows overlap with the expression patterns of *Plit_SoxN* in the ventral half, reaching almost as far ventrally, but is not as widely expressed in the regions of the VOs. This is especially the case for ganglia that are in an earlier developmental stage, in which the expression domains of *Plit_Pros* have not yet concentrated dorsally to the VOs (Fig. 12D). As these domains concentrate concentrically around the VOs of further developed ganglia in later stages, the overlap with *Plit_SoxN* becomes less noticeable (Fig. 12E).

Lastly, *Plit_Elav* has very little to no overlap with *Plit_SoxN*, with the latter mostly being confined to the regions surrounding the VOs, while the former mostly extends across the dorsal half of the ganglion, resulting in only a narrow strip of overlap in the center where the expression domains of the two genes meet (Fig. 12F). The posteriormost ganglion anlage initially shows only expression of *Plit_SoxN*, but as it develops further *Plit_Elav* starts being expressed at the dorsal pole. In such earlier stages of the ganglion anlagen, the regions of overlapping expression are thus much broader and more prominent (Fig. 12G).

Between *Plit_ASH2* and *Plit_Brat* very little to no overlapping expression patterns are detectable. The former is expressed in the ventralmost regions of the ganglion anlage, whereas the latter is expressed more or less ubiquitously but with significantly decreasing intensity towards the ventral pole (Fig. 12H). Especially in the posteriormost ganglion anlagen, it is apparent that the three stereotypical *Plit_ASH2*-positive cells completely lack *Plit_Brat* expression as they become recognizable (Fig. 12H).

By contrast, *Plit_Brat* shows much more overlapping expression with *Plit_Elav*. *Plit_Brat* is almost exclusively expressed in *Plit_Elav*-positive regions except for the ventral margin of the ganglion, in which *Plit_Elav* is absent (Fig. 12I). However, the overall extent of the *Plit_Brat* territory is much smaller in comparison, as its boundaries do not extend as far dorsally and are more concentrated around the central areas of each hemiganglion (Fig. 12I).

Gene expression at the cellular level and localisation to cells in division

In order to make inferences about the activity of genes within different cell types, gene expression needs to be examined at the cellular level.

The most notable pattern at the cellular level is that of the three cells in triangular formation, which was observed in the posteriormost ganglion anlage of several different instars in both of the *achaete-scute* homologs as well as in *Plit_Pros* (Fig. 13). Based on these expression profiles these likely represent the first cells adopting neural fate and are therefore referred to as neural precursor cells (NPCs). This general triangular pattern is relatively consistent, with two cells positioned towards the anterior margin of the ganglion, one closer to the midline (NPC3) and

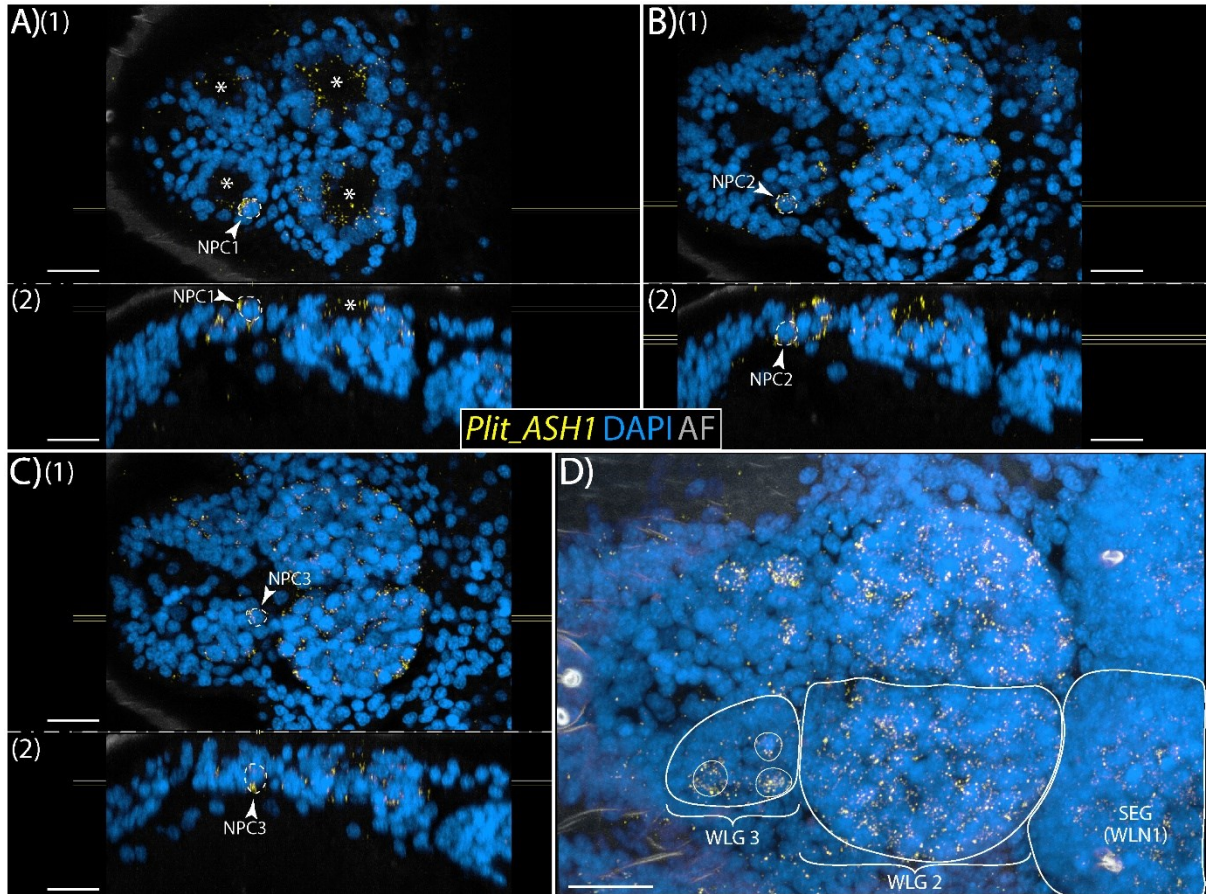


Figure 10. Triangular pattern of *Plit_ASH1*-positive cells in the posteriormost ganglion anlage of instar III. Gene expression patterns of *Plit_ASH1* (yellow) with nuclear counterstain via DAPI (blue) and autofluorescent signal of the cuticle (gray). Anterior is oriented towards the right. Virtual extended sections (1 – horizontal; 2 – sagittal) of instar III (A-C). 3D-rendering in ventral view (D). Outlines indicate the shape of the ganglia. Dashed line highlights cells NPC1-3. Note that although the cells NPC2 and NPC3 occupy the same (horizontal) plane, the former lies in the apical cell layer of the neuroectodermal depression, whereas the latter is located subapically in the ganglion anlage. Asterisks indicate the regions of the VOs central cavity. Scalebars = 15µm. *Abbreviations:* NPC – neural precursor cell, SEG – subesophageal ganglion, WLG – walking leg ganglion, WLN – walking leg neuromere.

the other further laterally (NPC1), posterior of which the third cell is located (NPC2). The distribution along the dorsoventral axis on the other hand is more variable. NPC1 is generally located ventrally and consistently is part of the apical cell layer making up the VOs. Conversely, NPC3 is typically situated further dorsally being part of the subapical cell mass. NPC2 was variably found either in the same horizontal plane as the two previous cells or lay between them,

accordingly these cells are either apical or subapical. As mentioned before these three cells do not emerge simultaneously, however based on the positions of these initially formed cells it can be inferred that expression is initiated first in NPC1, followed by NPC2 and finally NPC3. An additional cell was rarely seen directly dorsally of NPC1, however, not consistently enough to be included in this pattern (Fig. 13C).

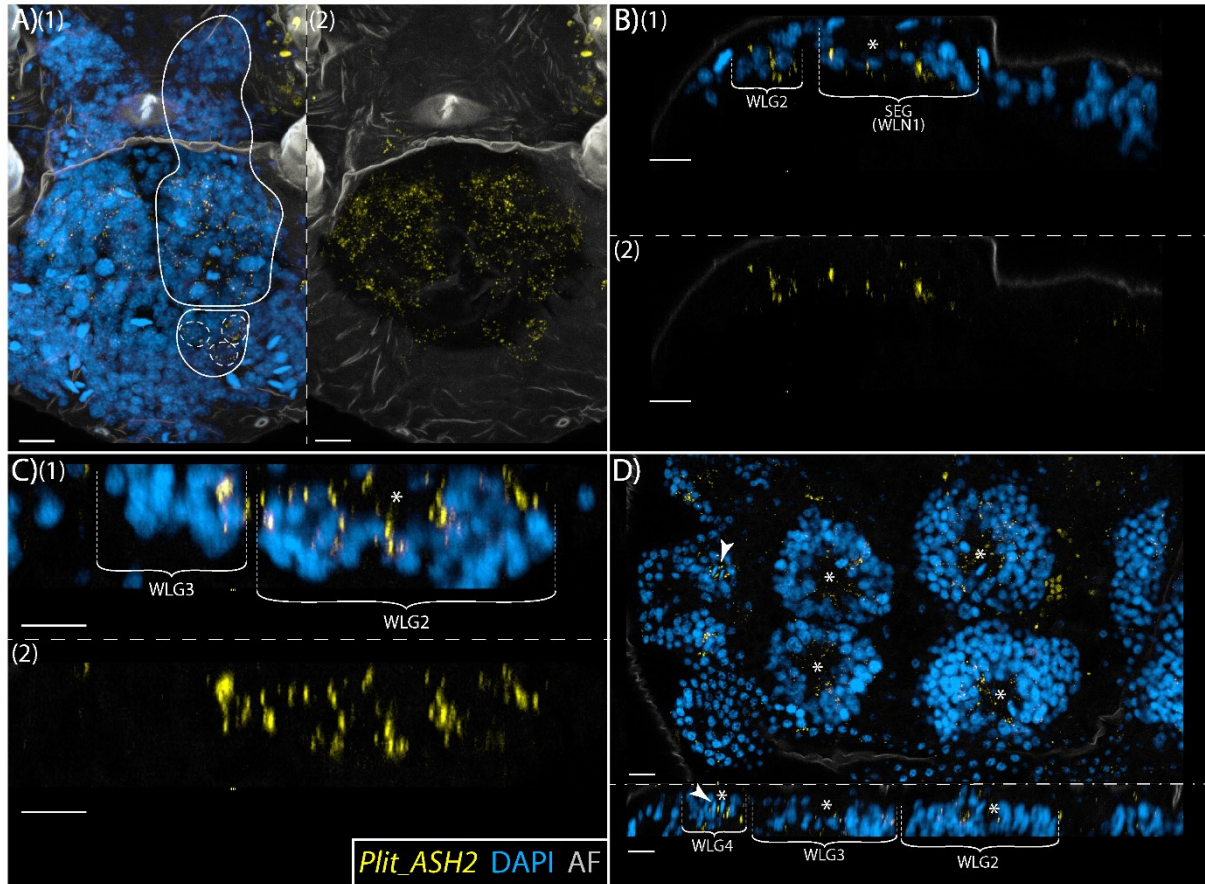


Figure 11. Expression of *Plit_ASH2* in the developing VNC. Gene expression patterns of *Plit_ASH2* (yellow) with nuclear counterstain via DAPI (blue) and autofluorescent signal of the cuticle (gray). 3D-renderings (A) and virtual extended sections across the horizontal (top) and/or sagittal (bottom) planes (B–D). (1) with, (2) without nuclear staining. Anterior in virtual sections is oriented towards the right, in 3D-renderings towards the top. **A–B:** instar II. Note the three cells in WLG2 showing expression. **C:** instar III. **D:** instar IV. Note the single dividing *Plit_ASH2*-positive cell (indicated via arrowheads) tangentially oriented in the apical cell layer of the VO. The outlines indicate the shape of the SEG and WLG2 anlage. The dashed line highlights single cells expressing *Plit_ASH2*. Asterisks indicate the positions of VOs central cavity. Scalebars = 10µm. *Abbreviations:* SEG – subesophageal ganglion, WLG – walking leg ganglion, WLN – walking leg neuromere.

The second notable pattern was the emergence of single cells or rather clusters of basal cells expressing both *Plit_Brat* and *Plit_Elav* at the dorsal pole of the primordial ganglion anlage, often skewed towards the anterior. In contrast to the previous, this pattern is far less consistent, not allowing the identification of individual recurring cells (data not shown). Due to their expression profiles, however, it can be inferred that these cells are the first differentiating neurons.

Another important feature contributing to the identification of different neurogenic cell types is the presence of cell divisions in cells expressing certain genes. A general observation regarding cell divisions is that most samples featured either no cell divisions or several divisions at once. Thus, divisions appear to happen in bursts, with periods of little to no occurrences followed by more or less simultaneous divisions. The mode of division can also be a determinant of different cell types, whereby an asymmetric division is characteristic of the proposed NSCs, while symmetric divisions are more indicative of INPs. This feature, however, could not be used reliably as most of the captured divisions were in metaphase making the detection of size differences in the daughter cells impossible. Even in cells during ana- or telophase, however, morphological size differences are – if present at all – not strongly pronounced and thus difficult to ascertain with confidence (Fig. 14).

Of the observed divisions, the majority occur in *Plit_SoxN*-positive cells within the apical cell layer of the VOs, with some possibly showing an asymmetric division mode (Fig. 14B). A significant number of these divisions showed additional expression of *Plit_ASH1* (Fig. 14C). *Plit_ASH2*-positive cells were also found in the same cell layer, but less often and usually with lower levels of expression (Fig. 14D). Additionally, single cases of *Plit_Pros*- and *Plit_Dpn-like*-positive divisions were observed, whereby the limited signal of the latter generally rendered the interpretation regarding cell divisions difficult (Fig 14E, F). The presence of cell divisions in this apical cell layer, expressing the aforementioned genes is congruent with the presence of large NSCs in the VOs of pycnogonids.

Plit_Brat positive divisions were also observed, but apart from individual divisions in the apical VOs showing only low expression, these were generally located in the subapical cell mass, which to a lesser extent also applies to *Plit_Pros* (Fig 14G). Both the location of these cells as well as the genes expressed in them might point to these cells representing the INPs.

Regions expressing *Plit_Elav* generally lacked any cell divisions, with the occasional exception of a subapical mitosis profile. This is in accordance with the assumption that these *Plit_Elav*-positive territories mainly contain post-mitotic neuronal cells.

Expression outside of CNS

Although not the focus of this thesis, some notable expression patterns outside the VNC are here reported, since none of the studied genes were expressed exclusively in CNS.

Plit_SoxN is expressed in a bilaterally symmetrical stripe in the ventral ectoderm, extending between the posteriormost ganglion anlage to the posterior pole of the animal (Fig. 15A).

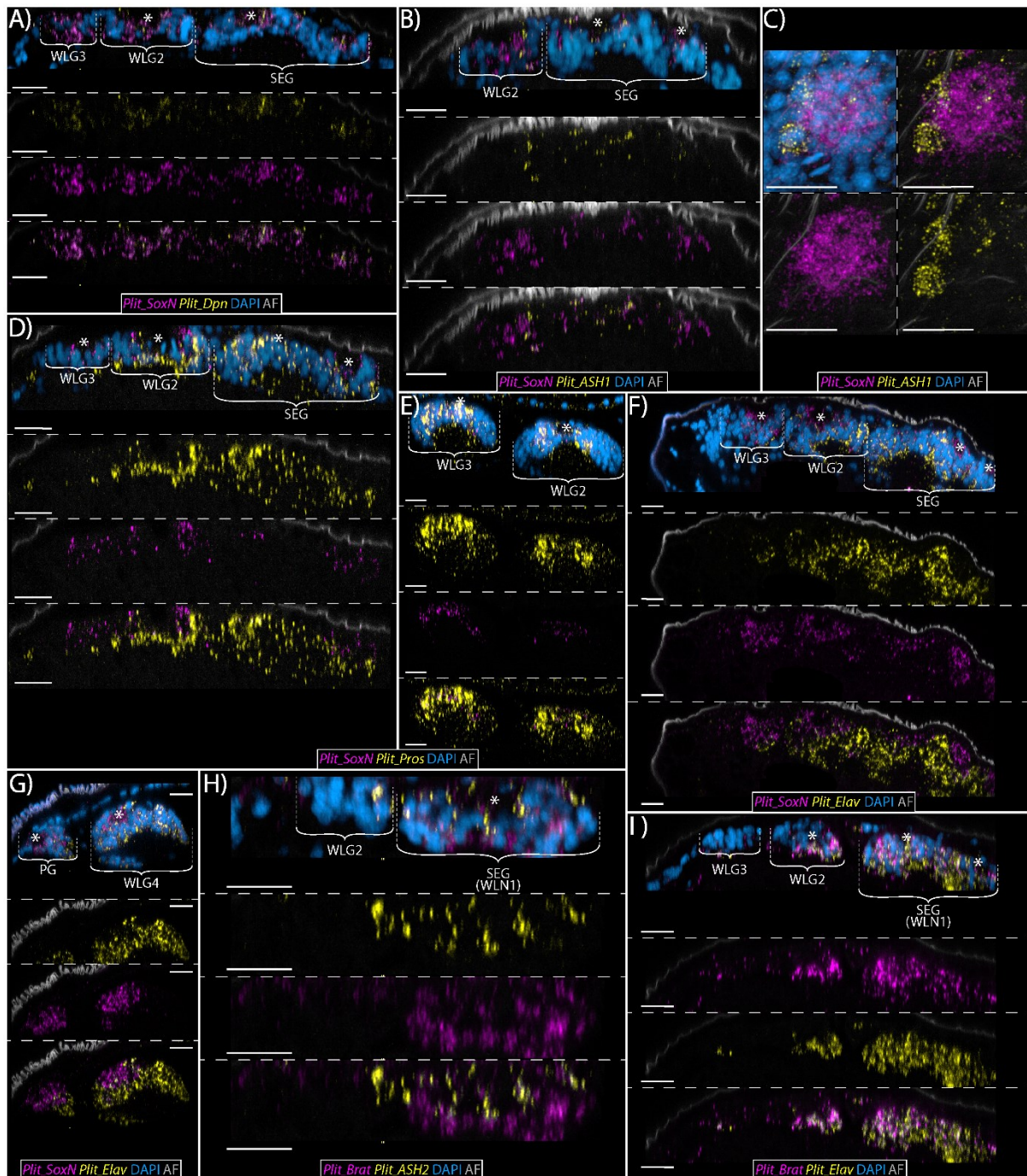


Figure 12. Co-expression of different neurogenic genes in the developing VNC. Gene expression patterns of various genes (magenta and yellow) with nuclear counterstain via DAPI (blue) and autofluorescent signal of the cuticle (gray). Virtual extended sections (MIP) across the sagittal plane. Anterior is oriented towards the right. Asterisks indicate the regions of the VOs central cavity. Scalebars = 15µm. *Abbreviations:* SEG – subesophageal ganglion, WLJ – walking leg ganglion.

Additional expression domains are present near the tip of the limb buds, as well as a ring of expression running around a proximal region of the limb buds (Fig. 15A').

Similar ring-shaped expression domains are present for *Plit_Dpn-like*. However, here multiple of these can be found along the length of the limb bud, with the last one almost reaching into

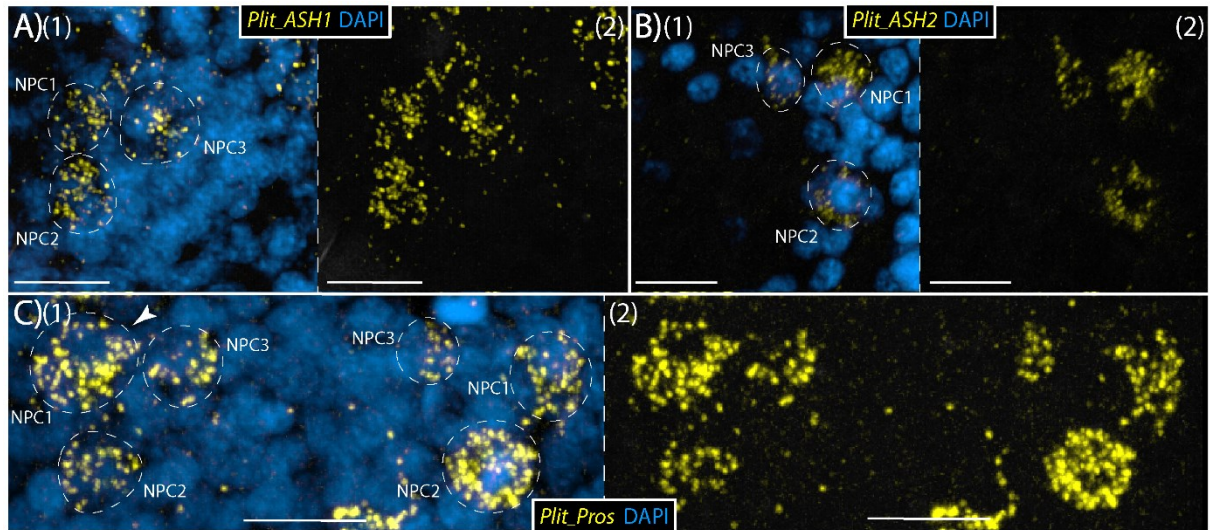


Figure 13. Triangular pattern observed in *Plit_ASH1*, *Plit_ASH2* and *Plit_Pros*. Gene expression patterns of *Plit_ASH1* (A; instar III), *Plit_ASH2* (B; instar II) and *Plit_Pros* (C; instar III) (yellow) with nuclear counterstain via DAPI (blue) and autofluorescent signal of the cuticle (gray). Virtual extended sections (MIP) across the horizontal plane. (1) with, (2) without nuclear staining. Anterior is oriented towards the top. Dashed circles highlighting the individual cells (NPC1-3) with increased expression. **A:** detail of a single hemiganglion of the WL3 anlage. The midline is situated toward the right. **B:** detail of a single hemiganglion of the WL2 anlage. The midline is oriented toward the left. **C:** detail of the WL3 anlage. The arrowhead indicates the position of a fourth cell located directly dorsally of NPC1. Scalebars = 10µm. Abbreviations: NPC – neural precursor cell.

the tip (Fig. 15B). These ring-shaped expression domains possibly demarcate the prospective leg segments.

Plit_Elav on the other hand, is expressed more broadly in single cells or cell clusters which are distributed regularly along the limb buds, reaching far down into their tips (Fig. 15C). Other notable expression domains are present in the larval appendages as well as the in the posteriormost region along the midline (Fig. 15C).

Plit_Brat generally shows quite broad expression across the entirety of the ectoderm, extending from the larval appendages to the posterior pole of the body, whereby the expression is much stronger and dense in the posterior half of the animal, including the limb buds and surrounding regions (Fig. 15D). *Plit_Brat* is expressed particularly strongly in the distal portion the ‘inner’ limb buds of the prospective WL2. (Fig. 15D).

By contrast, *Plit_Pros* is expressed strongly many single cells which are densely packed in the proboscis and line the ectoderm of the limb buds all the way into the tips. Additional *Plit_Pros*-expressing cells are visible in the ectoderm posterior of the VNC (Fig. 15E).

Both of the *achaete-scute* homologs show a similar pattern of single positive cells with increased expression, which are spread out diffusely across the regions of the limb buds (Fig. 15F, H). Additional *Plit_ASH2*-positive cells are present posterior of the VNC, similar to those of *Plit_Pros*. These individual cells dispersed in the limb buds and posterior regions, expressing

multiple genes with neurogenic function, plausibly represent neuronal or glial cells of the peripheral nervous system.

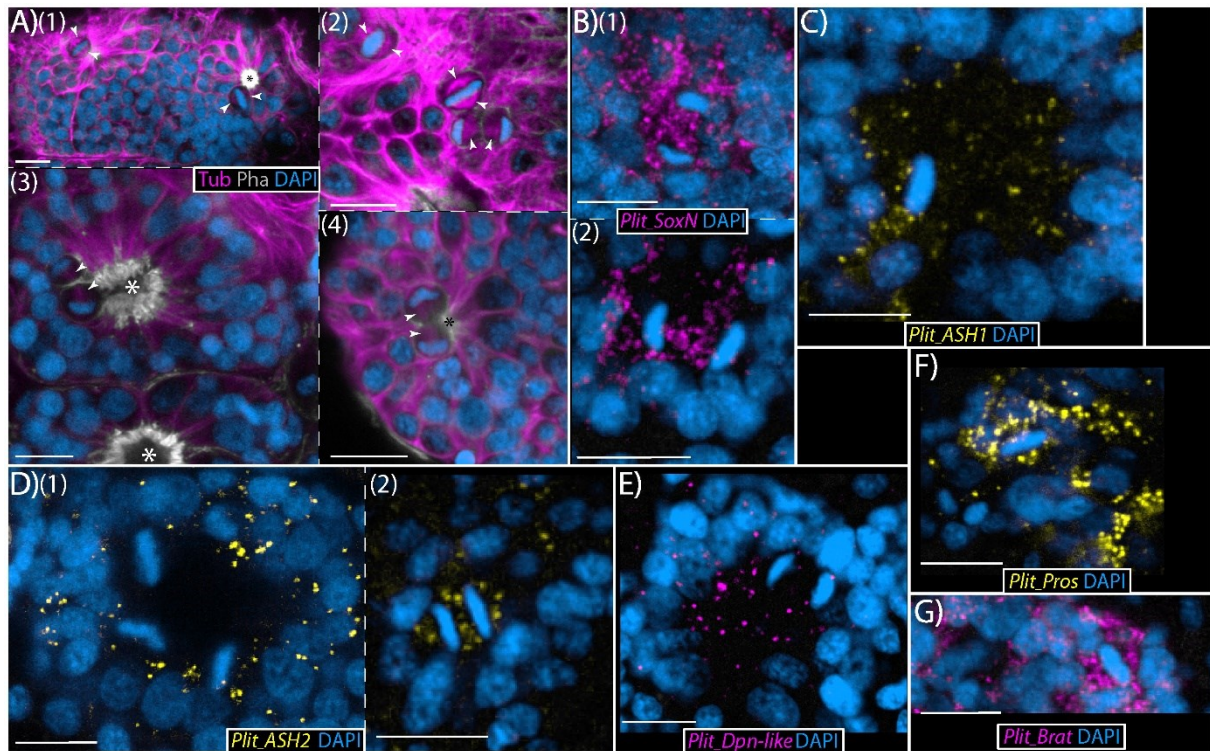


Figure 14. Overview of the observed cellular divisions in the VNC. Virtual extended sections (MIP) across the horizontal plane. Nuclear counter staining via DAPI (blue). **A:** tubulin (magenta) and phalloidin (grey) staining. Arrowheads highlighting the tubulin-stained spindle apparatus. **B-G:** HCR-FISH signal showing the gene expression patterns of various genes (magenta and yellow). Asterisks indicate the positions of the VO's central cavity. The white arrows highlight the cells undergoing cellular division (meta- or anaphase). Note the size difference of dividing nuclei in **A3**, **A4**, **B1** and **E**, which is characteristic for morphologically asymmetric cell divisions. Scalebars = 15µm.

Discussion

Postembryonic neurogenesis in *P. litorale* lacks an initial cell immigration phase

The process of embryonic neurogenesis in pycnogonids has been described to consist of two sequential, partially overlapping phases. During the first phase post-mitotic neural precursors migrate from specific cell immigration sites of the neuroepithelium. This is followed by the formation of metameric central invaginations lined by large NSCs, representing the so-called VO's of pycnogonids [9]. Reports of these large NSCs in the VO's date back to the first description of these structures by T.H. Morgan [46]. Based on their position and proliferative nature, they were almost unanimously concluded to play a NB-like function during the embryonic development of sea spiders [46, 59, 60]. Only more recently, Brenneis et al. [9], first recognized a second neural precursor cell type – the INP – which they concluded to represent the progeny of the asymmetrically dividing NSCs. Although these studies focused on another

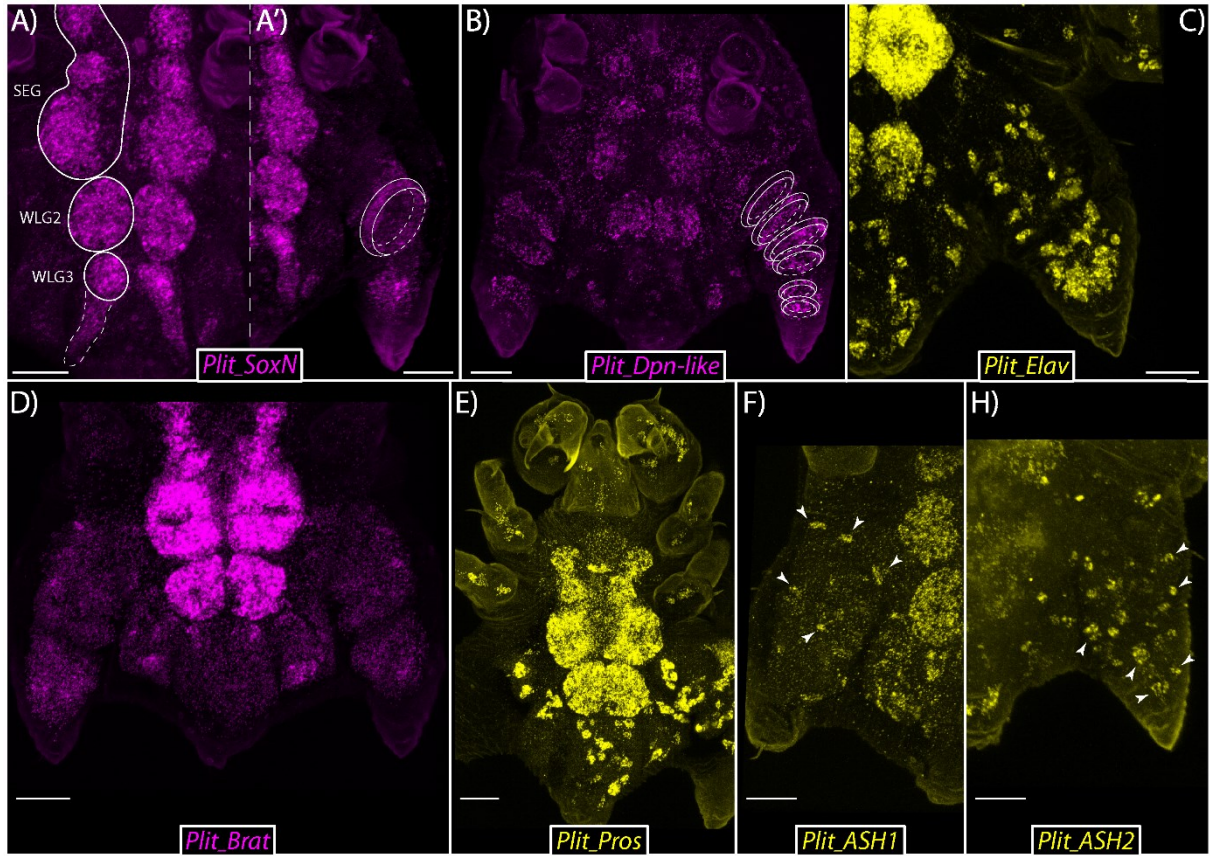


Figure 15. Expression patterns of different neurogenic genes outside of the VNC. 3D-renderings (MIP) of the gene expression patterns of instar III (A, B, D-F) and instar IV (C, H) in ventral view (anterior oriented towards the top). **A:** expression of *Plit_SoxN*. Outlines indicate positions of respective ganglia. Dashed line highlights stripe of expression posterior of the WLG3. **A':** expression of *Plit_SoxN* in a ring-shaped domain near the base of the limb bud. **B:** expression of *Plit_Dpn-like*. Note the presence of several ring-shaped domains along the limb bud. **C:** expression of *Plit_Elav* at the posterior pole and in the limb buds. **D:** expression of *Plit_Brat*. **E:** expression of *Plit_Pros*. **F:** expression of *Plit_ASH1*. **H:** expression of *Plit_ASH2*. Arrowheads indicate individual cells with notable expression. Scalebars = 30µm. Abbreviations: LB – limb bud, SEG – subesophageal ganglion, WLG – walking leg ganglion.

pycnogonid species, supplementary results regarding further pycnogonid species – including *P. litorale* – indicated similar neurogenic phases [9]. The described initial phase of cell immigration shows striking similarities to the neurogenic development of myriapods and euchelicerates. Accordingly, this morphological similarity may be expected to extend also to the expression patterns of key genes governing neurogenesis in pycnogonids. Namely the expression of *achaete-scute* homologs and *pros* in cells as they immigrate from the apical cell layer into more basal layers [17, 35, 36].

In contrast to earlier work, however, this study focuses on neurogenesis during the postembryonic development of a pycnogonid. Here, the three identified NPCs are the first cells of the primordial ganglion anlage of *P. litorale* to express both *pros* and the *achaete-scute* homologs. These cells, however, only emerge once the invaginations of the early VOs have already formed, with NPC1 -and variably also NPC2- even being part of their apical cell layer.

Though the two phases were described as not being strictly separated [9], the emergence of such expression patterns would nonetheless be expected to occur at an earlier point of development. Seeing as when these first cells emerge, the primordial ganglion anlage has already significantly increased in size, these findings strongly speak against such cells representing migrating neural precursors. Additionally, it is impossible to trace the NPCs as during further development both *achaete-scute* genes and *pros* start to be expressed ubiquitously throughout the entire ganglion, leaving the previously visible cells indiscernible. Regardless of gene expression, older studies on embryonic development report a thickening of the ventral neuroepithelium leading up to neuromere formation [46, 60], which was later explained to be the result of the initial migratory phase [9]. The ganglion anlagen formed during postembryonic development, however, feature no such thickening, with the neuroepithelial invagination forming the central cavity of the VOs usually denoting the onset of ganglion formation. It is important at this point to mention that this initial phase has previously been missed due to difficulties in identifying the cell internalisation sites.

Nonetheless, based on these findings there is no indication of an initial neurogenic phase featuring apico-basally immigrating cells that leads to a thickening of the ventral neuroepithelium during the postembryonic development of *P. litorale*. Instead the data point to the incipient formation of VOs with their NSCs which through division generate the cell material making up the growing ganglion.

SoxN, *achaete-scute* homologs and the putative *dpn* ortholog are expressed in the apical NSCs

The *SoxN* ortholog of *P. litorale* is first expressed broadly in the ventral neuroepithelium, prefiguring the regions of subsequent ganglion formation, which starts with the invagination of the neuroepithelium forming the central depression of the forming VOs. During this early neurogenic phase, *SoxN* is ubiquitously expressed across the early ganglion anlage (Fig. 16). This continues up to the detachment of the ganglion anlage from the neuroepithelium at which point expression starts to successively concentrate around the VOs, where it remains throughout anamorphic development (Fig. 16). This gene expression dynamic combined with the numerous observed divisions in the *SoxN*-positive apical cells of the VOs is in accordance with a role of *SoxB* family members in conferring neurogenic potential to ectodermal cells in pycnogonids, similar to other arthropods [11]. The continued expression of *SoxN* in the cells of VOs further indicates a role in NSC maintenance, possibly promoting self-renewal.

Both of the *achaete-scute* homologs show significant overlap with the expression domains of *SoxN*, generally also being expressed broadly in newly formed ganglia followed by concentration of the expression domains in the regions of the VOs (Fig. 17).

Despite the generally weak signal, the putative *dpn* ortholog still is generally confined to the VOs and the directly surrounding regions (Fig. 17). The presence of this (hexapod) NB-specific marker overlapping with the expression domains of the proneural genes *SoxN* and the *achaete-scute* homologs is in line with the previously described large NSCs lining the apical surface of the VOs in other pycnogonid representatives [9]. Further, it parallels the expression patterns observed in the NBs of pancrustaceans [22, 23, 29, 30].

Notably, however, both *SoxN* as well as *achaete-scute* family members are also expressed in the neuroepithelium of myriapods and euchelicerates [17], prefiguring the formation and immigration of typically post-mitotic neural precursors [10, 26]. The expression patterns and function of *dpn* during neurogenesis on the other hand has not been studied in these groups. Nonetheless, this means that the combined expression of these genes in the apical VOs of *P. littorale* does not qualify as an exclusive feature of arthropod neural stem cells.

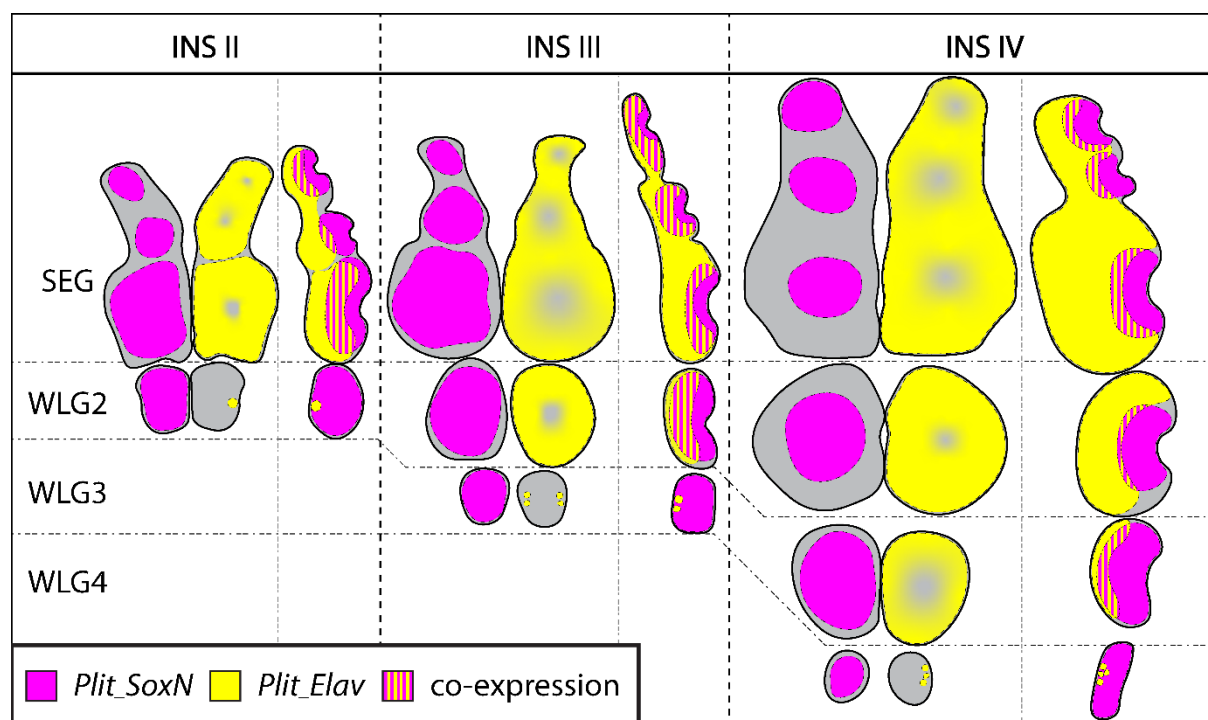


Figure 16. Schematic illustration of *Plit_SoxN* and *Plit_Elav* expression patterns. Schematic drawings of the VNC of instars II – IV in ventral (left) and lateral (right) views respectively. Anterior is oriented towards the top. In lateral views ventral is oriented towards the right. In ventral views the expression domains of *Plit_SoxN* are shown in magenta in the left half of the VNC, while *Plit_Elav* is shown in yellow on the right. In lateral views both expression patterns are depicted, with areas of co-expression being indicated by the hatched pattern.

The NSC divisions in *P. litorale* do not show a pronounced morphological asymmetry

The multitude of cell divisions observed in the apical cell layer during this study aligns with previous investigations [9, 53] and underscores the high mitotic activity of NSCs. However, a morphologically asymmetric mode of cell division as either explicitly described or implied in those studies could for the most part not be assessed to a satisfactory degree, in part due to persisting problems with the nuclear staining via DAPI, resulting in suboptimal signal of nuclei. Additionally, many of the observed divisions were captured during metaphase of the cell cycle, therefore rendering the morphological characterization of the division mode even more challenging. The handful of dividing cells observed in telophase, however, did not exhibit a pronounced morphological asymmetry. These findings, while not necessarily indicative of morphological asymmetry, do not exclude the possibility of molecular asymmetry, i.e. the asymmetrical segregation of genetic material and proteins into the resulting daughter cells during division. In line with this, previous studies have reported the absence of strong morphological asymmetry for NBs of the copepod *Tigriopus californicus* [61].

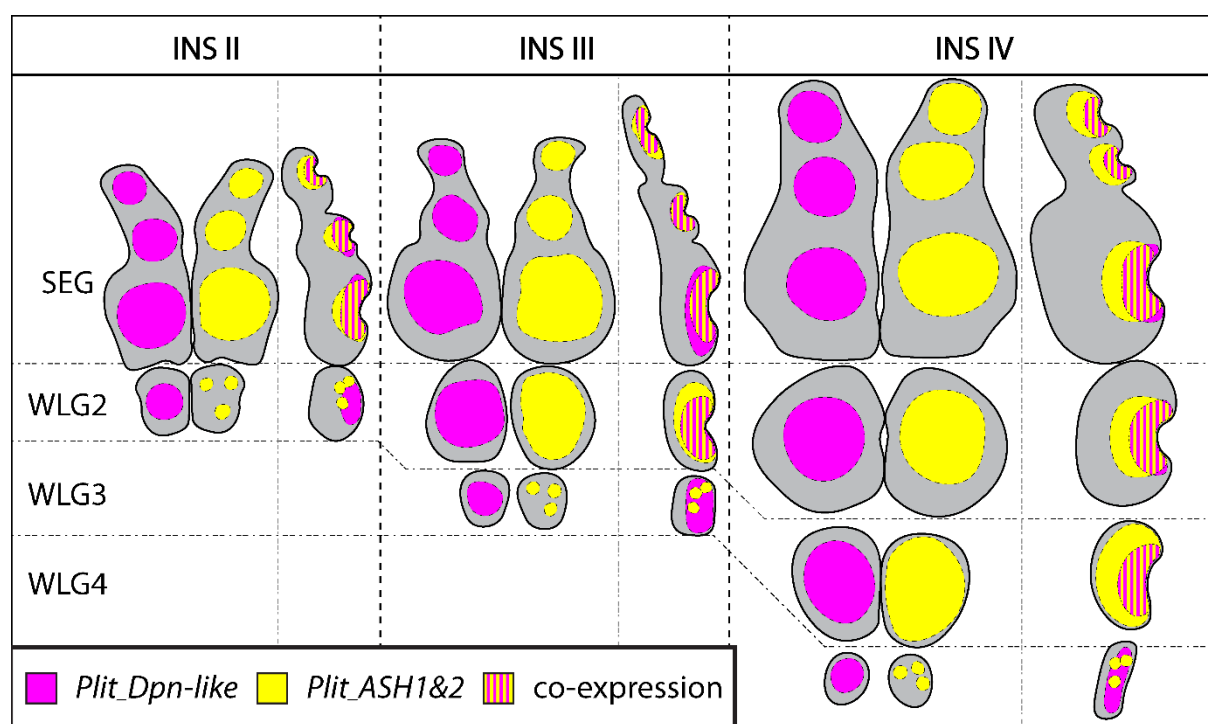


Figure 17. Schematic illustration of *Plit_Dpn-like* and *Plit_ASH1&2* expression patterns. Schematic drawings of the VNC of instars II – IV in ventral (left) and lateral (right) views respectively. Anterior is oriented towards the top. In lateral views ventral is oriented towards the right. In ventral views the expression domains of *Plit_Dpn-like* are shown in magenta in the left half of the VNC, while *Plit_ASH1&2* are shown in yellow on the right. In lateral views both expression patterns are depicted, with areas of co-expression being indicated by the hatched pattern.

What was recognisable, however, is the tangential orientation of dividing cells in the apical cell layer, as well as most other observed divisions. This is consistent with findings by Brenneis et al. [9], and contrasts an older study by Winter [59] who reported a radial mode of NSC division. As a consequence of this the resulting daughter cells remain initially part of the apical layer rather than being directly budded off into another cell layer. This initial apical position concurs with the previously described INPs that subsequently move into more basal layers where they divide symmetrically [9].

Pycnogonid INPs form a subapical layer expressing *pros* and *brat* reminiscent of pancrustacean GMCs

In hexapods, *pros* and *brat* act synergistically to drive the exit of NB daughter cells from the cell cycle and specify GMC fate [32]. In the growing ganglion anlagen of *P. litorale*, both genes show considerable regions of co-expression in selected cells of the apical VOs as well as in more dorsal/subapical cell layers (Fig. 18). If similar functions of these genes are assumed in pycnogonids, then the expression of *pros* and *brat* in the apical NSCs may act to suppress cell-cycle and self-renewal genes in their progeny, the INPs. Consequently, it would be expected that the gene products of *pros* and *brat* are asymmetrically segregated into the INP during NSC division. Since, however, the results of this study provide only insight into the regions of gene expression, but not the distribution of the encoded proteins these postulations cannot be tested. It would therefore be interesting in following studies to apply antibody-stainings against the *pros* and *brat* proteins, similar to previous studies done on NBs of *Drosophila* [29, 32]. In spiders on the other hand, the *pros* protein was previously shown to be present in all post-mitotic invaginating cells as they invaginate and remains so throughout their differentiation into neurons [36].

The expression of the two genes in the subapical layers on the other hand coincides with the location of previously described pycnogonid INPs, which is in accord with a role in the cell fate specification in the NSC progeny by activation of neural differentiation. The central concentration of *pros* and *brat* observed in further developed ganglia of later stages (Fig. 18), which extend from the subapical cell layers directly dorsal of the VOs towards the neuropil plausibly represent several layers of INPs as well as early stages of post-mitotic, differentiating neurons contributing to the increasing mass of the developing ganglion. The subsequent decrease in the expression domains of both genes coincides with the maturation of the ganglia, accompanied by a reduction of INPs with only few *pros*- and *brat*-positive cells remaining

dispersed throughout the ganglion. The presence of fewer neural precursor cells is concordant with the description of these ganglia in later stages only increasing proportionally in size [44]. Notably, *pros* is also expressed in the immigrating neural precursors of euchelicerates and myriapods [35, 36]. A corresponding interpretation of these expression patterns in pycnogonids would, apart from the previously discussed lack of an initial migratory phase, be poorly supported due to the condensation of expression domains in later developmental stages. The occurrence of divisions in *pros* and/or *brat* expressing cells, which were mainly observed in subapical layers, also indicates these representing INPs. Additionally, there is no evidence of these cells exhibiting an asymmetric mode of division, in line with such an interpretation.

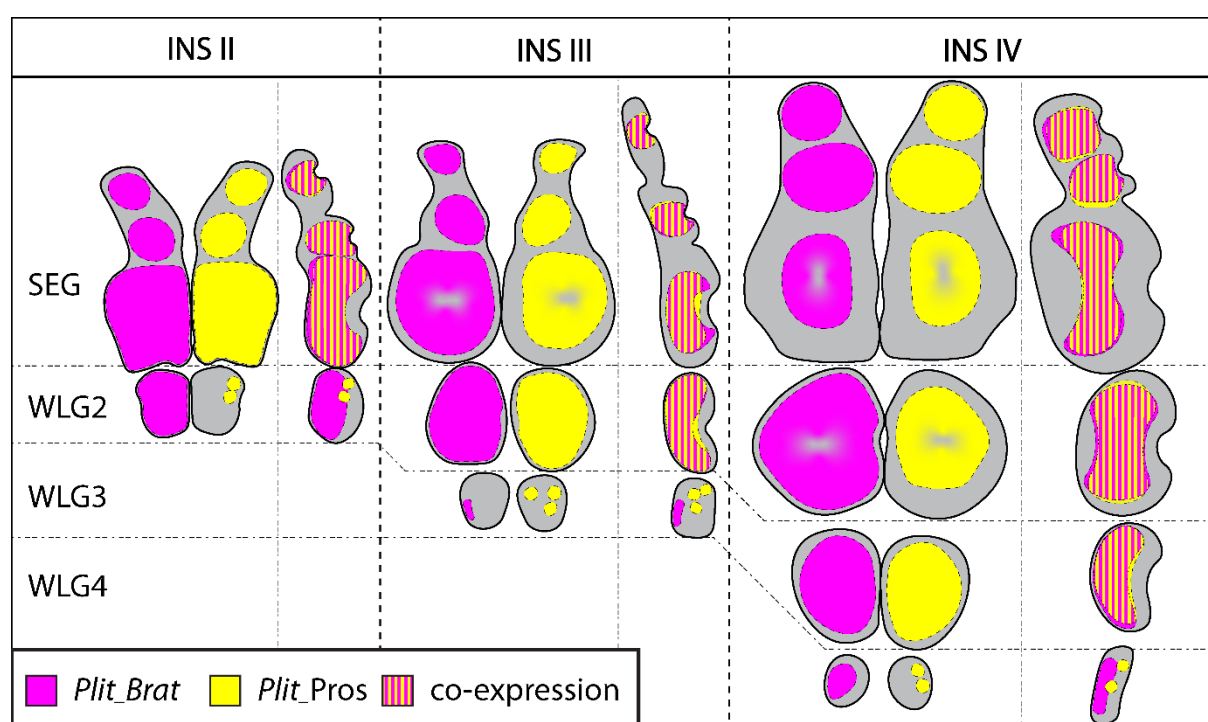


Figure 18. Schematic illustration of *Plit_Brat* and *Plit_Pros* expression patterns. Schematic drawings of the VNC of instars II – IV in ventral (left) and lateral (right) views respectively. Anterior is oriented towards the top. In lateral views ventral is oriented towards the right. In ventral views the expression domains of *Plit_Brat* are shown in magenta in the left half of the VNC, while *Plit_Pros* is shown in yellow on the right. In lateral views both expression patterns are depicted, with areas of co-expression being indicated by the hatched pattern.

Elav marks pycnogonid neurons from early differentiation to their maturation

Lastly, the neuron marker *elav* starts to be expressed at the dorsal margin of the developing ganglion, subsequently the expression domains expand further as the ganglion increases in size (Fig. 16). The presence of *elav*-positive cells at this early stages matches with previous descriptions of a basal layer of neurons and glial cells in the primordial ganglion anlage of pycnogonids during embryonic neurogenesis [9]. This successive expansion continues dorsally

until the gene is expressed ubiquitously throughout the ganglion apart from the apical cells of the VOs (Fig. 16), which is in line with expression of *elav* in both differentiating and mature neurons [36].

The partially overlapping expression domains of *elav* and *pros/brat* are again in agreement with previous investigations of hexapods, in which the latter two genes have been confirmed to be expressed transiently in immature neurons [29, 30, 32-34]. Conversely, the more dorsal regions, where co-expression is not observed, represent layers of more fully developed neurons. This is in line with the condensation of strongly *pros/brat*-positive domains in more advanced stages of ganglia, which is accompanied by a decrease of overlap with *elav*, as the previously formed neurons mature.

The virtual lack of cell divisions observed in *elav*-positive cells is another relevant feature indicating that the great majority of these cells represents post-mitotic neuronal cells.

The first neural precursors are specified in segmentally stereotypic positions in the neuroectoderm

A significant finding is the discovery of three cells that strongly express both *achaete-scute* homologs as well as *pros*, which emerge in a stereotypic spatio-temporal fashion in each hemiganglion primordium. At this early developmental stage of neurogenesis, all cells of the hemiganglion anlage strongly express *SoxN*, whereas the three individual cells themselves, however, do not show elevated expression. They are likely the first cells to adopt neural fate, possibly representing either NSCs or INPs. The emergence of these cells from *SoxN*-positive domains showing strong expression of the proneural *achaete-scute* genes as well as *pros* points to their NSC identity, as these genes are at least transiently expressed in the NB of hexapods [11, 33, 34]. Interestingly, similar co-expressions of *achaete-scute* homologs, *pros* and *brat*, among other genes, were previously described during early brain neurogenesis of the malacostracan *Procambarus virginalis* [24]. In this study, however, the authors concluded these cells to represent directly differentiating neural precursors, due to the self-renewal inhibiting function of *pros* and *brat* in combination with the occurrence of first axonal projections in the corresponding regions before the first NBs have fully emerged [24]. Similarly, the identification of the three described cells as NSCs is somewhat ambiguous, seeing as none of these exhibit expression of the proposed *dpn* ortholog, a feature characteristic of the hexapod NBs [29, 30]. Additionally, *Plit_dpn-like* is later expressed in the apical cells lining the VOs of *P. litorale*, further speaking against the NSC identity of these NPCs. Further, despite their proximity to the VOs, the location of these emerging cells do not aid in assigning them to a specific cell type.

Cell NPC1 is consistently found in the apical cell layer, in line with the position of NSCs, while NPC3 conversely is located in a subapical layer, which would rather be in concordance with INP identity. The expression of *pros* might also be indicative of these cells representing INPs, due to this gene being expressed both in hexapod NBs and GMCs [33, 34]. Such an interpretation is, however, conflicted by the expression of both *achaete-scute* homologs as well as the absence of *brat* expression characteristic for hexapod GMCs [29, 30, 32].

As previously discussed, the interpretation of these three NPCs as early immigrating post-mitotic cells is highly unfavourable, despite their expression patterns matching those of migrating neural precursors of Euehelicera and Myriapoda. The sequential emergence in combination with the fixed stereotypic pattern speak against such an interpretation. Further one would expect to be able to trace these cells as they migrate, which is incongruent with the results of this study. Lastly, on occasion, one of the three NPCs was observed dividing, strongly speaking against a strictly post-mitotic cell type. Though the division mode is not conclusively identifiable, these divisions are definitely oriented tangentially, so that the resulting daughter cells remain part of the same cell layer, rather than being delaminated into another layer. This was previously described as a characteristic feature of pycnogonid NSCs [9], which notably contrasts to the typically radial division of pancrustacean NBs.

With the currently available evidence the identity of these cells cannot be satisfactorily resolved. Additional data on the cell division behaviour of these cells, or even cell lineage studies might allow a more accurate interpretation to be reached.

Evolutionary inferences and future perspectives

This study provides first insights into the transcriptional profiles of neurogenic cell types driving postembryonic nervous system development in the pycnogonid *P. littoralis*. Beyond this, it reveals the previously unknown stereotypic emergence of the neural precursor cells in the primordial ganglion anlagen of pycnogonids.

The co-expression patterns of several of the candidate genes in the NSCs and INPs of pycnogonids roughly match those of NBs and GMCs of pancrustaceans. Despite this, several incongruences and uncertainties, such as i) the inability to conclusively detect asymmetric cell divisions, ii) the tangential orientation of apical divisions and iii) the identification of stereotypically emerging NPCs that could not conclusively be assigned to a cell type, remain.

To gain an even better understanding of these neurogenic cell, however, additional data are necessary. Firstly, antibody-stainings of the *pros* and *brat* proteins, similar to those on *Drosophila* NBs [29, 32] would provide crucial information on whether they are asymmetrically

segregated into only one daughter cell during division. Along with this the distribution of the scaffolding protein Miranda should be investigated in sea spiders, since in hexapods this protein directly interacts with Pros and Brat and is responsible for their asymmetric segregation [29, 32, 33].

Additionally, cell lineage studies might be applied to permit an unambiguous assignment of the identified NPCs. Combining such data with the gene expression patterns reported in this study will have the potential to conclusively resolve the different cell types underlying pycnogonid neurogenesis and enable a more detailed comparison with the pancrustacean NBs and GMCs and informed assessments of potential cell type homology.

References

1. Zhang, Z. (2011). Animal biodiversity: An introduction to higher-level classification and taxonomic richness. *Zootaxa*, 3148(1). <https://doi.org/10.11646/zootaxa.3148.1.3>
2. Giribet, G., Edgecombe, G.D. (2013). The Arthropoda: A Phylogenetic Framework. In: Minelli, A., Boxshall, G., Fusco, G. (eds) *Arthropod Biology and Evolution*. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-36160-9_2
3. Smarandache-Wellmann, C. R. (2016). Arthropod neurons and nervous system. *Current Biology*, 26(20), R960–R965. <https://doi.org/10.1016/j.cub.2016.07.063>
4. Kristan, W. B. (2016). Early evolution of neurons. *Current Biology*, 26(20), R949–R954. <https://doi.org/10.1016/j.cub.2016.05.030>
5. Boyan, G., Williams, J., & Herbert, Z. (2008). Fascicle switching generates a chiasmal neuroarchitecture in the embryonic central body of the grasshopper *Schistocerca gregaria*. *Arthropod Structure & Development*, 37(6), 539–544. <https://doi.org/10.1016/j.asd.2008.07.005>
6. Brenneis, G., & Richter, S. (2010). Architecture of the nervous system in mystacocarida (Arthropoda, crustacea)—An immunohistochemical study and 3D reconstruction. *Journal of Morphology*, 271(2), 169–189. <https://doi.org/10.1002/jmor.10789>
7. Richter, S., Loesel, R., Purschke, G., Schmidt-Rhaesa, A., Scholtz, G., Stach, T., Vogt, L., Wanninger, A., Brenneis, G., Döring, C., Faller, S., Fritsch, M., Grobe, P., Heuer, C. M., Kaul, S., Möller, O. S., Müller, C. H., Rieger, V., Rothe, B. H., Stegner, M. E.J., Harzsch, S. (2010). Invertebrate neurophylogeny: suggested terms and definitions for a neuroanatomical glossary. *Frontiers in Zoology*, 7(1), 29. <https://doi.org/10.1186/1742-9994-7-29>
8. Linne, V., & Stollewerk, A. (2011). Conserved and novel functions for Netrin in the formation of the axonal scaffold and glial sheath cells in spiders. *Developmental Biology*, 353(1), 134–146. <https://doi.org/10.1016/j.ydbio.2011.02.006>
9. Brenneis, G., Stollewerk, A., & Scholtz, G. (2013). Embryonic neurogenesis in *Pseudopallene* sp. (Arthropoda, Pycnogonida) includes two subsequent phases with similarities to different arthropod groups. *EvoDevo*, 4(1), 32. <https://doi.org/10.1186/2041-9139-4-32>
10. Stollewerk, A., & Simpson, P. (2005). Evolution of early development of the nervous system: a comparison between arthropods. *BioEssays*, 27(9), 874–883. <https://doi.org/10.1002/bies.20276>
11. Martín-Bermudo, M. D., Martínez, C., Rodríguez, A., & Jiménez, F. (1991). Distribution and function of the lethal of scute gene product during early neurogenesis in *Drosophila*. *Development*, 113(2), 445–454. <https://doi.org/10.1242/dev.113.2.445>
12. Ungerer, P., Eriksson, B. J., & Stollewerk, A. (2011). Neurogenesis in the water flea *Daphnia magna* (Crustacea, Branchiopoda) suggests different mechanisms of neuroblast formation in insects and crustaceans. *Developmental Biology*, 357(1), 42–52. <https://doi.org/10.1016/j.ydbio.2011.05.662>
13. Hein, H., & Scholtz, G. (2018). Larval neurogenesis in the copepod *Tigriopus californicus* (Tetraconata, Multicrustacea). *Development Genes and Evolution*, 228(2), 119–129. <https://doi.org/10.1007/s00427-018-0610-2>
14. Giribet, G., & Edgecombe, G. D. (2019). The phylogeny and evolutionary history of arthropods. *Current Biology*, 29(12), R592–R602. <https://doi.org/10.1016/j.cub.2019.04.057>
15. Ungerer, P., & Scholtz, G. (2007). Filling the gap between identified neuroblasts and neurons in crustaceans adds new support for Tetraconata. *Proceedings of the Royal Society B Biological Sciences*, 275(1633), 369–376. <https://doi.org/10.1098/rspb.2007.1391>
16. Dohle, W. (2001). Are the insects terrestrial crustaceans? A discussion of some new facts and arguments and the proposal of the proper name 'Tetraconata' for the monophyletic unit Crustacea + Hexapoda. *Annales de la Societe Entomologique de France*. 37. 85-103.
17. Stollewerk, A., Tautz, D., & Weller, M. (2003). Neurogenesis in the spider: new insights from comparative analysis of morphological processes and gene expression patterns. *Arthropod Structure & Development*, 32(1), 5–16. [https://doi.org/10.1016/s1467-8039\(03\)00041-0](https://doi.org/10.1016/s1467-8039(03)00041-0)
18. Stollewerk, A., Weller, M., & Tautz, D. (2001). Neurogenesis in the spider *Cupiennius salei*. *Development*, 128(14), 2673–2688. <https://doi.org/10.1242/dev.128.14.2673>
19. Kadner, D., & Stollewerk, A. (2004). Neurogenesis in the chilopod *Lithobius forficatus* suggests more similarities to chelicerates than to insects. *Development, Genes and Evolution*, 214(8). <https://doi.org/10.1007/s00427-004-0419-z>
20. Crémazy, F., Berta, P., & Girard, F. (2000). Sox Neuro, a new *Drosophila* Sox gene expressed in the developing central nervous system. *Mechanisms of Development*, 93(1–2), 215–219. [https://doi.org/10.1016/s0925-4773\(00\)00268-9](https://doi.org/10.1016/s0925-4773(00)00268-9)
21. Janssen, R., Andersson, E., Betnér, E., Bijl, S., Fowler, W., Höök, L., Leyhr, J., Mannelqvist, A., Panara, V., Smith, K., & Tiemann, S. (2018). Embryonic expression patterns and phylogenetic analysis of panarthropod sox genes: insight into nervous system development, segmentation and gonadogenesis. *BMC Evolutionary Biology*, 18(1). <https://doi.org/10.1186/s12862-018-1196-z>
22. Overton, P. M., Meadows, L. A., Urban, J., & Russell, S. (2002). Evidence for differential and redundant function of the Sox genes *Dichaete* and *SoxN* during CNS development in *Drosophila*. *Development*, 129(18), 4219–4228. <https://doi.org/10.1242/dev.129.18.4219>
23. Arefin, B., Parvin, F., Bahrapour, S., Stadler, C. B., & Thor, S. (2019). *Drosophila* neuroblast selection is gated by Notch, Snail, SoxB, and EMT gene Interplay. *Cell Reports*, 29(11), 3636–3651.e3. <https://doi.org/10.1016/j.celrep.2019.11.038>
24. Brenneis, G., Schwentner, M., Giribet, G., & Beltz, B. S. (2021). Insights into the genetic regulatory network underlying neurogenesis in the parthenogenetic marbled crayfish *Procambarus virginalis*. *Developmental Neurobiology*, 81(8), 939–974. <https://doi.org/10.1002/dneu.22852>

25. Paese, C. L. B., Leite, D. J., Schönauer, A., McGregor, A. P., & Russell, S. (2018). Duplication and expression of Sox genes in spiders. *BMC Evolutionary Biology*, 18(1). <https://doi.org/10.1186/s12862-018-1337-4>
26. Pioro, H. L., & Stollewerk, A. (2006). The expression pattern of genes involved in early neurogenesis suggests distinct and conserved functions in the diplopod *Glomeris marginata*. *Development Genes and Evolution*, 216(7–8), 417–430. <https://doi.org/10.1007/s00427-006-0078-3>
27. Bier, E., Vaessin, H., Younger-Shepherd, S., Jan, L. Y., & Jan, Y. N. (1992). deadpan, an essential pan-neural gene in *Drosophila*, encodes a helix-loop-helix protein similar to the hairy gene product. *Genes & Development*, 6(11), 2137–2151. <https://doi.org/10.1101/gad.6.11.2137>
28. Zhu, S., Wildonger, J., Barshow, S., Younger, S., Huang, Y., & Lee, T. (2012). The BHLH repressor deadpan regulates the self-renewal and specification of *drosophila* larval neural stem cells independently of notch. *PLoS ONE*, 7(10), e46724. <https://doi.org/10.1371/journal.pone.0046724>
29. Lee, C., Wilkinson, B. D., Siegrist, S. E., Wharton, R. P., & Doe, C. Q. (2006). Brat Is a Miranda Cargo Protein that Promotes Neuronal Differentiation and Inhibits Neuroblast Self-Renewal. *Developmental Cell*, 10(4), 441–449. <https://doi.org/10.1016/j.devcel.2006.01.017>
30. Reichardt, I., Bonnay, F., Steinmann, V., Loedige, I., Burkard, T. R., Meister, G., & Knoblich, J. A. (2017). The tumor suppressor Brat controls neuronal stem cell lineages by inhibiting Deadpan and Zelda. *EMBO Reports*, 19(1), 102–117. <https://doi.org/10.15252/embr.201744188>
31. Choksi, S. P., Southall, T. D., Bossing, T., Edoff, K., De Wit, E., Fischer, B. E., Van Steensel, B., Micklem, G., & Brand, A. H. (2006). Prospero Acts as a Binary Switch between Self-Renewal and Differentiation in *Drosophila* Neural Stem Cells. *Developmental Cell*, 11(6), 775–789. <https://doi.org/10.1016/j.devcel.2006.09.015>
32. Betschinger, J., Mechtler, K., & Knoblich, J. A. (2006). Asymmetric segregation of the tumor suppressor BRAT regulates Self-Renewal in *drosophila* neural stem cells. *Cell*, 124(6), 1241–1253. <https://doi.org/10.1016/j.cell.2006.01.038>
33. Knoblich, J. A., Jan, N., & Jan, Y. N. (1995). Asymmetric segregation of Numb and Prospero during cell division. *Nature*, 377(6550), 624–627. <https://doi.org/10.1038/377624a0>
34. Doe, C. Q., Chu-LaGriff, Q., Wright, D. M., & Scott, M. P. (1991). The prospero gene specifies cell fates in the *drosophila* central nervous system. *Cell*, 65(3), 451–464. [https://doi.org/10.1016/0092-8674\(91\)90463-9](https://doi.org/10.1016/0092-8674(91)90463-9)
35. Dove, H. L. (2003). Neurogenesis in the millipede *Glomeris marginata* (Myriapoda: Diplopoda). <https://kups.ub.uni-koeln.de/1045/>
36. Weller, M., & Tautz, D. (2003). Prospero and Snail expression during spider neurogenesis. *Development Genes and Evolution*, 213(11), 554–566. <https://doi.org/10.1007/s00427-003-0362-4>
37. Robinow, S., & White, K. (1991). Characterization and spatial distribution of the ELAV protein during *Drosophila melanogaster* development. *Journal of Neurobiology*, 22(5), 443–461. <https://doi.org/10.1002/neu.480220503>
38. Dunlop, J. A., & Arango, C. P. (2005). Pycnogonid affinities: a review. *Journal of Zoological Systematics & Evolutionary Research*, 43(1), 8–21. <https://doi.org/10.1111/j.1439-0469.2004.00284.x>
39. Regier, J. C., Shultz, J. W., Zwick, A., Hussey, A., Ball, B., Wetzer, R., Martin, J. W., & Cunningham, C. W. (2010). Arthropod relationships revealed by phylogenomic analysis of nuclear protein-coding sequences. *Nature*, 463(7284), 1079–1083. <https://doi.org/10.1038/nature08742>
40. Ballesteros, J. A., Santibáñez-López, C. E., Baker, C. M., Benavides, L. R., Cunha, T. J., Gainett, G., Ontano, A. Z., Setton, E. V. W., Arango, C. P., Gavish-Regev, E., Harvey, M. S., Wheeler, W. C., Hormiga, G., Giribet, G., & Sharma, P. P. (2022). Comprehensive species sampling and sophisticated algorithmic approaches refute the monophyly of Arachnida. *Molecular Biology and Evolution*, 39(2). <https://doi.org/10.1093/molbev/msac021>
41. Brenneis, G., Bogomolova, E. V., Arango, C. P., & Krapp, F. (2017). From egg to “no-body”: an overview and revision of developmental pathways in the ancient arthropod lineage Pycnogonida. *Frontiers in Zoology*, 14(1). <https://doi.org/10.1186/s12983-017-0192-2>
42. Minelli A, Fusco G. Arthropod Post-embryonic Development. In: Minelli A, Boxshall G, Fusco G, editors. *Arthropod Biology and Evolution Molecules, Development, Morphology*. Heidelberg: Springer Verlag; 2013. p. 91–122
43. Bain, B. A. (2003). Larval types and a summary of postembryonic development within the pycnogonids. *Invertebrate Reproduction & Development*, 43(3), 193–222. <https://doi.org/10.1080/07924259.2003.9652540>
44. Alexeeva, N., & Tamberg, Y. (2020). Anatomical changes in postembryonic development of *Pycnogonum litorale*. *Journal of Morphology*, 282(3), 329–354. <https://doi.org/10.1002/jmor.21308>
45. Brenneis, G. (2015). Pycnogonida (Pantopoda). In A. Schmidt-Rhaesa, S. Harzsch, & G. Purschke, *Structure and Evolution of Invertebrate Nervous Systems* (1st ed., pp. 419–427). Oxford University Press, Oxford. <https://doi.org/10.1093/acprof:oso/9780199682201.003.0035>
46. Morgan, T. H. (1891). Contribution to the embryology and phylogeny of the pycnogonids. <https://doi.org/10.5962/bhl.title.8208>
47. Tomaschko, K., Wilhelm, E., & Bückmann, D. (1997). Growth and reproduction of *Pycnogonum litorale* (Pycnogonida) under laboratory conditions. *Marine Biology*, 129(4), 595–600. <https://doi.org/10.1007/s002270050201>
48. Vilpoux, K., & Waloszek, D. (2003). Larval development and morphogenesis of the sea spider *Pycnogonum litorale* (Ström, 1762) and the tagmosis of the body of Pantopoda. *Arthropod Structure & Development*, 32(4), 349–383. <https://doi.org/10.1016/j.asd.2003.09.004>
49. Brenneis, G., Frankowski, K., Maaß, L., & Scholtz, G. (2023). The sea spider *Pycnogonum litorale* overturns the paradigm of the absence of axial regeneration in molting animals. *Proceedings of the National Academy of Sciences*, 120(5). <https://doi.org/10.1073/pnas.2217272120>
50. Klementz, B. C., Brenneis, G., Hinne, I. A., Laumer, E. M., Neu, S. M., Hareid, G. M., Gainett, G., Setton, E. V. W., Simian, C., Vrech, D. E., Joyce, I., Barnett, A. A., Patel, N. H., Harvey, M. S., Peretti, A. V., Gulia-Nuss, M., &

- Sharma, P. P. (2024). A novel expression domain of extradenticle underlies the evolutionary developmental origin of the chelicerate patella. *Molecular Biology and Evolution*, 41(9). <https://doi.org/10.1093/molbev/msae188>
51. Klementz, B. C., Brenneis, G., Laumer, E. M., Neu, S. M., Harvey, M. S., & Sharma, P. P. (2025). Evolution and homology of leg segments in Chelicerata: Evo-devo solutions to century-old challenges. *Arthropod Structure & Development*, 87, 101446. <https://doi.org/10.1016/j.asd.2025.101446>
 52. Brenneis, G. & Scholtz, G. (2014). The ‘Ventral Organs’ of Pycnogonida (Arthropoda) Are Neurogenic Niches of Late Embryonic and Post-Embryonic Nervous System Development. *PLoS ONE*, 9(4), e95435. <https://doi.org/10.1371/journal.pone.0095435>
 53. Brenneis, G., Scholtz, G. & Beltz, B. S. (2018). Comparison of ventral organ development across Pycnogonida (Arthropoda, Chelicerata) provides evidence for a plesiomorphic mode of late neurogenesis in sea spiders and myriapods. *BMC Evolutionary Biology*, 18(1). <https://doi.org/10.1186/s12862-018-1150-0>
 54. Kuehn, E., Clausen, D. S., Null, R. W., Metzger, B. M., Willis, A. D. & Özpolat, B. D. (2021). Segment number threshold determines juvenile onset of germline cluster expansion in *Platynereis dumerilii*. *Journal Of Experimental Zoology Part B Molecular And Developmental Evolution*, 338(4), 225–240. <https://doi.org/10.1002/jez.b.23100>
 55. Elagoz, A. M., Styfhals, R., Maccuro, S., Masin, L., Moons, L. & Seuntjens, E. (2022). Optimization of Whole Mount RNA Multiplexed in situ Hybridization Chain Reaction With Immunohistochemistry, Clearing and Imaging to Visualize Octopus Embryonic Neurogenesis. *Frontiers in Physiology*, 13. <https://doi.org/10.3389/fphys.2022.882413>
 56. Choi, H. M. T., Schwarzkopf, M., Fornace, M. E., Acharya, A., Artavanis, G., Stegmaier, J., Cunha, A., & Pierce, N. A. (2018). Third-generation in situ hybridization chain reaction: multiplexed, quantitative, sensitive, versatile, robust. *Development*, 145(12). <https://doi.org/10.1242/dev.165753>
 57. Bruce, H. S., Jerz, G., Kelly, S. R., McCarthy, J., Pomerantz, A., Senevirathne, G., Sherrard, A., Sun, D. A., Wolff, C., & Patel, N. H. (2021). Hybridization Chain Reaction (HCR) In Situ Protocol v1. <https://doi.org/10.17504/protocols.io.bunznvff6>
 58. Schwarzkopf, M., Liu, M. C., Schulte, S. J., Ives, R., Husain, N., Choi, H. M. T., & Pierce, N. A. (2021). Hybridization chain reaction enables a unified approach to multiplexed, quantitative, high-resolution immunohistochemistry and in situ hybridization. *Development*, 148(22). <https://doi.org/10.1242/dev.199847>
 59. Winter, V. G. (1980). Beiträge zur Morphologie und Embryologie des vorderen Körperabschnitts (Cephalosoma) der Pantopoda Gerstaecker, 1863: Entstehung und Struktur des Zentralnervensystems. *Journal Of Zoological Systematics & Evolutionary Research*, 18(1), 27–61. <https://doi.org/10.1111/j.1439-0469.1980.tb00726.x>
 60. Dogiel, V. (1913). Embryologische Studien an Pantopoden. *Zeitschrift für wissenschaftliche Zoologie.*, Bd, 107.
 61. Hein, H., & Scholtz, G. (2018). Larval neurogenesis in the copepod *Tigriopus californicus* (Tetraconata, Multicrustacea). *Development Genes and Evolution*, 228(2), 119–129. <https://doi.org/10.1007/s00427-018-0610-2>

Appendices

1: List of arthropod species whose published amino acid sequences were used as baits to identify the genes of interest as well as the respective accession numbers

	<i>Achaete-scute</i> homologs	<i>Brain tumor</i>	<i>Deadpan</i>	<i>Embryonic</i> <i>lethal abnormal</i> <i>vision</i>	<i>Prospero</i>	<i>SoxNeuro</i>
Chelicerata						
<i>Centruroides vittatus</i>	XP_067129253.1					
<i>Cupiennius salei</i>				JAC59325.1	CAE00181.1	
<i>Dermacentor albipictus</i>	XP_065299889.1					
<i>Dermacentor silvarum</i>	XP_037580238.1				XP_049526462.1	
<i>Galendromus occidentalis</i>				XP_028966459.1		
<i>Hadrurus spadix</i>					JAV47934.1	
<i>Ixodes scapularis</i>					XP_040064731.1	
<i>Ornithodoros turicata</i>	XP_064489817.1					
<i>Parasteatoda tepidariorum</i>	XP_015909772.1			LAA01731.1	XP_015918934.1	
<i>Rhipicephalus sanguineus</i>	XP_037525474.1					
Myriapoda						
<i>Glomeris marginata</i>	CAD60437.1					ABF21053.1
"Crustacea"						
<i>Chionoecetes opilio</i>				KAG0720924.1		
<i>Penaeus vannamei</i>						ROT84828.1
<i>Portunus trituberculatus</i>						UZP64753.1

<i>Procambarus clarkii</i>			XP_045618065.1			
<i>Procambarus virginalis</i>				UBR90702.1		UBR90693.1
Hexapoda						
<i>Anopheles darlingi</i>				ETN58395.1		
<i>Bemisia tabaci</i>			QAB02854.1			
<i>Bombyx mori</i>				ABM21482.2		
<i>Carabus blaptoides fortunei</i>						GLV39099.1
<i>Drosophila melanogaster</i>	NP_476824.1 NP_476803.1 NP_476694.1	AAF13929.1	NP_476923.1		BAA01464.1	CAB64386.1
<i>Drosophila tropicalis</i>				XP_068159935.1		
<i>Drosophila virilis</i>				EDW63036.2		
<i>Frankliniella occidentalis</i>			KAE8738467.1			
<i>Lasius niger</i>		KMQ95443.1				
<i>Leptinotarsa decemlineata</i>			AKG92780.1			
<i>Neodiprion pinetum</i>	XP_046489719.1					
<i>Pararge aegeria</i>				JAA80154.1		
<i>Periplaneta americana</i>		XP_069679402.1				
<i>Tribolium castaneum</i>	NP_001034537.1	XP_064215157.1	XP_967694.1		EFA07555.2	EFA04654.1
<i>Zophobas morio</i>	XP_063922435.1					

2: Probe sequences designed via Easy-HCR in situ probe generator (modified)

<i>Plit_ASH1_B3</i>				
Pair#	Initiator	Probe	Probe	Initiator
1	GTCCCTGCCTCTATATCT	CAATTGTAGCTTCGAT CAGACCGGA	GTCGTCTTGAGCACTGG AACTACGT	CCACTCAACTTTAACCCG
2	GTCCCTGCCTCTATATCT	CCACTTGTCTTGTCGT GGGCCAATA	GAGGAGTCCATCTGTTG TCAACAAC	CCACTCAACTTTAACCCG
3	GTCCCTGCCTCTATATCT	GTAAGTAGTTTCACGA GAACCATAAC	AAATTTATAGCGTCCTAA TTTCGAT	CCACTCAACTTTAACCCG
4	GTCCCTGCCTCTATATCT	GCAGATATTGTCGTAA CTCGAAGCA	GAAGTCCATAAGATCTT GTCCATTC	CCACTCAACTTTAACCCG
5	GTCCCTGCCTCTATATCT	GCAACGGAAGACGAT GGACATAGAG	GAAACGTAAGTTGGCGT CGGAGATC	CCACTCAACTTTAACCCG
6	GTCCCTGCCTCTATATCT	GATGACATGTCGTCGT CGTATTGTA	ATAGAGTGATATAATCGG GCTTGTT	CCACTCAACTTTAACCCG
7	GTCCCTGCCTCTATATCT	GATGTAGGAGACGGG CGAAGGCATG	AGCGTGACGATGGAAG CATTTAGA	CCACTCAACTTTAACCCG
8	GTCCCTGCCTCTATATCT	TTGGCTGGAGATCCAG CAGGAAGAC	GATTCCGGAGCGCTATA ATCACAAC	CCACTCAACTTTAACCCG
9	GTCCCTGCCTCTATATCT	AGAATTGGTATTCCGG TGAGGAATC	CCGATGCGGGAGATCCG CAAGATTC	CCACTCAACTTTAACCCG
10	GTCCCTGCCTCTATATCT	CGAGTCCAATACAGTG TGCAGATGC	TTCGGAATCGAAACTAC TGCTGCTA	CCACTCAACTTTAACCCG
11	GTCCCTGCCTCTATATCT	CCAACGACGTTATCTT CCTCTTGCA	TTTTCGTAGTCGGTTTCT GACCGTT	CCACTCAACTTTAACCCG
12	GTCCCTGCCTCTATATCT	ATTATTCGGAGAGGGT GGCGAATTT	ATTCGTATTCTCCTGTT GGCCATC	CCACTCAACTTTAACCCG
13	GTCCCTGCCTCTATATCT	TCGGTCGGTGAGTTAC TTGATTGCA	TTCATGACGACGAGCAG CGCAGACG	CCACTCAACTTTAACCCG
14	GTCCCTGCCTCTATATCT	ACTGACTGGTACGCG GTGAGGTTAT	TCACGATGTTGGGTCGA CTTCACTA	CCACTCAACTTTAACCCG
<i>Plit_ASH2_B4</i>				
Pair#	Initiator	Probe	Probe	Initiator
1	CCTCAACCTACCTCCAAC	GTGATCGAATAAACGA GGTCGTCGG	TTGCTCGTTTCTTTCAAT TTTCGAT	TCTCACCATATTCGCTTC
2	CCTCAACCTACCTCCAAC	CAGCATTCAATTATAATA ATAATTCA	GAAACCCGTCCTTCCAT CATCCTTA	TCTCACCATATTCGCTTC
3	CCTCAACCTACCTCCAAC	TATCACATTATATTGTA CCGTAAAA	TATTAATTATAACAATGA TGACTAG	TCTCACCATATTCGCTTC

4	CCTCAACCTACCTCCAAC	CGTAAAGGTCCCGTTC GTTTTGTTG	CGGATCACATCAACCAT GCGCTAAG	TCTCACCATATTCGCTTC
5	CCTCAACCTACCTCCAAC	GTTATTCGATCCGAGA CTCGGCGAC	CATTGGACGTTTCAGAG AGACGTCT	TCTCACCATATTCGCTTC
6	CCTCAACCTACCTCCAAC	GTCACGTGATCGGCGC CATTTTGT	GAACTGGACATTGAATA CGTCGGAC	TCTCACCATATTCGCTTC
7	CCTCAACCTACCTCCAAC	GGTAATAGTTATTGTA GATGGGACT	TATAATTTGTAATTTGTT GGTCGTA	TCTCACCATATTCGCTTC
8	CCTCAACCTACCTCCAAC	AGATCCGCACGATCCT GGCGAACGG	TTGTTCCGGCGGCGGTCT CGTACTCG	TCTCACCATATTCGCTTC
9	CCTCAACCTACCTCCAAC	ATGATGTCCGACTTCC GGTTCTCTT	CCGCACATTCCGGTTC GTCCTTGA	TCTCACCATATTCGCTTC
10	CCTCAACCTACCTCCAAC	TGGCCGAATACGTCTT CTTCTTGCC	TTTTCGATGCGGGCCGC GGATGATT	TCTCACCATATTCGCTTC
11	CCTCAACCTACCTCCAAC	ACACTTCAGTCCGTAA TAGACACCT	CTGATCTTGTGTCGCG TCTCCACC	TCTCACCATATTCGCTTC
12	CCTCAACCTACCTCCAAC	ATCATGACCGACGAG ATGACTTCCA	TCCTGAACACCTGAGTC TTGTGACG	TCTCACCATATTCGCTTC

Plit_Brat_B1

Pair#	Initiator	Probe	Probe	Initiator
1	GAGGAGGGCAGCAAACGG	ATCCTTACTGGCAAGG ACGACGGAA	GGTGTAACGGTAGATGT AAAGTCTA	GAAGAGTCTTCCTTTACG
2	GAGGAGGGCAGCAAACGG	TTCACCTTACTCTCGA GCGCGTTCA	AGCGATACATCGAAACA TTGGGCGT	GAAGAGTCTTCCTTTACG
3	GAGGAGGGCAGCAAACGG	AGTTGAAATTGTTGTG GTTGTGCGC	ACGCTCCGTCTGAGCA AATATCGT	GAAGAGTCTTCCTTTACG
4	GAGGAGGGCAGCAAACGG	AGTGGGCGCGGTTATC GGAAATGAA	TGCCTTCGTAGTTGAAG ACCTTGAC	GAAGAGTCTTCCTTTACG
5	GAGGAGGGCAGCAAACGG	AACTTCTGCAGGACG TTGCCAATCT	GGGAACTCGAGGTGTTT AGAGCAAC	GAAGAGTCTTCCTTTACG
6	GAGGAGGGCAGCAAACGG	ATTCCACGACGATGAT CCTACCCTT	CGAAGATGATTACTCTC ATCACCTT	GAAGAGTCTTCCTTTACG
7	GAGGAGGGCAGCAAACGG	AACGAACTGGCCGTA CTGATTGTAG	TTGGAGGACGTTGGCGC CGAATTTA	GAAGAGTCTTCCTTTACG
8	GAGGAGGGCAGCAAACGG	TGACGACGATGTCGCC AGATGTCTT	GAATCTGGTGTGTCGGA CTACGTTT	GAAGAGTCTTCCTTTACG
9	GAGGAGGGCAGCAAACGG	CACACTCGCCGAACT GGAACCTGAA	GATACAACAACTGGCTG TCACGCTT	GAAGAGTCTTCCTTTACG
10	GAGGAGGGCAGCAAACGG	ACGCCGAACTCGCCA AACTTACAGT	CTCGGTTTCGGTAAACTG ACCTTCCA	GAAGAGTCTTCCTTTACG

11	GAGGAGGGCAGCAAACGG	ACTTTGGAGGAAACA TGGAAGTCGA	AGATCATCTTGTGGCGC TTTATCTG	GAAGAGTCTTCCTTTACG
12	GAGGAGGGCAGCAAACGG	TAGAGGCCGTAGAATC GTTGGGCAA	TGAGTTTCGATGACAGA TCTATCAA	GAAGAGTCTTCCTTTACG
13	GAGGAGGGCAGCAAACGG	AGATGCTGCCACCGTT GGACCATT	CGCAACCATTCTGGAAG ATGTCCGT	GAAGAGTCTTCCTTTACG
14	GAGGAGGGCAGCAAACGG	TTGGCGTAAACGGAC CGAGACAGTT	ACGGATTGAGATCGTTG ATGGTTGT	GAAGAGTCTTCCTTTACG
15	GAGGAGGGCAGCAAACGG	TGATTTCGCTGAAGACG TGTCCATTA	CGAACTGAAACGTTTAG TCAGAATG	GAAGAGTCTTCCTTTACG
16	GAGGAGGGCAGCAAACGG	GTTCACGCCATTCTGA TGACGGATA	TTTAAACGGCCCGTTTCG ACATCTCG	GAAGAGTCTTCCTTTACG
17	GAGGAGGGCAGCAAACGG	ATCGCCTGATAGTTTG ATACGAATT	CCGAATGTGTTCTTAC GCCAACCT	GAAGAGTCTTCCTTTACG
18	GAGGAGGGCAGCAAACGG	TAATTCAGCAACGTCA TAAACCGAT	TCCATCGTCTGATTGGG TTCCGGAT	GAAGAGTCTTCCTTTACG
19	GAGGAGGGCAGCAAACGG	TTAGACGTTTCGACAA AGTTACAAGT	AAACCTCGACGTTGCTC GAATGTTT	GAAGAGTCTTCCTTTACG
20	GAGGAGGGCAGCAAACGG	GTTAAGCATCGATAAA GACATTAGC	TTTGTGATCGCCTCCT GTACCTTC	GAAGAGTCTTCCTTTACG
21	GAGGAGGGCAGCAAACGG	AAAGTCTCGTGCTTCC GTTCTTCCA	GCATTGAAGATGCTCTC CAACTCTT	GAAGAGTCTTCCTTTACG
22	GAGGAGGGCAGCAAACGG	AGACTTGTGGTACTGG GTACGTAAC	CTGGAAAGTTTCGTTAA TATCGTTC	GAAGAGTCTTCCTTTACG
23	GAGGAGGGCAGCAAACGG	TTTCTGAGATCAGATG CCTTCATCT	GTCGTGTGCTCGACTGT CTTGTAAG	GAAGAGTCTTCCTTTACG
24	GAGGAGGGCAGCAAACGG	CAACTTGTTTTGTGACC AATGTCGTT	TTTCGGACATCTGCTGA GTGATTAC	GAAGAGTCTTCCTTTACG
25	GAGGAGGGCAGCAAACGG	GTGATCCATCACCGTA CAATCTTTA	GTAATCGAAGTCGTGTA AACCTTTC	GAAGAGTCTTCCTTTACG
26	GAGGAGGGCAGCAAACGG	GACCAATTTCAAATCT TCTCTGTTG	CTTATGTTTAGTGCAGTA GATAGGC	GAAGAGTCTTCCTTTACG
27	GAGGAGGGCAGCAAACGG	CGATGACCTTCGAAGC AATGCATGT	TGTTGAAGCTCGCCAA CGTCATAA	GAAGAGTCTTCCTTTACG
28	GAGGAGGGCAGCAAACGG	GTGTGTAAGGTCGAAC GACGAGGAT	TTTACATCCAGTACAGC TCAACGAT	GAAGAGTCTTCCTTTACG
29	GAGGAGGGCAGCAAACGG	GTAAGCAGCCAATACC TTGTAACGT	AAATATTACTGATAGCGC AGTCGGA	GAAGAGTCTTCCTTTACG
30	GAGGAGGGCAGCAAACGG	TATCTTATCTGCATTAG ACTCGTTC	CGTTTCGTAGCTACATC GAGGACAT	GAAGAGTCTTCCTTTACG
31	GAGGAGGGCAGCAAACGG	ACATAATAATACTTTG GGTAGGGTG	CAGGCATGGTTGACAAT AGGTATGT	GAAGAGTCTTCCTTTACG

32	GAGGAGGGCAGCAAACGG	GCCAAGTTAACTCTT CATTATCGC	GTGTCTTGACATATTCCG CATTAG	GAAGAGTCTTCCTTTACG
33	GAGGAGGGCAGCAAACGG	ATCGCCCAATAACGAA CCTAAAGAA	ATTACCTGTACACATACC AATATCC	GAAGAGTCTTCCTTTACG
<i>Plit_Dpn-like_B4</i>				
Pair#	Initiator	Probe	Probe	Initiator
1	CCTCAACCTACCTCCAAC	TGTCTGACGACTCGCG ACCTTGCGA	AGTCTTCGTCCGAAC TA TCTGCATC	TCTCACCATATTCGCTTC
2	CCTCAACCTACCTCCAAC	GGCTGCGCTGGTCGA ACAATTACCA	CCAATGGTTAGTGTCCG GCGGAGAT	TCTCACCATATTCGCTTC
3	CCTCAACCTACCTCCAAC	TCGATGTAGGCCAGAT GATTGGAT	TTGGTCGTTTCCGAATG GTAAAATT	TCTCACCATATTCGCTTC
4	CCTCAACCTACCTCCAAC	GTCGCGTATTCGACGG AAACTTGAA	CGTGTTTGGCGACGGGC TTCCGAAT	TCTCACCATATTCGCTTC
5	CCTCAACCTACCTCCAAC	GAGTTGGATTTATTCG GACTGAATT	GTCGTAGTCGTCTCGG CGACTGAT	TCTCACCATATTCGCTTC
6	CCTCAACCTACCTCCAAC	CGACCGGACTGCGAA AAGAAATGGA	TGTCGAAGAAACCGGA CGCGTTCGG	TCTCACCATATTCGCTTC
7	CCTCAACCTACCTCCAAC	CGCTCGTAAAGGCGG AGAAGATAAA	CGATGACGACGAACAA CCGAAACCG	TCTCACCATATTCGCTTC
8	CCTCAACCTACCTCCAAC	CGAAACTGTCCGAG ATCGGACGGA	CCGTGGATAACGTGAAC GGCGGTGA	TCTCACCATATTCGCTTC
9	CCTCAACCTACCTCCAAC	CAGATCTCCTCCTCCG GCTATTCGT	CGTCGGACTAGTCGAAG AAGCGAAC	TCTCACCATATTCGCTTC
10	CCTCAACCTACCTCCAAC	TTTGAAAATACGAA GACACGTCGT	GACGACCGCGTAATAT TTCGGGTC	TCTCACCATATTCGCTTC
11	CCTCAACCTACCTCCAAC	CCATGCCGAACATTTT GCCCCTTC	CATTCGACAGTCTACCC TGCAACAG	TCTCACCATATTCGCTTC
12	CCTCAACCTACCTCCAAC	GCCATCGAGACTGGC AGCTATTTGC	AGCCTGACTATCGGAAA TGTTGTCTG	TCTCACCATATTCGCTTC
13	CCTCAACCTACCTCCAAC	CTGGCCAATGTCGGTA AGAACGACG	GCAAACGGATGGAATTG CGGAACGA	TCTCACCATATTCGCTTC
14	CCTCAACCTACCTCCAAC	AGTTCATGCTCGTCAT GCAAATGTT	AAGACGACAACGTGGG CGTCGGTAT	TCTCACCATATTCGCTTC
15	CCTCAACCTACCTCCAAC	CCGTCCATTCCGTCCA TTCTGGCGA	TAGTTGAGAAGACGTTG CTTCAGTC	TCTCACCATATTCGCTTC
16	CCTCAACCTACCTCCAAC	TCCACACGGCCGGGT CACTCGATAT	TTTTGCGAGAAGCCTGAC CTAAACTT	TCTCACCATATTCGCTTC
17	CCTCAACCTACCTCCAAC	GTGCACGCAAATGAAT GCGTAATAA	TTTTGCGAGGGCATCTT ATATTCGA	TCTCACCATATTCGCTTC

18	CCTCAACCTACCTCCAAC	TCACGGCTGTGAACG GTCTCGTGAG	TTTATAGTCGCGGTGTTG TTGGATGA	TCTCACCATATTCGCTTC
19	CCTCAACCTACCTCCAAC	AGCTACCGATGACAC GGATGACTTA	TTTAAAGGTACAACACA CGTTTCGA	TCTCACCATATTCGCTTC
<i>Plit_Pros_B5</i>				
Pair#	Initiator	Probe	Probe	Initiator
1	CTCACTCCCAATCTCTAT	TTTGAAGTAGTCCGGA ACGTTATCG	TTCCAGTTGTTCCAAGA AATTCGGA	CTACCCCTACAAATCCAAT
2	CTCACTCCCAATCTCTAT	GACTGTTTCGATGACGT ATCGGAAGT	ATTATCGAGCGGAAGAA CTCGCGCA	CTACCCCTACAAATCCAAT
3	CTCACTCCCAATCTCTAT	CATTTGTATGTAATAG AACTCTCGG	GCTGAGAGCCTGTCTTG CGTACTTT	CTACCCCTACAAATCCAAT
4	CTCACTCCCAATCTCTAT	CGACGAGTAGCCAAA GTAAAGGTCA	TTTCCGGAGATGCATCG GAGTCAGA	CTACCCCTACAAATCCAAT
5	CTCACTCCCAATCTCTAT	TTTCTCCTTTGGCGTT ATGGGATTG	TGTGGCACTCACTGAAA TCGTCTTT	CTACCCCTACAAATCCAAT
6	CTCACTCCCAATCTCTAT	GGATGATAGGGCGAG AGTCCGATT	TCTTCTGTCTTCACG GTTGGCTG	CTACCCCTACAAATCCAAT
7	CTCACTCCCAATCTCTAT	TCTCGTGTCTGGTCACT TTGGTTCGT	GAGATTACGGATCGTTC TCGACGAC	CTACCCCTACAAATCCAAT
8	CTCACTCCCAATCTCTAT	TTGGCAAACCGCCTCC GTACATGAT	GTAAATTCGGCGACGGA TGACTCGA	CTACCCCTACAAATCCAAT
9	CTCACTCCCAATCTCTAT	CGGAAACGGTTTGATC GGAGTCGAT	CATCTTGGAACCGATG GAAAGATG	CTACCCCTACAAATCCAAT
10	CTCACTCCCAATCTCTAT	TTCAACATCGAACCGA CCGTTTCGC	TCAGTGAACGGACTGA CCCGATGAT	CTACCCCTACAAATCCAAT
11	CTCACTCCCAATCTCTAT	TCATCACATTATTGGAT GGAGTCGT	ACGACGACGACAACGA CAACGAAGA	CTACCCCTACAAATCCAAT
12	CTCACTCCCAATCTCTAT	AACGCGGCCACCAGA TCTCTATTGA	ACCTTTGTTCTGGGCGA TTTCCGGT	CTACCCCTACAAATCCAAT
13	CTCACTCCCAATCTCTAT	GCGTCTGAAATAACG ACCTGGTGTT	GATTATTGGCATCCATGA GTTTTGG	CTACCCCTACAAATCCAAT
14	CTCACTCCCAATCTCTAT	ACGATCCTCGGCAAC GAAGTCGAAA	GTCACGTAATTGGCCAC GATATTGT	CTACCCCTACAAATCCAAT
15	CTCACTCCCAATCTCTAT	CAGCCTGTGGCAATCC GAAAGGAAT	CATTGGTCAAAAGGGCG GCTGCATT	CTACCCCTACAAATCCAAT
16	CTCACTCCCAATCTCTAT	ACTGGCCACGCCCAAT TCACTTTCT	ATTACGTTTCGATTTTCGG ACGATTC	CTACCCCTACAAATCCAAT
17	CTCACTCCCAATCTCTAT	GCTTCGTCGATAAACA ACGACGGGT	TCTTGTGCTCGAATAC AAGACGAC	CTACCCCTACAAATCCAAT

18	CTCACTCCCAATCTCTAT	TCTCTGATGGACATTA CCATTAGGC	TTTTGGTTGGCGTTGGT GGTACTGA	CTACCCTACAAATCCAAT
19	CTCACTCCCAATCTCTAT	ATTCATCTTCTCCTTC TCTTCTTC	TAAGAACACTTACGTTT TGTTGTCG	CTACCCTACAAATCCAAT
20	CTCACTCCCAATCTCTAT	GTTGTTGCTGCTGATC GTCCCTCTT	CTTACTGGTATCGCTGA CGTTACCA	CTACCCTACAAATCCAAT
21	CTCACTCCCAATCTCTAT	TGTTGTACTTTTCCTG CATGACCGA	CATCGTTCGCTCCGTTG GTCAACAA	CTACCCTACAAATCCAAT
22	CTCACTCCCAATCTCTAT	CTTAAGACTTTCTTA TCCGTCTTC	CTGTTCTCGCATCCACT TCAACTGT	CTACCCTACAAATCCAAT
23	CTCACTCCCAATCTCTAT	TTTCCTCTTCTTACAA CCGTAAACC	GACATCGTGTGCTGAG GCTGGTAT	CTACCCTACAAATCCAAT
24	CTCACTCCCAATCTCTAT	TTGGTTACGATGTTTT CGACTCGCG	TGCTGAGGGGAATTCGT CATCACCA	CTACCCTACAAATCCAAT
25	CTCACTCCCAATCTCTAT	GCGATCGTTACCTGTT GATACATCT	TTGATCGTCAGTCGGCG ATGACTCA	CTACCCTACAAATCCAAT
26	CTCACTCCCAATCTCTAT	TGTTCCAACCTGGTAA CTACTGAAT	ATGTTGACTAAGTTGCT ACGTTTCT	CTACCCTACAAATCCAAT
27	CTCACTCCCAATCTCTAT	TAGCATTGGTAGGAGT AGGAGGAGG	TTTTACATGTACCAGTTG TAGACCT	CTACCCTACAAATCCAAT
28	CTCACTCCCAATCTCTAT	TCCTCCTCGTCTATGA CTAGAACAC	GAGCAGCTGTTACCCGG ACTCAAAA	CTACCCTACAAATCCAAT
29	CTCACTCCCAATCTCTAT	AACTCGCCGTTATTCC GAGACAATC	CGACTAGGATGACCCGT AGACGATT	CTACCCTACAAATCCAAT
30	CTCACTCCCAATCTCTAT	ACTGACCTTTCTCCAA CTGGTCCTG	TATTACCCTGTAGGATAC CGCGAAG	CTACCCTACAAATCCAAT
31	CTCACTCCCAATCTCTAT	TCATCGAAACGTCCGG ATCGAAAC	GCATCAGCTCATCTCCG TCCATGAT	CTACCCTACAAATCCAAT
32	CTCACTCCCAATCTCTAT	GCGACGAAACCGTTG CTTCCGAAAT	GTCAAATCGTTCAACAT CTTGCTAC	CTACCCTACAAATCCAAT
33	CTCACTCCCAATCTCTAT	GCGAGACTCTTTATAA CCTCAGCGT	TCCACTCTCTGACGACT CCTCTTCT	CTACCCTACAAATCCAAT

Plit_SoxN_B2

Pair#	Initiator	Probe	Probe	Initiator
1	CCTCGTAAATCCTCATCA	TTCTGTCATGGTGGA AAACAAAAA	GCACTGGCTGTAAAAAT TCTTTTC	ATCATCCAGTAAACCGCC
2	CCTCGTAAATCCTCATCA	TATTCGTTGACCAGTA ATGAGCCGA	TAAACTTACACACATTC GATGATTG	ATCATCCAGTAAACCGCC
3	CCTCGTAAATCCTCATCA	CGGCACGTGAACAGA ATGTTCTGAT	AATATCGTTCTACATGTG TGTTAAC	ATCATCCAGTAAACCGCC

4	CCTCGTAAATCCTCATCA	GCTTGTGCATTAGGAT CAGCAGTTC	CCGTAATGGCCATACT AGCTAATC	ATCATCCAGTAAACCGCC
5	CCTCGTAAATCCTCATCA	TACGTAAATCACTCGG ACAGCCCGG	CAGGTAGATACATGCTA ATCATCTC	ATCATCCAGTAAACCGCC
6	CCTCGTAAATCCTCATCA	GGCTGACAGGCGAAC CACTTCCGA	TCATCATCTGAGGCGCG GATGTTGG	ATCATCCAGTAAACCGCC
7	CCTCGTAAATCCTCATCA	CATGGGTGAATAAGTT GTCATACCG	AATAATCTGTCCGTTAG ACATCGAA	ATCATCCAGTAAACCGCC
8	CCTCGTAAATCCTCATCA	AAATAAGATGACATTG CCGGACTAC	ATCGGCGAATTATATGCA CTACTGT	ATCATCCAGTAAACCGCC
9	CCTCGTAAATCCTCATCA	AACGCGAATAAAGTC CGCCGTTTCC	ACGACATTGCGACGGG GTCATGTC	ATCATCCAGTAAACCGCC
10	CCTCGTAAATCCTCATCA	AGCATGTGCATCATGC ATCATCGAA	TACGGCAGCGTGTTGGT AAGCGGCA	ATCATCCAGTAAACCGCC
11	CCTCGTAAATCCTCATCA	TGATAAACATCGCGAG AGACCTGTT	CCGTTGGCATATAACC ATTCTCT	ATCATCCAGTAAACCGCC
12	CCTCGTAAATCCTCATCA	ATTCGGATTGCCAGGT TGACCATGA	TTTCTGGTTGTTTTIATT CTGGATG	ATCATCCAGTAAACCGCC
13	CCTCGTAAATCCTCATCA	GACTATTGTGAATATG AGTTTGATG	CTCCACCTCCTCCACCA CCGGAATT	ATCATCCAGTAAACCGCC
14	CCTCGTAAATCCTCATCA	CGTGTIATTTGATGGC GGCGCTGCA	ACCGTGAATCGACGATG GCGGCAGT	ATCATCCAGTAAACCGCC
15	CCTCGTAAATCCTCATCA	TGATGAGGGTACGCTG CCGGTGTTA	GCATGATGGGTCTGATG CGGATGAT	ATCATCCAGTAAACCGCC
16	CCTCGTAAATCCTCATCA	ATACCAGATGGAGGTA GAGAAACGT	GCAGCGGCCTTCAAAG AGTCCGTCT	ATCATCCAGTAAACCGCC
17	CCTCGTAAATCCTCATCA	GTACAGGTCCATATAG ATAAGAAAC	CGGTGGGGAATACAGCG TGACGGTT	ATCATCCAGTAAACCGCC
18	CCTCGTAAATCCTCATCA	GATATAATCTGGATTCC GATATTGA	AAAAACGTACCATAAGA ATTCTGTC	ATCATCCAGTAAACCGCC

3: Sequences of probes designed by Molecular Instruments (Los Angeles, USA)

<i>Plit_SoxN</i>		
Sequence	# of probe pairs	Amplifier
UAACAUGUGUGUGAUCUAUCUUUgAAAAAAUUGgAUUCUUgUUUAAA AAAAAgAAAAgAUUAUUUUUgAUCUCgUgAUUgAAUUUAAAgUUUCU UAUCUAUAUgACCUGUACUCAACCgUCACgCUgUAUUCACCgCAUU ACgUUUCUCUACCUCCAUCUgUAUgAgACgACUCUUUgAAgCCgCUg CCCAAUAACACCgCgCgUACCCUCAUCAUCAUCCgCAUACgACC CAUCAUgCgUCUgCgCgCCCAUCAAUAACACgACACUgCCgCCAUCg UCgAUUCACgUgUgUCAUCAUCAAACUCAUAUUCACAAUAgUCCgAAUU CCgUgUgUgAggAggUgUgUgUCAUCAUgUgUgCAUCCUgCAUCCgAAU	20	B5

AgCAUCCAgAAUAAAAACAACCAgAAUUCCgAUCgUgUUAAGCggCCCAUgAACgCAUUUAUggUAUggUCAAgAggUCAgCggCggAAgAUggCUCAAgAgAAUCCUAAgAUgCAUAAUUCggAAAUAAGCAAgCgAUUgggCgCgACgUggAAgUUACUgUCCgAAgCUgAAAAgAggCCAUUCAUCgACgAAgCCAAGAggUUAAGggCCgUUCACAUGAAAgAACAUCCCgAUUAUAAAUAACaggCCUAgAAggAAgACgAAgACgUUAAUgAAgAAggAUAAAUAUCCgUUgACggCagCAAUgUUACCgAgUgAUggCCggUCgUCgggggUUCAACAggUCUCUCgCgAUgUUUAUCAUCAgAUgAAUggUUAAUgCCCAACggUUAUUCgAUgAUgCAUgAUgCACAUCgUCgCCgCUUACCAACACgCUgCCgUAgCggCCggCggUUUCggUUCUCAAggAAACggCggACUUUAUUCgCgUUACgACAUGACCCCgUCgCAAUgUCgUCUAgUAgUCCggCAAUgUCAUCUUAUUUgAACAgUAgUgCAUAUAAUUCgCCgAUggCCggUAUgACAACUUAUUCACCCAUGAAUUCgAUgUCUAACggACAgAUUAUUAUAAUCCgAAAgUggUUCgCCUgUCAgCCCgCCAACAUCCgCgCCUCAgAUgAUgAggAgACCgggCUgUCCgAgUgAUUUACgUACAgAgAUgAUUAgCAUgUAUCUACCUgAUggAACUgCUgAUCCUAAUgCACAAgCCAgAUUAgCUAgUAUgggCCAUAUACggCCAUAUAgAAAUUCUgUUCACgUgCCgCCgUUAACACACAUGUAgAACgAUUUUUUUA		
<i>Plit_Elav</i>		
Sequence	# of probe pairs	Amplifier
gUUgUUAUUUAgUUUCgAgCACUACgUUUCggUCUAAAgUAAgAUUCAgUUAUUUUUACCGUAgCCUggCCAgCUgACgUUAAAUUUgUUCAUAUUUAACAAUgUUUCggAUUUUUUUUAUUUAUUUCCgUUCAgUCgUUUAUUCCCCUAgUAgUUUACgggCagCUCgUgUgUUUgUUACgUUAUUCCCAAUUACAACgAgCgUUUUUCAUAUAAUUUgCgACgCgUUAACgAACgACgACUgUUAUUUAACAUAUACgUggAUUUCgUCUAAUgUUCUgUgAUgUAUAACUUUACgUgAgAUAgAgAAAAUCUAAAAUAgAAAAUgAUCCAAGUCCAAAgAgAgAgAgAgAAACgUCgCCAUCAAUUUgCUgCACUUUCUgCCAAUUUAAUUAAAAAgAAAAUCCgAUUAUUUUUAUUUUUAUUUUAAACUCggCACgAAgAgAAgAUUgCAAAACUggACgUUAUAACgACggCAAgAAAUUACCUgAACAUgAACggAgUUggAAUggAUAAACggUUgCCAAcAgACUgUCCgAAUggAggAAUACAUGCUgUAgUACAgCAAAGUAUACAUAUgCUggAAgCggAgAUgACAgCAAgACAAAUUUUAUCgUCAAUUAUCUACCACAgACAAUgACCCAAGAAgAUCUACgAAgUCUUUUCUCgUCgAUUggCgAAgUCgAAUCUgUgAAAUgUAUAAgAgACAAAgUCACAgACgCUgCUggUAUCUCAgACCCUgggACCAUUCAAUUCAAAggUCAAAgUCUgUUUAUggUUUUgUUAUUUAUgUAAgAgCUgAAgAUgCCgAAAAAgCagUUAgUACgUUAAAUggAUUAAGAUUACAgAAUAAAACCAUAAAggUAUCUUUCgCAAgACCAAgUAgUgAggCCAUAUAAAggAgCAAAUUUgUACgUCAgCggAUUACCCAAAUcAgUgACCCAACAggAAUUGgAAAAUUUgUUCACACCgUACggAAgAAUUUAUACgUCACgUAUUCUUUgUgUAUACgUAACUgUUUCUggUUCUgAUgUUUCUACAggCCUgUCUAAAggggUCggAUUCAUUAgAUUUgACCAGCgUCACgAAgCUgAgAgAgCAAUUAACAAgUUAACAAACAgAUUCCAgAUggUUgCgUCgAACAgAUCACCgUCAAgUUUgCCAAUAAUCCgAgUAACA AUCCAAAggCAAUACCgCCgCUCgCCgCCUACCUgUACCCACAAAgAAggUUCgCUggUCCUAUUAUCAUCCUACCGUgCUUCAggUAUAUUCUCUgUCCCAAgUAUCAAggUAUUCACCgAUggCUggAgAUCUCCUACCAAUACUUUgAUggCUggUAACACgAUCggUggUUCUggUUggUgCAUCUUCgUUUACAAUUUAgCACCggAgACUgAAgAgAAUAUUCUgUggCagCUgUUUggUCCgUUCggAgCCgUUCAAAAGCgUCAAAGUgAUCCgCgAUCUUCAgACAAAUAAAUgCAAgggUUUCggCUUUgUCACCAUgACCAAUUACgACgAAUCCgUCUgCgUAUUCAgUCgCUCAACggUUAUgCACUCggUAAUCgAgUUCUACAggUUUCUUUAAAACCAUAAgUgCAAgUCgUAA	20	B2

4: Modified HCR-FISH protocol - *Pycnogonum litorale*

- Gradual rehydration of methanol-stored samples on ice
 - 75% MetOH/PBST for 3 min
 - 50% MethOH/PBST for 3 min
 - 25% MethOH/PBST for 3 min
- Washing of samples in PBST on ice
 - 1x 10 min
 - 3x 5 min each
- Removal of larval/walking legs & sonication in ultrasonication bath (in PBS)
- Permeabilisation of samples
 - In 500 µl of Detergent Solution for 30-40 min on orbital shaker (@RT)
- Pre-hybridisation of rehydrated and permeabilised samples in water bath @37°C
 - First in 500 µl of pre-warmed 5xSSCT:Probe Hyb Buffer mix (50:50) for 30 min
 - Then in 400-500 µl of pre-warmed Probe Hyb Buffer for 30-45 min
- Preparation of probe solution
 - 1-2 µl of each 1 µM probe stock solution added to 180-190 µl of pre-warmed Probe Hyb Buffer
 - Keep prepared probe solution @37°C once prepared
- Probe hybridisation in water bath @37°C
 - Remove pre-hyb solution and add probe solution
 - Incubation of samples overnight → 16-24 h
- After incubation as much of probe solution as possible aspirated
 - Saved for re-use (@-20°C)
- Washing of samples to remove excess probes
 - 4x 1 ml Probe Wash Buffer for 15 min each (@37°C)
 - 2x 1 ml 5xSSCT for 5-10 min each on orbital shaker (@RT)
- Pre-amplification of samples (@RT)
 - First in 800 µl 5xSSCT:Amp Buffer (50:50) for 30 min
 - Then in 800 µl Amp Buffer for 30-45 min
- Preparation of hairpin mixtures
 - Hairpin stocks thawed on ice in the dark and spun down
 - 2 µl of each 3 µM hairpin stock (per gene & reaction to be run) pipetted into a separate tube
 - Tubes heated for 90 seconds @95°C and left to cool down in the dark
 - 2 µl of each snap-cooled hairpin (h1 & h2 per gene in each experiment) added to 90 µl of Amp Buffer @RT and briefly spun down
- Pre-amplification solution removed & hairpin mixture added
 - Incubation of samples overnight in the dark → 16-24 h
- After incubation as much of hairpin solution as possible aspirated
 - Saved for re-use (@-20°C)
- Washing of samples on orbital shaker (@RT in the dark)
 - 2x 1 ml 5x SSCT for 5 min each
 - 2x 1 ml 5x SSCT for 30 min each

- Nuclear staining of samples on orbital shaker (@RT in the dark)
 - 1 ml 5x SSCT with DAPI (1 µg/ml) added to samples
 - Left to incubate for 1-2 h
 - Afterwards homogeneity of staining checked under an epifluorescence stereomicroscope
 - If needed the samples were incubated in 0.1 µg/ml DAPI in 50% glycerol/PBS overnight @4°C
- Washing of samples (@RT in the dark)
 - 1-2x 1ml 5x SSCT for 5 min each
- 5x SSCT replaced with 50% glycerol/PBS
 - Store @4°C until imaging

5: Overview of the different HCR experiments performed in the study, including the different gene combinations and fluorochromes used.

HCR No 1		
<u>Genes</u>	<u>Amplifiers</u>	<u>Fluorochrome used</u>
<i>SoxN</i> (singleplex)	B5	A647
<i>Elav</i> (singleplex)	B2	A647
<i>SoxN</i>	B5	A594
<i>Elav</i>	B2	A647
HCR No 2		
<u>Genes</u>	<u>Amplifiers</u>	<u>Fluorochrome used</u>
<i>Brat</i> (singleplex)	B1	A647
<i>ASH1</i> (singleplex)	B3	A647
<i>ASH2</i> (singleplex)	B4	A647
<i>Pros</i>	B5	A647
<i>SoxN</i>	B2	A594
HCR No 3		
<u>Genes</u>	<u>Amplifiers</u>	<u>Fluorochrome used</u>
<i>Dpn</i> -like (singleplex)	B4	A647

<i>SoxN</i>	B2	A647
<i>Pros</i>	B5	A594
<i>Brat</i>	B1	A647
<i>Elav</i>	B2	A594
<i>ASH1</i>	B3	A647
<i>SoxN</i>	B5	A594
<i>ASH2</i>	B4	A647
<i>Brat</i>	B1	A594