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Characterization of Larval Neurons in *Nematostella vectensis*

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Ana Brandic BSc

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Abstract

The cnidarian model organism *Nematostella vectensis*, the starlet sea anemone, undergoes a subtle type of metamorphosis from the free-swimming planula stage to the sessile polyp stage where the ability to swim is lost. The nervous system of *Nematostella* consists of a diffuse nerve net. Single cell RNA sequencing data published by Cole et al., 2024 proposed that in *Nematostella* larva-specific neurons exist which disappear after transition into the polyp stage. There were three larval-specific neural states (N1.L1, N1.L2 and N1.L3) and one larval-specific secretory state (S1.L1) described of which only some cells of the larval neural state N1.L3 persist into the adult polyp. Here we aim to initiate the process of characterizing these larval neurons. Therefore, the fundamental work to generate seven different larval neural specific transgenic reporter lines was performed. Focusing on one representative gene candidate per larval state we show that these neurons are truly only expressed in *Nematostella* larvae. This was quantitatively verified by fluorescence live-cell imaging and additionally confirmed through immunostaining assays. Using in situ hybridizations of various marker genes of the three larval neural states we confirm expression in larvae but show that there is no expression of the larval neural states N1.L1 and N1.L2 in primary polyps. Moreover, we show that in addition to gene expression at the larval stage, there is one marker gene specific for the larval neural state N1.L3 that is also expressed in the primary polyp.

Zusammenfassung

Der zu den Cnidaria gehörenden Modellorganismus *Nematostella vectensis*, eine Seeanemone, durchläuft eine leichte Form von Metamorphose von der frei-schwimmenden Planula-Larve bis hin zum sessilen Polypen, wobei die Fähigkeit zu schwimmen verloren geht. Das Nervensystem von *Nematostella* besteht aus einem diffusen Nervennetz. Cole et al., 2024 veröffentlichte Single-Cell RNA Sequencing Daten, die prognostizieren, dass in *Nematostella* larvenspezifische Neuronen existieren, die nach dem Übergang ins Polypenstadium nicht mehr vorhanden sind. Es wurden drei larvenspezifische neuronale Zustände (N1.L1, N1.L2 und N1.L3) und ein larvenspezifischer sekretorischer Zustand (S1.L1) beschrieben, von denen nur einige Zellen des larvalen neuronalen Zustands N1.L3 im adulten Polypen bestehen bleiben. In dieser Arbeit setzen wir die ersten Schritte zur Charakterisierung dieser larvalen Neuronen. Dazu wurde die grundlegende Arbeit zur Erzeugung von sieben verschiedenen Larven-spezifischen neuronalen transgenen Reporterlinien durchgeführt. Anhand eines repräsentativen Gens pro Larvenstadium zeigen wir, dass diese Neuronen tatsächlich nur in *Nematostella*-Larven exprimiert werden. Dies wurde quantitativ durch Fluorescence-Live-Cell-Imaging verifiziert und zusätzlich durch Immunostaining-Experimente bestätigt. Mittels In-situ-Hybridisierungen verschiedener Marker-Gene der drei larvalen neuronalen Zustände bestätigen wir die Expression im Larvenstadium, zeigen aber, dass Gene der larvalen neuronalen Zustände N1.L1 und N1.L2 nicht in primären Polypen exprimiert werden. Darüber hinaus zeigen wir, dass es zusätzlich zur Genexpression im Larvenstadium ein für den larvalen neuronalen Zustand N1.L3 spezifisches Marker-Gen gibt, das auch im primären Polypen exprimiert wird.

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1 Introduction

1.1 Why study the cnidarian *Nematostella vectensis*?

Cnidarians with their phylogenetic position as the sister group to bilaterians share a great part of the gene set with bilaterians (Putnam et al., 2007; Schwaiger et al., 2014). The cnidarian *Nematostella vectensis*, the starlet sea anemone, has been established as a model organism with a sequenced genome, stable transgenics and gene-editing technologies available (Putnam et al., 2007; Renfer et al., 2009; Ikmi et al., 2014; Rentzsch, et al., 2008). Neuron-specific promotors for reporter genes not only allow the visualization of neuronal morphology but also the conditional ablation of neurons which gives insights into their function (Nakanishi et al., 2012; Gavgani et al., 2025). Additionally, with the ability to perform in situ hybridization (ISH) an expression overview of specific genes in the whole organism can be achieved (Wolenski et al., 2013). Due to its high capacity to generate neurons throughout their lifetime and during regeneration many studies in *Nematostella* have been performed in order to understand cnidarian neurogenesis (Rentzsch et al., 2017).

1.2 Life cycle and morphology

The anthozoan *Nematostella* lives in estuarine environments like brackish water and evolves from a swimming planula larva which derives from sexual reproduction to a sessile polyp (Fig. 1A) (Hand et al., 1992; Layden et al., 2016).

The polyp has a tube-shaped body with a single opening that is surrounded by a ring of tentacles for catching prey. The inner tissue layer (gastrodermis) bears folds, forming so called mesenteries that contain gonadal tissue and longitudinal musculature that allows the animal to quickly contract along the long axis (Rentzsch et al., 2019).

In culture the life cycle of *Nematostella* lasts approximately 4-6 months. Spawning can be induced reproducibly and fertilization occurs in the external environment allowing accessibility for experimental research (Fritzenwanker et al., 2002; Stefanik et al., 2013).

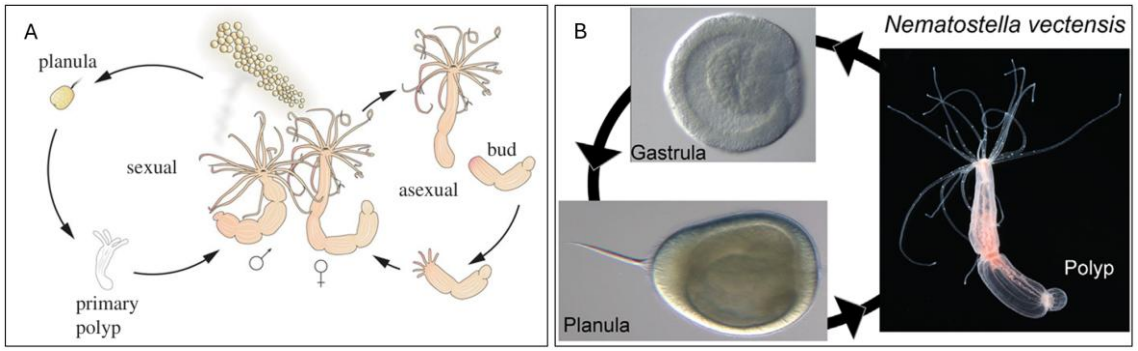

Figure 1. Life cycle and developmental stages of *Nematostella vectensis*. (A) Life cycle of *Nematostella vectensis*, showing sexual and asexual reproduction. Modified from Kelava et al., 2015. **(B)** Developmental stages (gastrula, planula and polyp) of *Nematostella vectensis*. Modified from Sinigaglia et al., 2015.

The zygote undergoes cleavages and forms a blastula, followed by gastrulation via invagination and then the planula stage with the free-swimming larva (Fig. 1B) (Fritzenwanker et al., 2007). During this stage planulae elongate and form the pharynx and the mesenteries. They undergo a gradual metamorphosis where they lose their ability to swim and form first tentacle buds and then the four tentacle primary polyp. This development from fertilized egg to primary polyp takes approximately 6 days. The juvenile polyp grows to the sexual mature adult polyp in a few months dependent on the available nutrients (Layden et al., 2016; Fritzenwanker et al., 2007; Lee et al., 2007).

Besides bilaterians cnidarian ciliated larvae are unique for evolving an apical sensory organ with integrated neurons (Gilbert et al., 2022; Marlow et al., 2009). The apical organ is at the apical pole and processes environmental cues for modulation of larval swimming (Sinigaglia et al., 2015; Iwao et al., 2002). In addition to locomotion, it is also considered to be involved in settlement and metamorphosis (Sinigaglia et al., 2015). Even though there are neuron-lacking animals like sponges that show that their larvae still have sensory and secretory cells in the anterior region, the neuronal coordination of motile cilia is proposed to increase the efficiency of sensory to motor transformation (Gilbert et al., 2022; Brauchle et al., 2018; Jekely, 2011). The morphology of the cnidarian larvae apical organ is similar to the bilaterian larvae (Sinigaglia et al., 2015; Marlow et al., 2014). It contains apical tuft cells that form a spot and larval-specific neurons which form a ring around this spot (Gilbert et al., 2022). Nevertheless, it is proposed that the common ancestor of bilaterians and cnidarians did not have an apical organ with a ciliary tuft, but rather traits evolved independently (Gilbert et al., 2023).

Nematostella larvae swim in the direction of the apical organ and also settle on the aboral side, even though the apical organ disappears after metamorphosis into the polyp stage (Rentzsch et al., 2008). They do not exhibit positive or negative phototaxis, however it was shown that swimming of *Nematostella* larvae could be induced by ultraviolet and violet light, and light with longer wavelength causes the larvae to swim down to the substrate which might lead to greater dispersal range in shallow environments (Lilly et al., 2024).

1.3 Nervous system

The nervous system of *Nematostella* consists of two interconnected neuronal networks, an ectodermal diffuse nerve net and mesendodermal condensed neurite longitudinal tracts that run along the mesenteries (Marlow et al., 2009; Havrilak et al., 2017; Nakanishi et al., 2012). The nervous system is comprised of two major cell type classes of neurons: sensory cells and ganglion cells (Kelava et al., 2015; Rentzsch et al., 2017). Although in contrast to bilaterians neurogenesis in *Nematostella* occurs both in the ectoderm and in the endoderm and mesoderm, the known underlying molecular regulation is similar to vertebrates, including SoxB and bHLH transcription factors for further differentiation (Richards et al., 2014; Magie et al., 2005; Bosch et al., 2017).

Using developmental single-cell RNA sequencing it was shown that neurons, gland cells and cnidocytes all derive from a common multipotent progenitor population with the conserved transcription factor gene SoxC as a key upstream regulator (Steger et al., 2022). In *Nematostella* there is continuous differentiation of those neuroglandular cells from the embryo to the adult animal (Steger et al., 2022), making the model organism a great candidate to investigate the structure and molecular composition of the ancestral nervous system that gave rise to the cnidarian and bilaterian nervous systems (Layden et al., 2016).

In *Nematostella* neurogenesis likely starts at the blastula stage in an aboral territory and at early gastrulation the oral cap is still lacking neurons (Fig. 2A (a); Kelava et al., 2015). After gastrulation neurons appear in the oral half of the planula larvae and in the endomesoderm (Fig. 2A (b); Kelava et al., 2015). In the apical organ, also referred to as the sensory centre at the most aboral region, no neurons that are marked with *SoxB(2)* can be found even after the neurogenesis switch (Kelava et al., 2015). *SoxB(2)* expressing

cells generate sensory neurons, ganglion neurons and cnidocytes, furthermore the production of neurons is strongly reduced with the knockdown of *SoxB(2)*, therefore marking *SoxB(2)* a marker for a general neural progenitor population (Richards et al., 2014). It is still unknown which mechanism is responsible for this early neurogenesis switch from aboral to oral during embryonic development (Kelava et al., 2015).

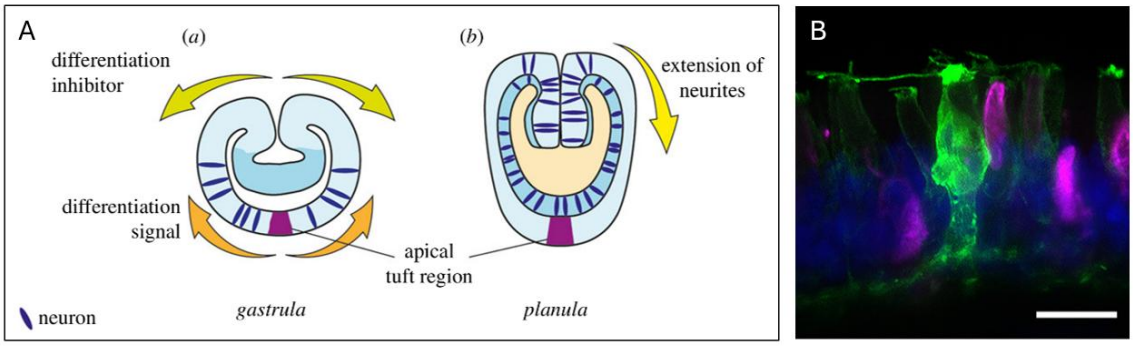

Figure 2. Early neurogenesis switch and neuron morphology in *Nematostella vectensis*. (A) Modified from Kelava et al., 2015. Early neurogenesis switch from (a) the gastrula stage where individual neurons appear in the aboral half, excluding future apical organ (magenta) to (b) the planula stage which has more localized character where many neurons appear at the oral side and in the endoderm. **(B)** Modified from Rentzsch et al., 2019. Labelled neurons in the body wall of transgenic late planula larva: (green) sensory neuron and (magenta) capsules of cnidocytes. Orientation: apical surface of the ectoderm is at the top. Scalebar: 20 µm.

The two different cell types of neurons differ in their morphology. Sensory neurons have an upright position in the epithelium and an apical cilium (Fig. 2B; Rentzsch et al., 2019), whereas the somata of ganglion neurons (equivalent to interneurons) are located basally within the epithelium. Nevertheless, both sensory and ganglion neurons extend neurites on their basal side to form a basiepithelial meshwork. Cnidocytes, a cnidarian specific neural cell type, resemble pre-synaptic sites with a cnidocil, a hairlike sensory structure, and the cnidocyst, an extrusive organelle that rapidly discharges for catching prey (Rentzsch et al., 2019; Ozbek et al., 2009). Due to this ultrafast exocytosis which is mechanosensory and calcium dependent and their common lineage with sensory and ganglion neurons cnidocytes are considered neural cells (Layden et al., 2016; Steger et al., 2022).

1.4 Larval neurons

Based on a newly generated gene annotation of *Nematostella vectensis*, called Nv2 (Zimmermann et al., 2023), and re-mapping of previously published sequence data (Steger et al., 2022; Cole at al., 2023) a new wildtype single cell transcriptome was published by Cole et al., 2024. It shows that neural and gland cell types cluster together into a large neuroglandular population with 47 distinct cell states (Fig. 3A; Cole et al., 2024) of which 18 states are identified as neurons (Cole et al., 2024; Steger et al., 2022). These neurons can be divided into insulinoma (INSM) positive (N1) and INSM negative (N2) populations (Steger et al., 2022). The zinc finger transcription factor Insulinoma-associated protein 1 (Insm1) is expressed in sensory cells, ganglion cells, and gland-secretory cells in *Nematostella* that derive from *SoxB(2)*-positive progenitor cells and is required for neural and endocrine cell development in vertebrates (Tournière et al., 2022).

Cole et al., 2024 describes 3 larval neural states (N1.L1, N1.L2 and N1.L3) and one larval secretory state (S1.L1) that appear at gastrulation and mostly disappear once the planula transitions into the tentacle-bud stage on day 5 (Fig. 3B). Since a few cells of the cluster N1.L3 are still found in the single cell transcriptome of tentacle and body wall adult samples, this cell type may persist into the adult polyp (Cole et al., 2024). All other larval clusters (N1.L1, N1.L2 and S1.L1) are proposed to only be found in gastrula and planula stages of *Nematostella* (Cole et al., 2024). Moreover, it is suggested that N1.L2 localizes to the aboral pole due to co- expression of *six3/6* or that it might form part of the apical organ (Cole et al., 2024). The cell state that corresponds to the apical organ in the aboral domain occurs in the 1-5d samples and disappears again in the 8d and 16d polyp samples (Cole et al., 2024).

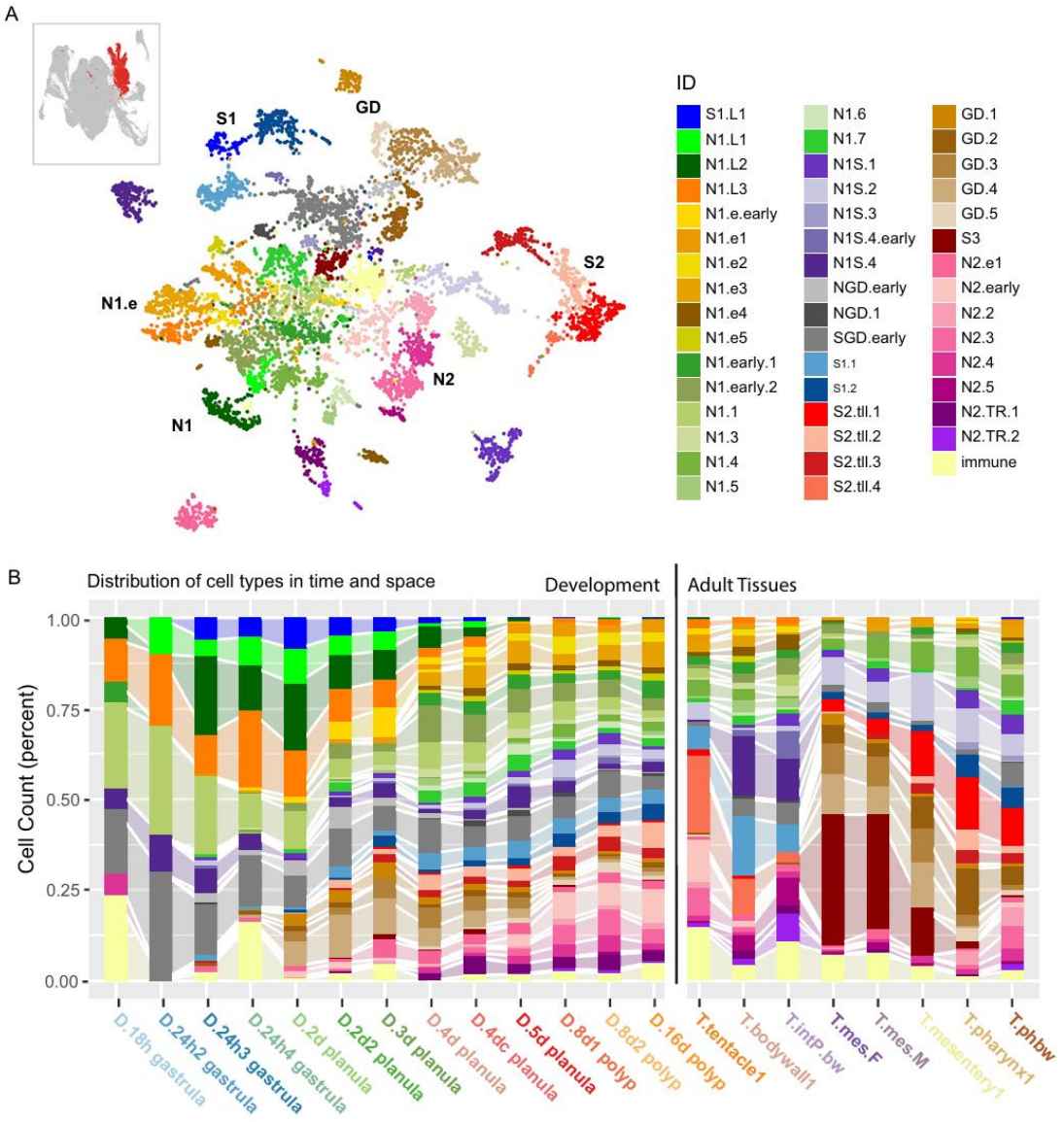

Figure 3. Neuroglandular population in *Nematostella vectensis*. Cole et al., 2024. **(A)** Clustering (UMAP) of 47 distinct cell states of neuroglandular population. **(B)** Relative contribution of each cell state across all samples showing 3 larval neural states (N1.L1, N1.L2 and N1.L3) and one larval secretory state (S1.L1) largely disappearing in primary and adult polyp stages.

1.5 Aim of the project

The overall aim of this project is to validate the larval specific neuronal clusters in *Nematostella vectensis* that are based on previous single-cell RNA sequencing. For each cluster I choose the most cluster-specific genes to clone their promotors into memEGFP and NTR2.0 containing vectors. These constructs were then microinjected into fertilized wildtype *Nematostella* eggs to generate seven different transgenic reporter lines labelling larval specific neurons.

Since the generation time of the microinjected animals exceeded the time of my project, no transgenic F1 animals could be generated yet. Nevertheless, I intend to show that the generated constructs do in fact label larval specific neurons of *Nematostella* and cannot be found in the primary polyp stage. For this purpose, I microinjected one representative construct per larval cluster and fluorescently live-cell imaged the mosaic F0 planula larvae. To quantify the success rate, I microinjected a higher number of fertilized eggs and counted the presence of specific GFP signal over the first eight days after fertilization. Additionally, I performed immunostaining assays to confirm that the GFP-labelled cells are present in the larvae and not in the primary polyp.

Lastly, I carried out in situ hybridizations of genes belonging to the three larval neural states (N1.L1, N1.L2 and N1.L3) to get insights on why they might cluster separately and identify possible differences and similarities of expression patterns between each cluster and among clusters themselves.

2 Materials and Methods

2.1 Animal culture

The animals were obtained from the colonies established in the Department of Neuroscience and Developmental Biology at the University of Vienna. *Nematostella vectensis* adult polyps are kept at 18°C in the dark in 16‰ artificial seawater (*Nematostella* Medium, NM) in either cylinders or boxes, keeping females and males separate. They are fed five times a week with *Artemia salina* and induced every three weeks for spawning.

For induction the animals are kept at 25°C in light for nine hours and transferred to 18°C in light for a minimum of three hours until no egg packages are released anymore. The egg packages are fertilized by leaving them in NM containing sperm for 30 minutes.

In order to use the fertilized or unfertilized egg packages for experiments they were de-jellied by incubation in 3% L-Cysteine solution (pH 7.4) for 10 to 15 minutes on a shaker at room temperature (RT) and washed five times in fresh NM. For short-time storage until the desired animal stage is fixed, they were kept at 21°C in the dark.

2.2 Fixation

The animals were collected after 24h, 48h, 72h, 4d, 5d, 6d and 7d after fertilization for in situ hybridization and after 1d, 3d and 8-9d for immunostaining. The 6 and 7 days old primary polyps were let to relax for 30min and then treated with 2 mL 1M $MgCl_2$ for 20min at RT to inhibit contraction of the animal. All embryos were fixed in 3.7% formaldehyde (in PTW) at RT for 1h on a rotor, only fixation for immunostaining was performed at 4°C. Afterwards they were washed four times with PTW, once with PBS and once with dH_2O.

For in situ hybridization they then were dehydrated with methanol (MeOH), first in a 50% MeOH solution (in dH_2O), then in 100% MeOH. They were stored in fresh MeOH at -20°C. There were no dehydration steps carried out for fixed animals used for immunostaining. These were stored in PTW at 4°C after washing four times in PTW.

2.3 Cloning and probe synthesis for in situ hybridization

The RNA extraction was performed on 3 days old stages of *Nematostella* by using 1mL trizol for 10min, adding 0.2mL chloroform, inverting 10 times to homogenize and centrifuge at 12.000xg at 4°C for 15min. For RNA precipitation the equivalent volume of isopropanol was added, incubated for 10min at RT and centrifuged at 12.000xg at 4°C for 15min. The formed pellet is washed in ice-cold 75% ethanol (5min centrifugation at 7.500xg at 4°C), resuspended in RNase-free water and can be stored at -70°C. For additional control the RNA concentration is measured using the NanoDrop spectrophotometer.

The RNA is reversed transcribed into cDNA using *SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (Invitrogen)* with previously random annealed hexamers. During reverse transcription 2 U/µL RNase OUT is added and incubated at 37°C for 20min.

The genes of interest were amplified by polymerase chain reaction (PCR) using an annealing temperature of 60°C and 35 cycles, separated by gel electrophoresis on a 1% agarose gel and purified by the *Monarch® DNA Gel Extraction Kit (NEB).*

Component	Final Concentration	Volume
Q5 Reaction Buffer (5x)	1x	2 µL
dNTPs (10 mM)	200 µM	0.2 µL
Q5 DNA-Polymerase	0.02 U/µL	0.1 µL
Forward Primer (10 µM)	0.2 µM	0.5 µL
Reverse Primer (10 µM)	0.2 µM	0.5 µL
cDNA	10 pg – 1 µg	0.2 µL
MQ H_2O	-	To total of 10 µL

Table 1. Q5 PCR reaction mix for in situ hybridization.

The products were cloned into pJET1.2/blunt vectors and transformed into competent *E.coli* which were grown on ampicillin agar plates overnight. The plasmids were purified using the *innuPREP Plasmid Mini Kit 2.0 (IST Innuscreen)* and sent to sequencing to confirm the correct orientation of the insert. The selected clones were re-grown to PCR-

amplify the targets from plasmids using SP6 and T7 primers (2-4 ng/µL plasmid DNA, annealing temperature of 55°C and 35 cycles).

Step	Temperature [°C]	Time [m:ss]
Initial Denaturation	98	1:00
Denaturation	98	0:15
Annealing	55 – 60	0:30
Extension	72	30 s/kb
Final Extension	72	2:00
Hold	10	

Table 2. Q5 PCR thermocycler program.

RNA probe synthesis was performed using the *HiScribe® T7 High Yield RNA Synthesis Kit (NEB)* or *HiScribe® SP6 High Yield RNA Synthesis Kit (NEB)* respectively.

Component	Volume
Template DNA	500 ng
Enzyme Mix	0.5 µL
DIG labeling mix	0.75 µL
Transcription buffer	0.5 µL
RNase OUT	0.5 µL
DTT	0.5 µL
RNase-free water	To total of 5 µL

Table 3. Probe synthesis for in situ hybridization.

After incubation at 37°C overnight the probe was purified using DNaseI for 30min at 37°C and precipitated via LiCl solution at -20°C overnight. This was followed by centrifugation for 30min at maximum speed at 4°C. The pellet was washed twice with ice-cold 70% Ethanol (EtOH), centrifuged again for 5min and resuspended in RNase-free water. The final product was then diluted with deionized formamide to a final concentration of 50ng/µL for storage at −20°C.

2.4 In situ hybridization

In situ hybridization was performed using stored animals at -20°C in MeOH which were re-hydrated into PTW. The embryos and polyps were digested by 10 µg/mL Proteinase-K for 20 min at RT without shaking. This digest was stopped by washing twice with glycine. Charged residues were blocked using triethanolamine and acetic hydride, followed by washing twice in PTW. The animals were re-fixed in 3.7% formaldehyde in PTW and washed five times in PTW.

For the pre-hybridization step (62°C for 2h) a 5 mg/mL Hybe-Mix with Torula RNA was used. Hybridization was performed at 62°C with 1 ng/µL Digoxigenin(DIG)-RNA probe in Hybe-Mix overnight. All animals were then washed with Hybe-Mix/2xSSCT.

An additional incubation step in 1U/µL RNAseT1 in 2xSSC (1:1000) at 37°C was added for animals older than 4 days, before all animals were again washed with 0.1x SCCT. The samples were blocked for 1h using blocking solution (0.5% Roche Blocking Reagent in 1xMAB, pH 7.5). The probes were detected with a 1:4000-diluted alkaline phosphatase (AP) coupled α-Digoxigenin antibody (α-Dig-AP) overnight and then washed ten times in PTW and three times in AP-buffer.

After staining with 1.5µL/mL BCIP (5-Bromo-4chloro-3-indolyl-phosphate) and 1µL/mL NBT (4-Nitro blue tetrazolium chloride) at RT in the dark, the animals were rinsed with dH_2O and transferred into 100% EtOH for one hour. Finally, they were washed twice in PTW, left in PTW for one hour and then transferred to 86% Glycerol for later imaging.

2.5 Cloning for transgenic reporter lines

The promotor region of the genes of interest together with the restriction sites for Asc-I and Pac-I were amplified by PCR using an annealing temperature of 60°C and 35 cycles, separated by gel electrophoresis on a 1% agarose gel and purified by the *Monarch® DNA Gel Extraction Kit (NEB).*

Component	Final Concentration	Volume
Q5 Reaction Buffer (5x)	1x	10 µL
dNTPs (10 mM)	200 µM	1 µL
Q5 DNA-Polymerase	0.016 U/µL	0.4 µL
Forward Primer (10 µM)	0.2 µM	1 µL
Reverse Primer (10 µM)	0.2 µM	1 µL
gDNA	-	3 µL
MQ H_2O	-	To total of 50 µL

Table 4. Q5 PCR reaction mix for transgenic reporter lines.

The restriction sites of both the gene products and the vectors were digested with the corresponding restriction enzymes for 3h at 37°C. To prevent ligation of the vector to itself, it was incubated with Shrimp Alkaline Phosphatase (rSAP) for 30min at 37°C, followed by a 5min heat inactivation of the enzyme at 65°C. The product of the restriction digest was then again separated by gel electrophoresis on a 1% agarose gel and purified by the *Monarch® DNA Gel Extraction Kit (NEB).*

The resulting gene of interests with sticky ends were cloned into digested NTR2.0-P2A-memEGFP vectors using the *NEB Ligation Protocol with T4 DNA Ligase* and transformed into competent *E.coli* which were grown on ampicillin agar plates overnight. The plasmids were purified using the *innuPREP Plasmid Mini Kit 2.0 (IST Innuscreen)* and sent to sequencing to confirm the correct sequence. The animals were microinjected with an Injection-Mix containing 50ng/µL of plasmid.

Component	Volume
CutSmart Buffer (NEB) (10x)	1 µL
Dextran, Alexa Fluor 568 (250 ng/µL)	1 µl
I-SceI (5 U/µL)	0.3 µL
Plasmid	50 ng/µL
Nuclease-free water	To total of 10 µL

Table 5. Injection mix for microinjections.

2.6 Immunostaining

After miroinjecting the animals with 50ng/µL plasmid for each gene of interest, they were grown and fixed at following time points: 24h, 72h and 7d-8d (primary polyp stage). They were washed four times with PTW for 10 min, incubated for 1h with blocking buffer (3% BSA and 5% Sheep Serum in PTW) on a shaker at RT and left incubating overnight with 200µl primary antibody (*mouse anti GFP*) in blocking buffer (1:250) at 4°C.

The next day they were washed four times in PTW for a total of 2h, again incubated with blocking buffer for 1h on a shaker at RT and transferred to 200µL secondary antibody (*Alexa Fluor™ 488 goat anti-mouse IgG*) (1:200) and DAPI (1:1000) diluted in blocking buffer for incubation overnight at 4°C.

The immunostaining was finished on the third day by washing 6 times with PTW for 20min, adding 100µL *VECTASHIELD® Antifade Mounting Medium* for incubation overnight and later mounting for imaging.

2.7 Primers

Gene ID	Gene	Primer ID	Primer Sequence
NV2.4915	TAAR6-like-2	NV2.4915_F1_AB	CGACTTAATTAAAGACTC GACAGCACAGTGAGC
		NV2.4915_R1_AB	CTTAGGCGCGCCCCTTT CTGACTGAAGGTGCGCT
NV2.13777	PRLHR-like-2	NV2.13777_F1_AB	CGACTTAATTAACTAAGTC AGAGGACGCAAACTC
		NV2.13777_R1_AB	CTTAGGCGCGCCTGAAG GATCAGCTGTCGCCATA
NV2.23614	NV2.23614	NV2.23614_F1_AB	CGACTTAATTAAACAGTG CGGGTATGGATAGACA
		NV2.23614_R1_AB	CTTAGGCGCGCCTGCAA TAGTTCCACGACATGATG
NV2.16064	USOM5-like-29	NV2.16064_F1_AB	CGACTTAATTAAATACGGT GCCCAATACGAGGAG
		NV2.16064_R1_AB	CTTAGGCGCGCCTCTCG GATATAGAGAAACCGTT
NV2.21840	NV2.21840	NV2.21840_F1_AB	CGACTTAATTAACACCCC TGACTTACTCGATTCT
		NV2.21840_R1_AB	CTTAGGCGCGCCGAAAC CGCGATACAAGATGGCA
NV2.1446	NV2.1446	NV2.1446_F1_AB	CGACTTAATTAAAAACCTT GGCGCCTCTGAGAAA
		NV2.1446_R1_AB	CTTAGGCGCGCCTAATGA GACACAGCGTCAAAACC
NV2.21375	C209A-like-1	NV2.21375_F1_AB	CGACTTAATTAACGCACT TGGAATTGTCGTGGAA
		NV2.21375_R1_AB	CTTAGGCGCGCCAGGAG CGAATGTAACAGGATT

Table 6. Primers for transgenic reporter lines.

Gene ID	Gene	Primer ID	Primer Sequence
NV2.19361	NV2.19361	H_NV2.19361_F1_AB	GCAGCAACAAGTAAACACAA
		H_NV2.19361_R1_AB	TCAATACCAAAGCATGACAG
NV2.13777	PRLHR-like-2	H_NV2.13777_F1_AB	ATGGCGACAGCTGATCCTTC
		H_NV2.13777_R1_AB	CAGTAGAGTCTGTCTTAGTG
NV2.23614	NV2.23614	H_NV2.23614_F1_AB	ATACCCCGAATCATCAAAAC
		H_NV2.23614_R2_AB	GCATGAAGCGGTCTCTTGTACG
NV2.16064	USOM5-like-29	H_NV2.16064_F1_AB	CCGGTACGCACTCAGTACCT
		H_NV2.16064_R1_AB	AACACCATGTCGGCCTATTG
NV2.21840	NV2.21840	H_NV2.21840_F1_AB	ACGGTCGGGTGATCGTTCTT
		H_NV2.21840_R1_AB	GAGTGTCACCAGATATGAAG
NV2.1446	NV2.1446	H_NV2.1446_F3_AB	GGAACTCTTGACAACCATCGCA
		H_NV2.1446_R3_AB	TGTTACAGATGGCAAAGCATTATGG

Table 7. Primers for in situ hybridization.

2.8 Solutions and Recipes

Buffers and Reagents	**Ingredients**
3% L-Cysteine solution	30 mL NM (*Nematostella* medium) 1 g L-Cysteine adjust pH to 7.4 with 1 M NaOH (2-3 drops)
10x PBS	18.6 mM NaH_2PO_4 84.1 mM Na_2HPO_4 1.750 M NaCl adjust pH to 7.4 if necessary
PTW	1x PBS 0,1% Tween20 detergent
20x SSC(T)	0.3 M Na citrate 3 M NaCl pH 7 SSCT = SSC + 0.1% Tween20
Hybe Buffer	50% formamide 5x SSC (pH 4.5) 100µg/mL Heparin 0.1% Tween20 1% SDS 5 mg/mL Torula RNA fill up with dH_2O
1x MAB	100mM maleic acid 150mM NaCl pH 7.5 (adjust with NaOH)
Alkaline Phosphatase (AP) Buffer	100mM NaCl 50mM $MgCl_2$ 100mM Tris (pH 9.5) 0.1% Tween20 fill up with dH_2O

Blocking Solution (In situ hybridization)	0.5% Roche Blocking Reagent in 1x MAB pH 7.5 fill up with dH_2O
Blocking Buffer (Immunostaining)	3% BSA (Bovine Serum Albumin, heat shock fraction, pH 7) 5% Sheep Serum in PTW

Table 8. Ingredient list of buffers and reagents.

3 Results

3.1 Transgenic reporter lines

3.1.1 Generation of larval neural specific transgenic reporter lines

The nervous system of *Nematostella* consists of two interconnected neuronal networks, an ectodermal diffuse nerve net and mesendodermal condensed neurite longitudinal tracts, and can be divided into two different cell type classes: sensory cells and ganglion cells (Kelava et al., 2015; Rentzsch et al., 2017, Marlow et al., 2009).

The wildtype single cell transcriptome of *Nematostella* (Steger et al., 2022) was recently annotated by Cole et al., 2024, based on a newly published genome of *Nematostella* (Nv2) (Zimmermann et al., 2023). Cole et al., 2024 shows that neural and gland cell types cluster together into a large neuroglandular population with 47 distinct cell states. Furthermore, there were 3 larval neural states (N1.L1, N1.L2 and N1.L3) and one larval secretory state (S1.L1) described that appear at gastrulation and mostly disappear once the planula transitions into the tentacle-bud stage on day 5 (Cole et al., 2024).

To validate these larval specific neuronal clusters in *Nematostella vectensis* transgenic reporter lines have been designed. For each cluster I chose the most specific genes based on expression profiles of up-regulated genes across each neuroglandular cluster (Fig. 4).

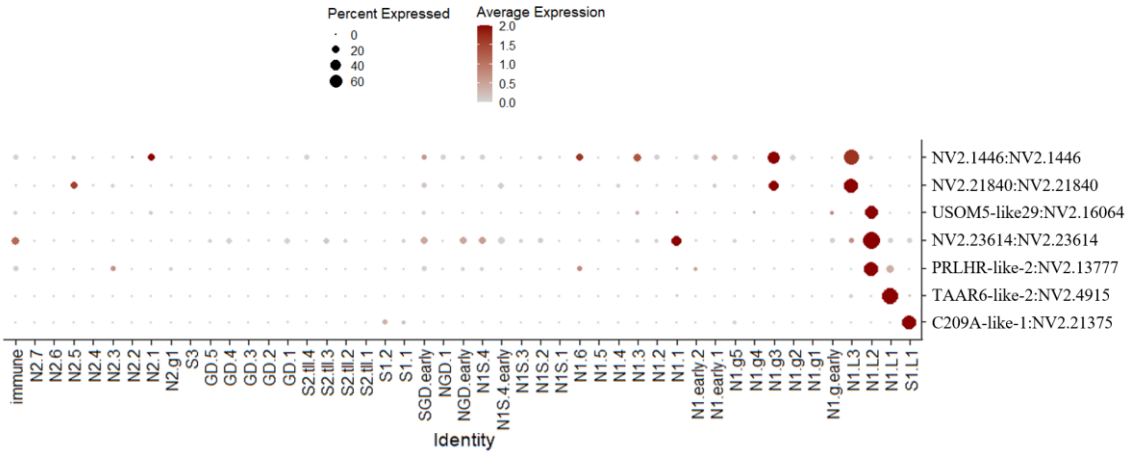

Figure 4. Genes of interest of larval neural and secretory states for transgenic reporter lines. Dot plot representation of expression profiles of up-regulated genes of interest across each neuroglandular cluster. Originally provided by Linda Kloss, additionally modified.

N1.L2 could be separated into two subclusters depending on the settings of the clustering parameters. Therefore, I generated three different constructs for the transgenic reporter lines of larval neural state N1.L2; *NV2.13777* and *NV2.23614* cluster in the subclusters respectively, *NV2.16064* showed similar expression in both subclusters. Since the two top candidates for cluster N1.L3 (*NV2.21840* and *NV2.1446*) are also expressed in different clusters, especially N1.g3, we proceeded with both.

The larval neuron specific (LNS) promotor of each gene of interest was cloned into a NTR2.0 and memEGFP containing vector with the restriction sites *PacI* and *AscI* (Fig. 5B) Nitroreductase (NTR) catalyzes the reduction of a non-toxic prodrug Metronidazole (MTZ) into a cytotoxic product that induces cell death (Gavgani et al., 2025). This system can be used for targeted cell ablation in these transgenic reporter lines in order to determine their function. memEGFP is separated by the self-cleaving peptide P2A and the whole construct was flanked by *I-SceI* cutting sites for microinjection.

These constructs for seven different transgenic reporter lines labelling larval specific neurons (Fig. 4) were cloned and microinjected into fertilized wildtype *Nematostella* eggs. Since the generation time of the microinjected animals exceeded the time of my project, no transgenic F1 animals could be generated yet. At this moment the F0 animals have been raised and started to produce eggs or sperm but still need to be screened to obtain the F1 generation. The F0 animals are regularly induced and kept in cycle and will be screened by a different member of the Technau group.

3.1.2 Analysis of microinjected *Nematostella* larvae and primary polyps

For this project I intend to show that the generated constructs do in fact label larval specific neurons of *Nematostella* and therefore cannot be found in the primary polyp stage. To accomplish this, I chose one representative construct per larval cluster which was the most cluster-specific (Fig. 5A) and microinjected two rounds of 250-300 animals with each of the four constructs.

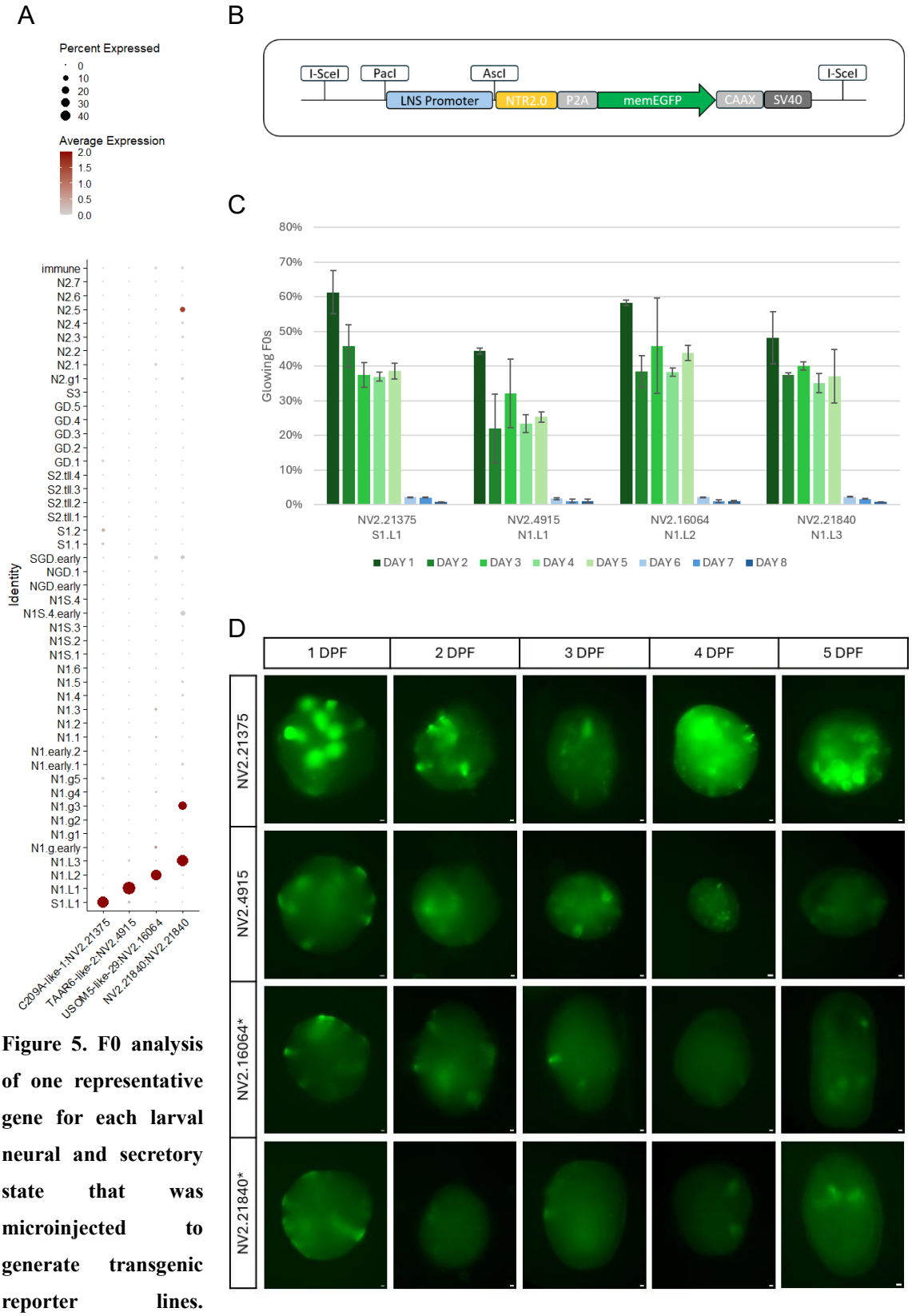

Figure 5. F0 analysis of one representative gene for each larval neural and secretory state that was microinjected to generate transgenic reporter lines. **(A)** Dot plot representation of expression profiles of up-regulated genes of interest across each neuroglandular cluster. Originally provided by Linda Kloss, additionally modified. **(B)** Scheme of the microinjected constructs. The larval neuron specific (LNS) promotor with the restriction sites *PacI* and *AscI*

was cloned into a NTR2.0 (ablation) and memEGFP (separated by the self-cleaving peptide P2A) containing vector. The construct was flanked by *I-SceI* cutting sites for microinjection. **(C)** The graph depicts the percentage of the counted number of microinjected animals that are showing a GFP-signal when fluorescently live-cell imaged over 8 days after fertilization and microinjection. n = 80-160. Two rounds of microinjections were counted separately; bars represent an average of these results. Both the larval stage (green) and the polyp stage (blue) were normalized against autofluorescence using *Nematostella* wildtype. **(D)** Fluorescence live-cell imaging of with the GFP-construct microinjected animals (F0s). For the genes *NV2.21375* and *NV2.4915* the images show the same animal respectively; the asterisk (*NV2.16064* and *NV2.21840*) indicates that the images for each time point were taken of different animals. Scalebar: 10 µm. dpf = days post fertilization.

Fluorescence live-cell imaging of the microinjected F0 generation shows a mosaic expression where the transgene is only expressed in some of the cells of the animal. Nevertheless, for every reporter gene (*NV2.21375*, *NV2.4915*, *NV2.16064* and *NV2.21840*) a GFP-signal in every time point between one and five days after fertilization can be detected (Fig. 5D). Planula larvae are difficult to capture via fluorescence live-cell imaging due to their ability to swim. Before imaging the dishes containing the animals, they were put on ice to slow down swimming of the planula larvae, but no reagents were used to ensure survival; this had a negative effect on the resolution of the images. However, an ectodermal expression of the microinjected constructs labelling larval specific neurons could still be observed.

In order to verify whether the chosen reporter genes are truly only expressed in *Nematostella* larvae and not in the primary polyp, I counted the presence of GFP signal in the microinjected animals over the first eight days after fertilization (Fig. 5C). The graph depicts the percentage of the counted number of animals which were previously microinjected with the corresponding reporter gene construct that are showing a GFP-signal ("Glowing F0s") when fluorescently live-cell imaged. There were two rounds of microinjections performed separately; the average of the counted results is represented by the bars. Both the larval stage and the polyp stage were normalized against autofluorescence using *Nematostella* wildtype. The percentage of planula larvae (green) that express the microinjected construct drastically reduces from over 20-40% on day 5 after fertilization to 0-2% on day 6 after fertilization when the larvae transition through subtle metamorphosis to the primary polyp (blue) (Fig. 5C). This absence of GFP

expression in the primary polyp which was observed through fluorescence live-cell imaging needed to be validated using immunostaining assays against GFP.

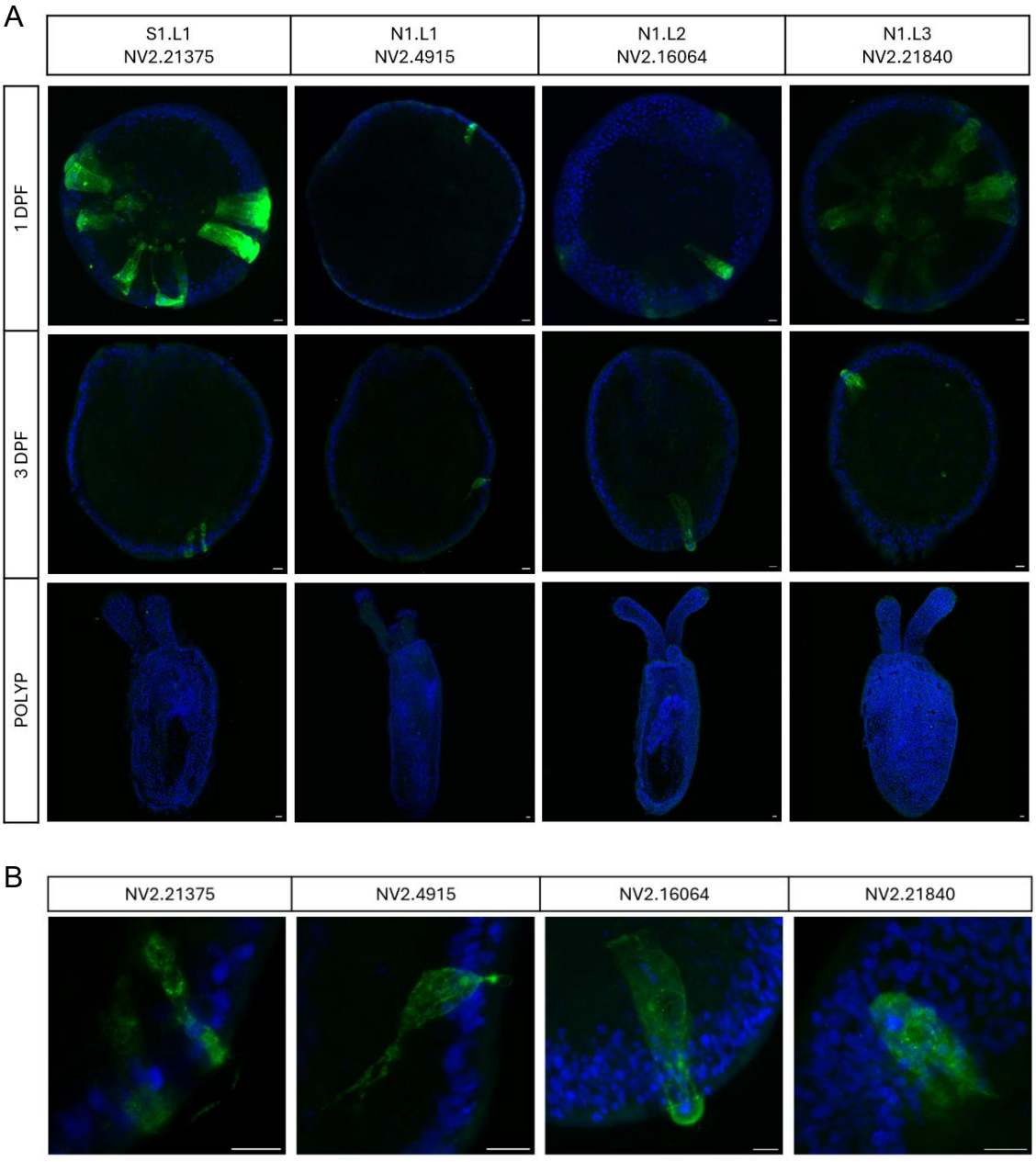

Figure 6. Immunostaining of one representative gene for each larval neural and secretory state that was microinjected to generate transgenic reporter lines. F0 generation. Scalebar: 10 µm. dpf = days post fertilization. **(A)** Merged images of GFP-labelled larval specific neurons (green) and DAPI staining (blue) of planula larvae (1dpf and 3dpf) and primary polyps (8-9dpf). **(B)** Close-up of GFP-labelled cells corresponding to imaged planula larvae (3dpf).

The reporter gene constructs were microinjected once more, and the larvae were fixed at one and three days after fertilization; for fixation of primary polyps the same animals from the quantitative F0 analysis were used. Immunostaining against GFP (green) confirmed that the expression of the larval neuron specific reporter genes occurs only in the planula larvae and not in 8-9 days old primary polyps (Fig. 6A). Moreover, we show that distinct cells are labelled which sit upright in the epithelium and have an apical cilium (Fig. 6B). Since in mosaic transgenic F0 *Nematostella* larvae often only one or a few cells integrate the microinjected construct not much can be concluded regarding interaction of neurons. Future immunostaining of the transgenic F1 generation might give more insight into neural connections and the basiepithelial meshwork that is formed.

3.2 Larval neural cluster analysis by in situ hybridization

In situ hybridizations of specific genes belonging to the three larval neural states (N1.L1, N1.L2 and N1.L3) were performed to compare the expression pattern between each cluster and among clusters themselves. In choosing the genes of interest for designing the probes for in situ hybridization I again prioritized cluster specificity based on expression profiles of up-regulated genes across each neuroglandular cluster (Fig. 7).

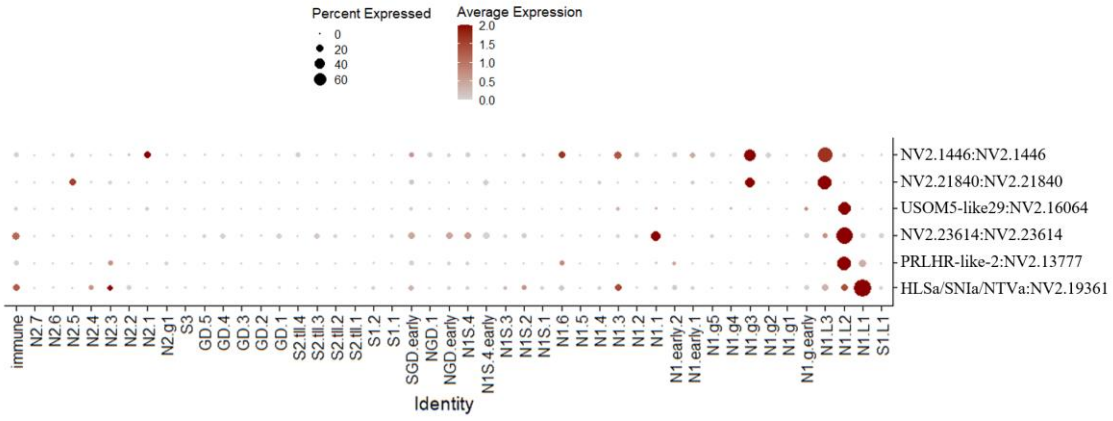

Figure 7. Genes of interest of larval neural states for in situ hybridization. Dot plot representation of expression profiles of up-regulated genes of interest across each neuroglandular cluster. Originally provided by Linda Kloss, additionally modified.

Due to the previously mentioned tendency of larval neural state N1.L2 to subcluster into two clusters when changing parameter settings all three gene candidates were examined. *NV2.13777* and *NV2.23614* cluster in their subclusters, respectively, whereas *NV2.16064*

is expressed similarly in both subclusters. Additionally, I sought to compare the two most larval neural state N1.L3 specific gene candidates (*NV2.21840* and *NV2.1446*) since they show a similar expression profile even though they are both also expressed in different clusters like N1.g3.

Based on single cell RNA sequencing data we expect that the three larval neural states appear at gastrulation and mostly disappear once the planula transitions into the tentacle-bud stage on day 5 after fertilization (Cole et al., 2024; Steger et al., 2022).

3.2.1 Larval neural state N1.L1

For the larval neural state N1.L1 in situ hybridization of the cluster specific gene *NV2.19361* was performed. The expression pattern of *NV2.19361* is ectodermal and occurs in the mid body of the larvae one day after fertilization, it broadens with the elongation of the planula during day 2 (Fig. 8). On day 3 the expression appears to be restricted from the mid boy towards the aboral side, which switches on day 4 to the oral side, but with much less signal. As expected, there is no expression of this larval neuronal gene present in the primary polyp (6 and 7 days after fertilization).

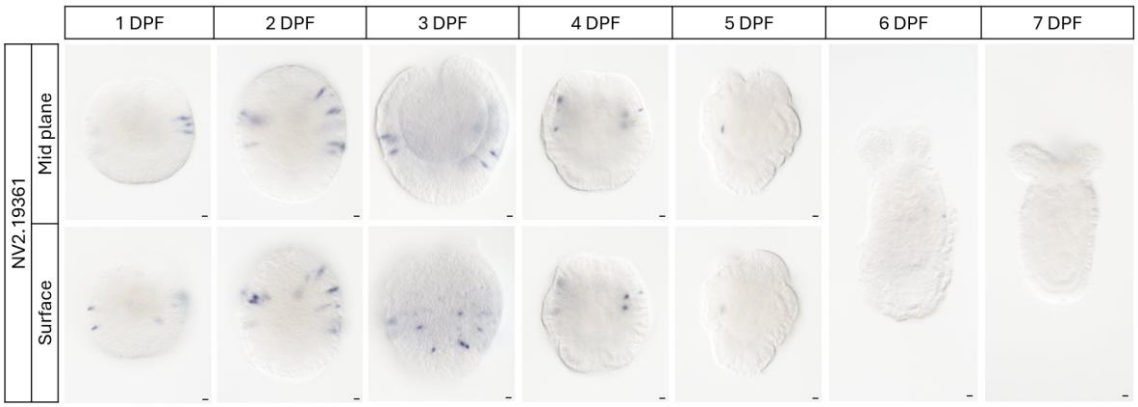

Figure 8. In situ hybridization in *Nematostella* of larval neural state N1.L1. NV2.19361. Orientation: oral to aboral (top to bottom). Scalebar: 10 µm. dpf = days post fertilization.

3.2.2 Larval neural state N1.L2

The larval neural state N1.L2 appeared to be more diverse and sometimes clustered into two different subclusters in the single cell RNA sequencing analysis. Therefore, three different genes were chosen (*NV2.13777*, *NV2.23614* and *NV2.16064*).

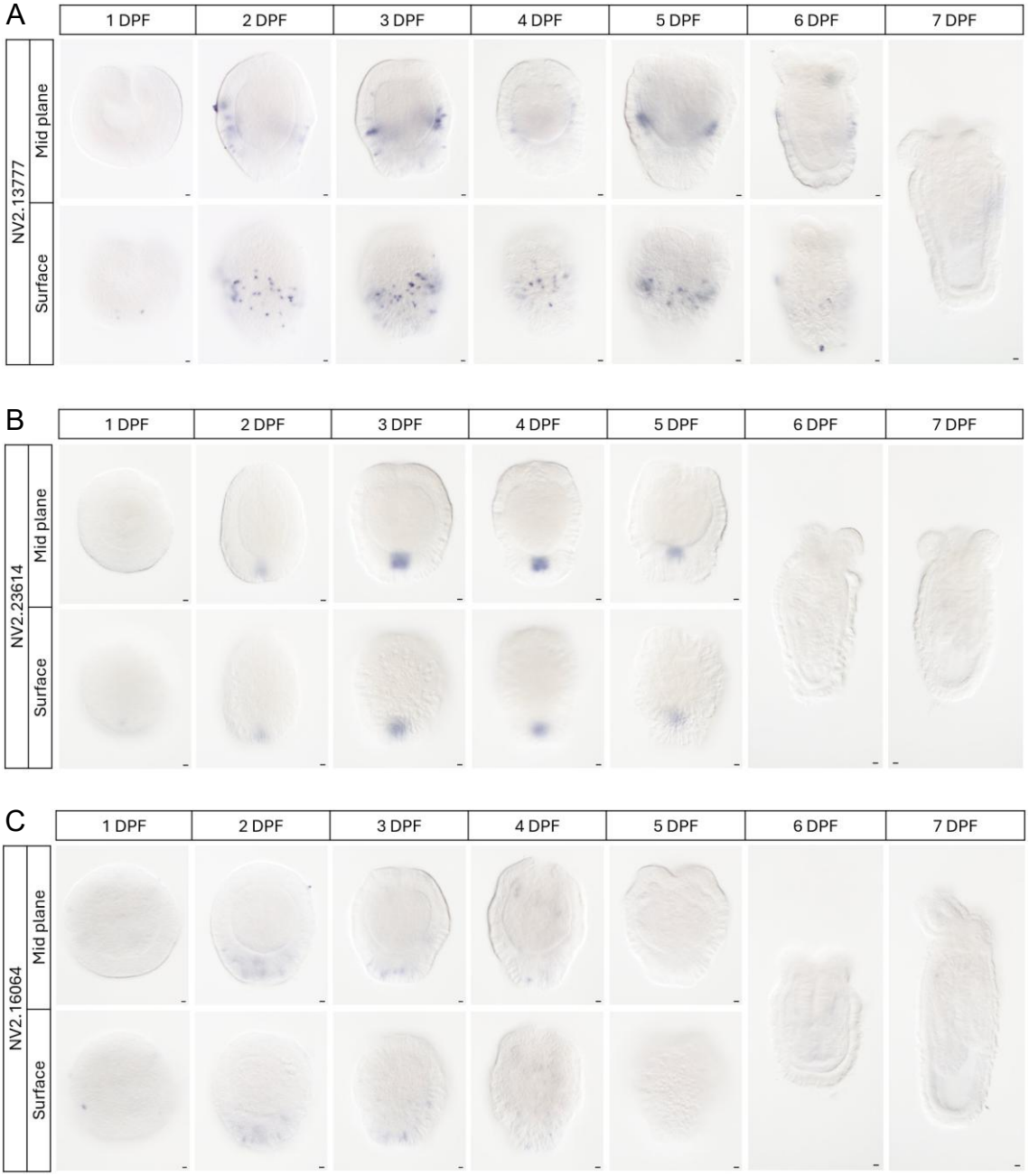

Figure 9. In situ hybridization in *Nematostella* of larval neural state N1.L2. (A) *NV2.13777*. **(B)** *NV2.23614*. **(C)** *NV2.16064*. Orientation: oral to aboral (top to bottom). Scalebar: 10 µm. dpf = days post fertilization.

The single cell RNA sequencing data analysis of *NV2.13777* and *NV2.23614* showed them clustering in two separate subclusters, whereas *NV2.16064* was expressed in both subclusters. Furthermore, the Technau group recently proposed that N1.L2 localizes to the aboral pole due to co-expression of *six3/6* or that it might form part of the apical organ (Cole et al., 2024). The cell state that corresponds to the apical organ in the aboral domain occurred only in the 1-5d samples of single cell RNA sequencing data and disappeared again in the 8d and 16d polyp samples (Cole et al., 2024).

In situ hybridization showed ectodermal expression of *NV2.13777* that starts two days after fertilization and spans towards the aboral half of the animal (Fig. 9A). It is barely present on day 6 after fertilization and disappears in the primary polyp on day 7.

NV2.23614 expression is constricted to the apical organ of the larvae and only being expressed between day 2 and 5 (Fig. 9B). *NV2.16064*, which is assigned to both subclusters, is expressed in the aboral ectoderm between day 2 and 4 and cannot be found in the primary polyp (Fig. 9C).

All three genes share the characteristics of predominantly aboral expression and no localization on the oral pole can be observed. Whereas the expression pattern of *NV2.13777* is broader and more localized in the midbody, extending aborally, but excluded from the most aboral domains and therefore also the apical organ (Fig. 9A), *NV2.23614* expression is restricted to the apical organ (Fig. 9B). The gene *NV2.16064,* which is expressed equally in both subclusters, represents a combination of both expression patterns for the reason that it is restricted more towards the aboral pole but still has a broader expression pattern than *NV2.23614* also outside of the apical organ domain (Fig. 9C). Considering these results, we propose that the sub-clustering may be based on the localization of the larval neurons rather than on them having a different cell state identity.

3.2.3 Larval neural state N1.L3

In contrast to the larval neural states N1.L1 and N1.L2, may the larval neural state N1.L3 persist into the adult polyp since a few cells are still found in tentacle and body wall adult samples (Cole et al., 2024). In situ hybridizations of two cluster specific genes *NV2.21840* and *NV2.1446* were performed.

Even though the single cell data showed a similar expression for both genes, in situ hybridizations suggest contradicting expression patterns (Fig. 10A,B).

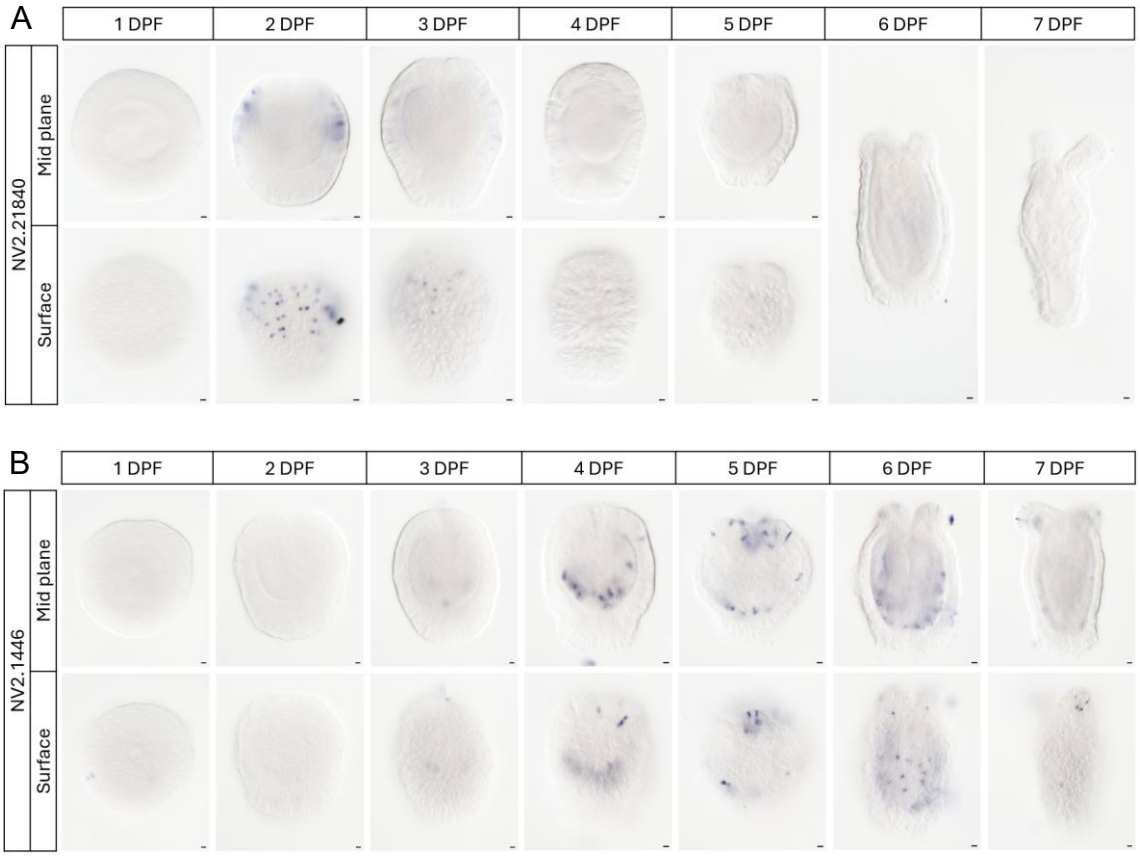

Figure 10. In situ hybridization in *Nematostella* of larval neural state N1.L3. (A) *NV2.21840*. **(B)** *NV2.1446*. Orientation: oral to aboral (top to bottom). Scalebar: 10 µm. dpf = days post fertilization.

NV2.21840 is strongly expressed two days after fertilization with staining still present on day 3 in single cells of the mid-body of the planula larva (Fig. 10A). By contrast, *NV2.1446* expression starts only on day 4 after fertilization and continues into the primary polyp (Fig. 10B). *NV2.1446* expression is the strongest between day 4 and day 6 after fertilization.

Besides the temporal complementarity to *NV2.21830*, *NV2.1446* expression also differs starkly by its spatial pattern: it is expressed in an aboral mesodermal domain as well as an ectodermal domain in the tentacle buds and tentacles of the primary polyp (Fig. 10B). Although the staining of *NV2.1446* does get weaker on day 7, it is still present in the primary polyp.

3.2.4 Conclusion of the larval neural cluster analysis

The performed in situ hybridizations verified previously published single cell RNA sequencing data that proposed larval specific neurons in *Nematostella vectensis*. As expected, larval neural states N1.L1 and N1.L2 specific genes are only expressed in the planula larvae of *Nematostella* and disappear in the primary polyp after subtle metamorphosis occurs (Fig. 11). Cole et al., 2024 additionally stated that cells clustered in the larval neural state N1.L3 are mostly larval specific but some may persist into the adult polyp. Here we showed that one gene specific to N1.L3 does in fact label larval neurons (Fig. 10A) but another gene with similar expression profile (Fig. 7) is still present in the primary polyp (Fig. 10B) which is consistent with the single cell RNA sequencing data.

Previous studies proposed that neurogenesis likely starts at the late blastula stage in an aboral territory with the oral cap lacking neurons and only after gastrulation neurons appear in the oral half of the planula larvae and in the endomesoderm (Kelava et al., 2015). This neurogenesis switch cannot be observed in the performed in situ hybridizations because nearly all genes of interest did not show any staining in the gastrula stage (Fig. 11). Solely *NV2.19361*, belonging to the larval neural state N1.L1, exhibited an expression pattern 24h after fertilization which was localized neither at the oral nor aboral pole but rather at the midbody. Additionally, no expression at the most oral part of the planula larvae can be observed.

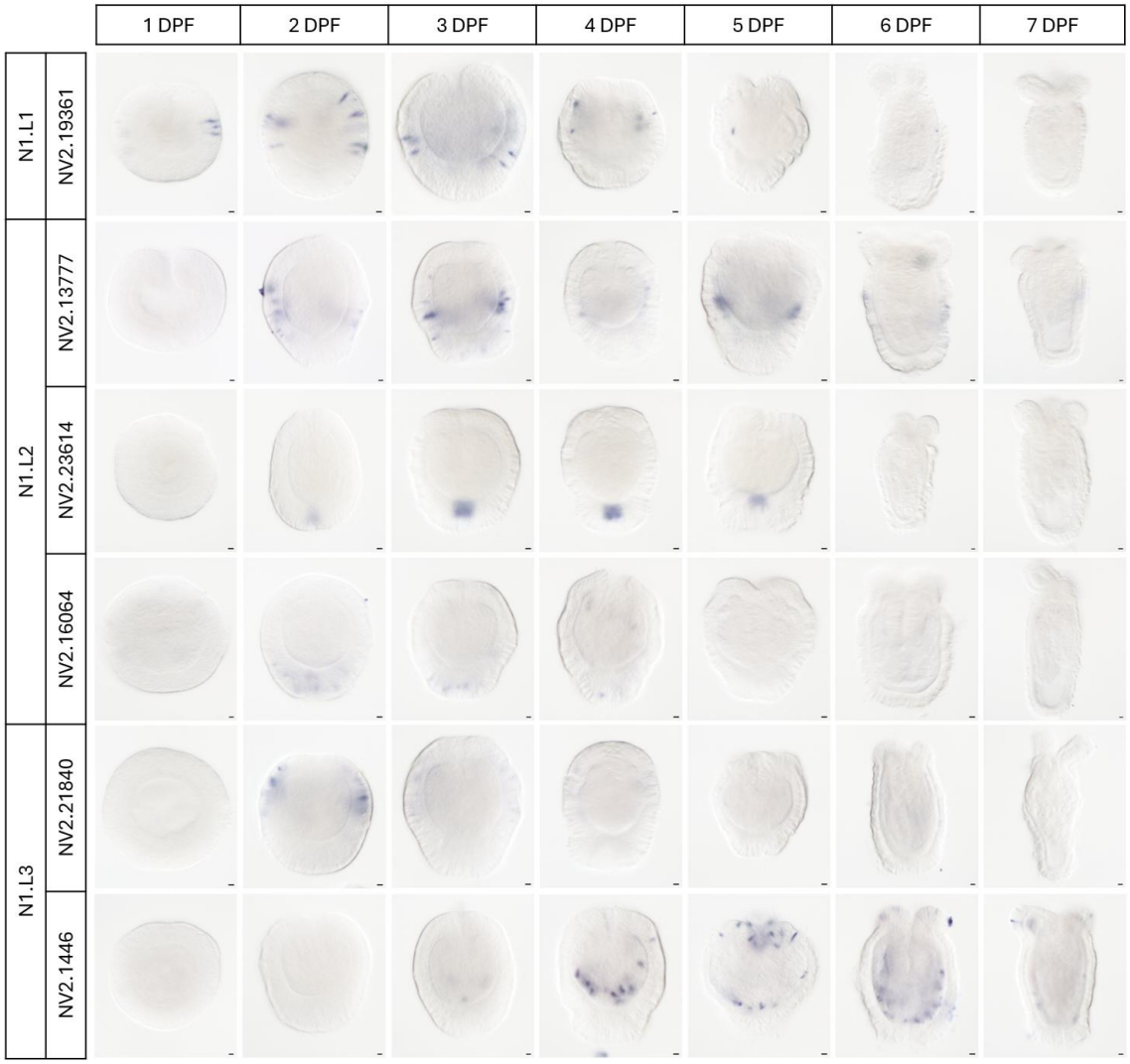

Figure 11. Overview of in situ hybridization in *Nematostella* of larval neural states N1.L1, N1.L2 and N1.L3. Orientation: oral to aboral (top to bottom). Scalebar: 10 µm. dpf = days post fertilization.

The expression pattern of larval neural state N1.L2 was suggested to localize to the aboral pole due to co- expression of *six3/6* or that it might form part of the apical organ (Cole et al., 2024). This aboral expression of genes from the larval neural state N1.L2 can be observed in *NV2.23614* and *NV2.16064*; only *NV2.13777* is mainly expressed in the midbody of the planula larvae (Fig. 11). Nevertheless, some cells are still expressed aborally in *NV2.13777*, whereas no oral expression is visible.

Additionally, at least one gene (*NV2.23614*) of larval neural state N1.L2 does localize with the apical organ (Fig. 9B) which is characterized by the specific spot-like expression at the most aboral domain (Rentzsch et al., 2008; Gilbert et al., 2022). Single cell RNA sequencing data predicted that the cell state that corresponds to the apical organ occurs in

the 1-5d samples and disappears again in the 8d and 16d polyp samples (Cole et al., 2024). This is validated by the in situ hybridization result of *NV2.23614* where the expression occurs precisely only during the planula stage and vanishes after metamorphosis into the primary polyp (6 days after fertilization).

In conclusion, the presented outcomes are congruent to the expectations based on previously published single cell transcriptomes data. Nevertheless, we do recognize the need for further validation of the larval neural states through additional in situ hybridizations of different larval neural markers. Moreover, the generation of transgenic reporter lines will be beneficial for additional confirmation of the larval neural states.

4 Discussion

4.1 Key findings

Single cell RNA sequencing data published by Cole et al., 2024 proposed that in *Nematostella vectensis* neurons exist which are only present in the *Nematostella* larvae and disappear after metamorphosis into the polyp stage. There were three larval neural states (N1.L1, N1.L2 and N1.L3) and one larval secretory state (S1.L1) described of which only some cells of the larval neural state N1.L3 persist into the adult polyp (Cole et al., 2024).

This project aimed to characterize these larval neurons. Therefore, the fundamental work to generate seven different larval neural specific transgenic reporter lines was performed. Focusing on one representative gene candidate per larval neural and secretory state we showed that the constructs that were microinjected into wildtype *Nematostella* fertilized eggs only label cells in *Nematostella* larvae (one to five days after fertilization). This was quantitatively verified in a larger scale fluorescence live-cell imaging experiment that counted the presence of larvae or primary polyps which showed a GFP-signal after microinjection with the designed constructs containing larval neuron specific promotors together with memEGFP. Moreover, immunostainings of planula larvae (1 and 3 days after fertilization) and primary polyps (8 to 9 days after fertilization) were performed to confirm the observed signal excluding the possibility of autofluorescence and get insights into the morphology of the labelled cells.

Furthermore, in situ hybridizations of various genes belonging to the three larval neural states (N1.L1, N1.L2 and N1.L3) were performed to get insight on the question why they might cluster separately and identify differences and similarities of expression patterns between each cluster and among clusters themselves. My results confirmed that there indeed was no expression of genes belonging to the larval neural states N1.L1 and N1.L2 in primary polyps but only in *Nematostella* larvae. The diversity regarding expression patterns of larval neural state N1.L2 is congruent with the fact that larval neural state N1.L2 did subcluster into two separate clusters depending on the parameter settings of the single cell data cluster analysis. Evaluating the expression patterns of the genes of

interest regarding the larval neural state N1.L2 we propose that the sub-clustering may be based on the sightly varied localization of the larval neurons rather than on them having a different cell state identity. We additionally confirmed that there is one gene specific to the larval neural state N1.L3 that is also expressed in the tentacle bud and in the tentacles of the primary polyp, aligning with some N1.L3 specific cells still being present in tentacle and body wall adult samples of the single cell data. Nevertheless, we presented that there are also genes belonging to the larval neural state N1.L3 that are restricted to the *Nematostella* larvae.

In conclusion, we verified the presence of neurons which are only expressed in the *Nematostella* larvae and not in the primary polyp and confirmed previously published single cell RNA sequencing data describing these larval neural states.

4.2 Limitations

Gene constructs for seven different transgenic reporter lines labelling larval specific neurons were cloned and microinjected into fertilized wildtype *Nematostella* eggs. Since the generation time of the microinjected animals exceeded the time of my project, no transgenic F1 animals could be generated yet.

Although we showed that the designed constructs for the transgenic reporter lines label larval neurons, there are limitations in investigating F0 generations. F0 animals have a mosaic expression because the modified gene is not consistently expressed in all cells. Furthermore, epichromosomal expression directly from the plasmid can be possible due to the lack of integration into the genome. This leads to difficulties in interpretating their expression or phenotype. Additionally, since for the most part only a few cells express the transgenic gene, no conclusions can be made on the overall expression pattern of the whole organism and especially in this case no connections and network can be observed between transgenic neurons themselves.

To quantitatively prove that the larval neural and secretory state specific genes are only expressed in *Nematostella* larvae they were counted while fluorescence live-cell imaging which may result in not reliable signals due to autofluorescence of the larvae and polyps. Therefore, both the larval stages and the polyp stages were normalized against

autofluorescence using *Nematostella* wildtype. Moreover, immunostainings against GFP were performed for further validation.

During the larval neural cluster analysis that was performed using in situ hybridizations we encountered several obstacles that could not be resolved in a timely manner during this project resulting in fewer representative neural markers per cluster. Furthermore, the in situ hybridization expression patterns that could be observed would benefit from stronger staining to reliably conclude that no expression occurs in the primary polyp of *Nematostella*. Therefore, we recognize the need for further validation of the larval neural states through additional in situ hybridizations of larval neural markers to be able to make more generalized statements about possible differences and similarities of expression patterns between each cluster and among clusters themselves.

Lastly, while we aim to characterize larval neurons in *Nematostella* we acknowledge that this study does not include experiments to investigate the function of larval neurons, and the reason why they disappear in the *Nematostella* primary polyp. Based on the fact that *Nematostella* larvae are free-swimming and lose this ability after transition into the primary polyp, we propose that larval neurons might contribute to planula swimming. Nevertheless, by including NTR2.0 into the transgenic construct that was microinjected, we set the ground work for further ablation experiments for studying the function of these larval neurons.

4.3 Outlook

As previously mentioned, no transgenic F1 animals for investigating larval neurons could be generated yet. At this moment the F0 animals have been raised and started to produce eggs or sperm but still need to be screened in order to obtain the F1 generation. The F0 animals are regularly induced and kept in cycle and will be screened by a different member of the Technau Group.

The presented experimental tools that were performed on F0 transgenic animals will be repeated after obtaining the F1 generation. With no limitations regarding mosaic *Nematostella* larvae and primary polyps a broader picture of the expression profile of larval neural and secretory state specific genes can be observed. Furthermore, the connectivity of these larval neurons can be studied. Due to the integration of NTR2.0 into

the transgenic construct cell ablation experiments can be easily performed adding a non-toxic prodrug.

In addition to ablation experiments, the function of larval neurons can be studied using shRNA or morpholino knock-down experiments which have already been established in *Nematostella*.

Lastly, behavioral experiments which have been established in a different model organism have been planned to track the swimming of the *Nematostella* larvae. This will potentially clarify whether ablating larval neurons of specific larval neural states will influence *Nematostella* larvae swimming behavior and therefore give more insight into the function of larval neurons.

5 References

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6 Supplementary Figures

6.1 Clustering of the neuroglandular population

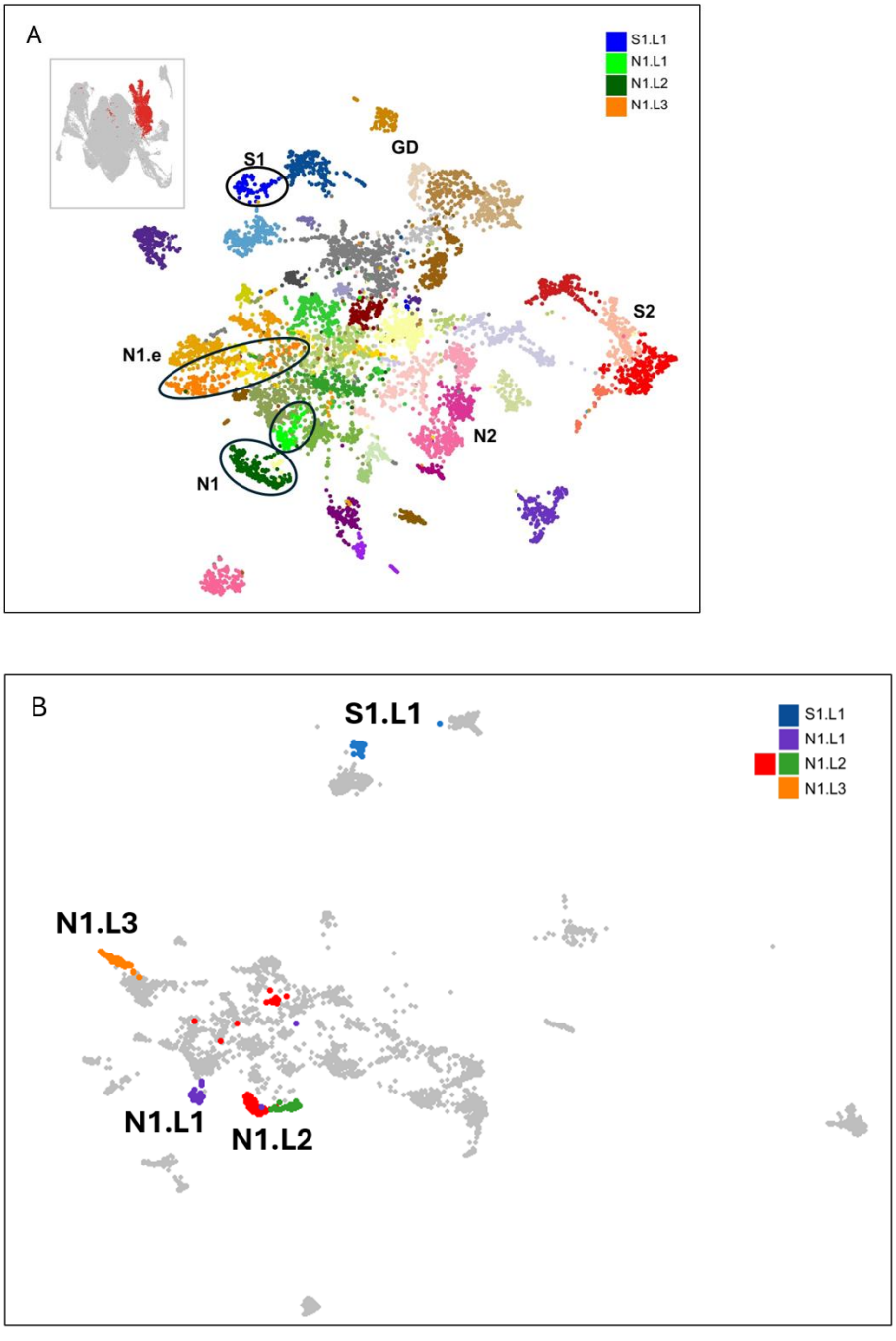

Figure 12. Two different clustering (UMAP) results of distinct cell states of neuroglandular population in *Nematostella*. (A) Modified from Cole et al., 2024. Clustering shows three larval neural states (N1.L1, N1.L2 and N1.L3) and one larval secretory state (S1.L1). **(B)** Modified from Linda Kloss, unpublished data. N1.L2 clusters into two separate subclusters (red and green).

6.2 Expression profiles of upregulated genes in the larval specific states

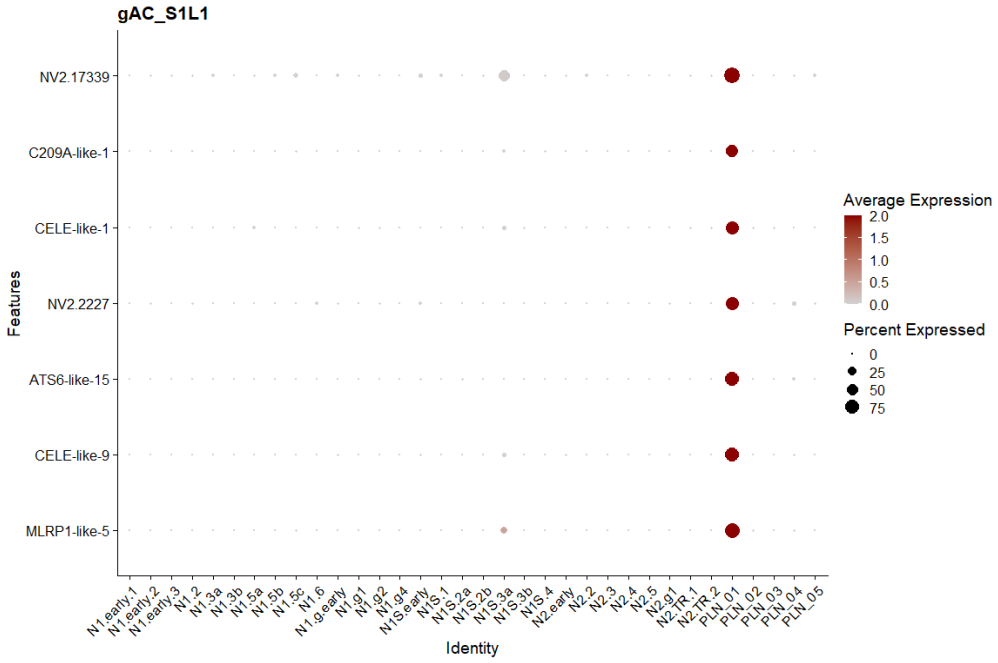

Figure 13. Dot plot representation of expression profiles of up-regulated genes of the larval secretory state S1.L1. Provided by Linda Kloss.

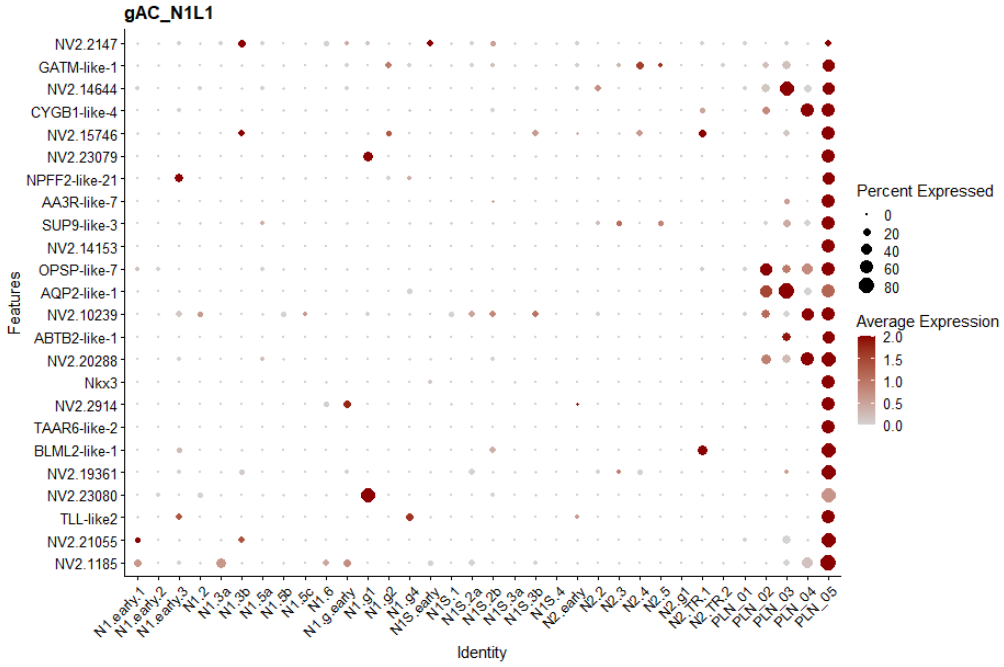

Figure 14. Dot plot representation of expression profiles of up-regulated genes of the larval neural state N1.L1. Provided by Linda Kloss.

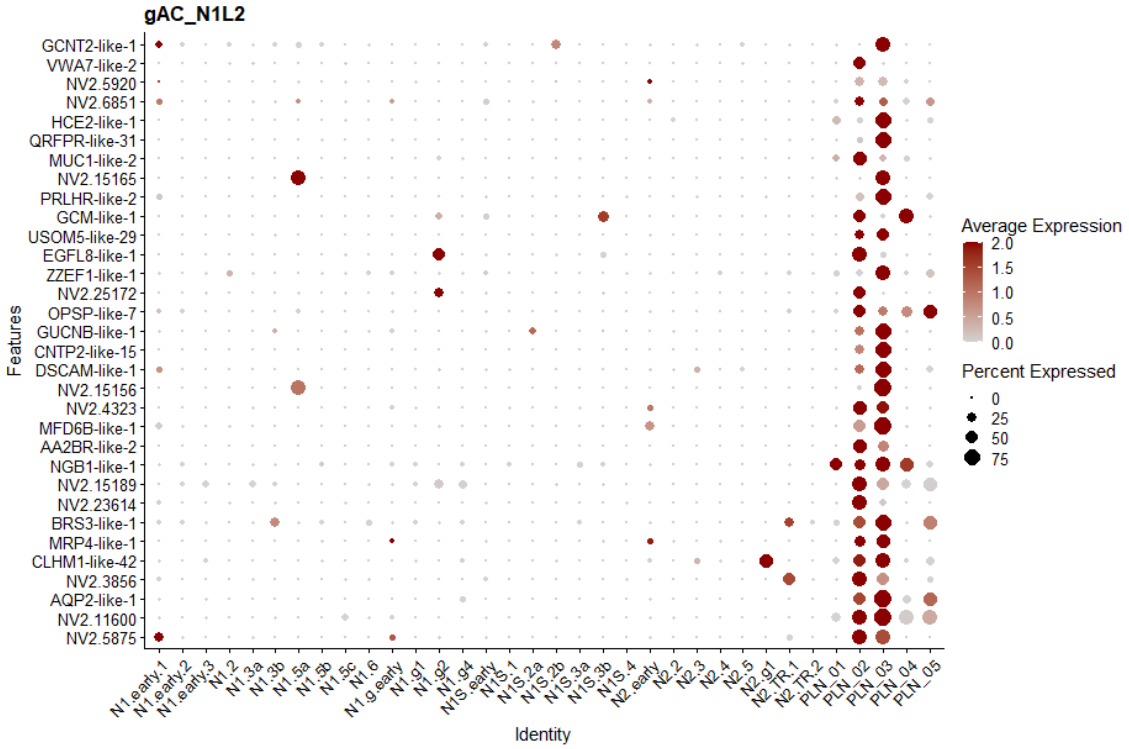

Figure 15. Dot plot representation of expression profiles of up-regulated genes of the larval neural state N1.L2. Provided by Linda Kloss.

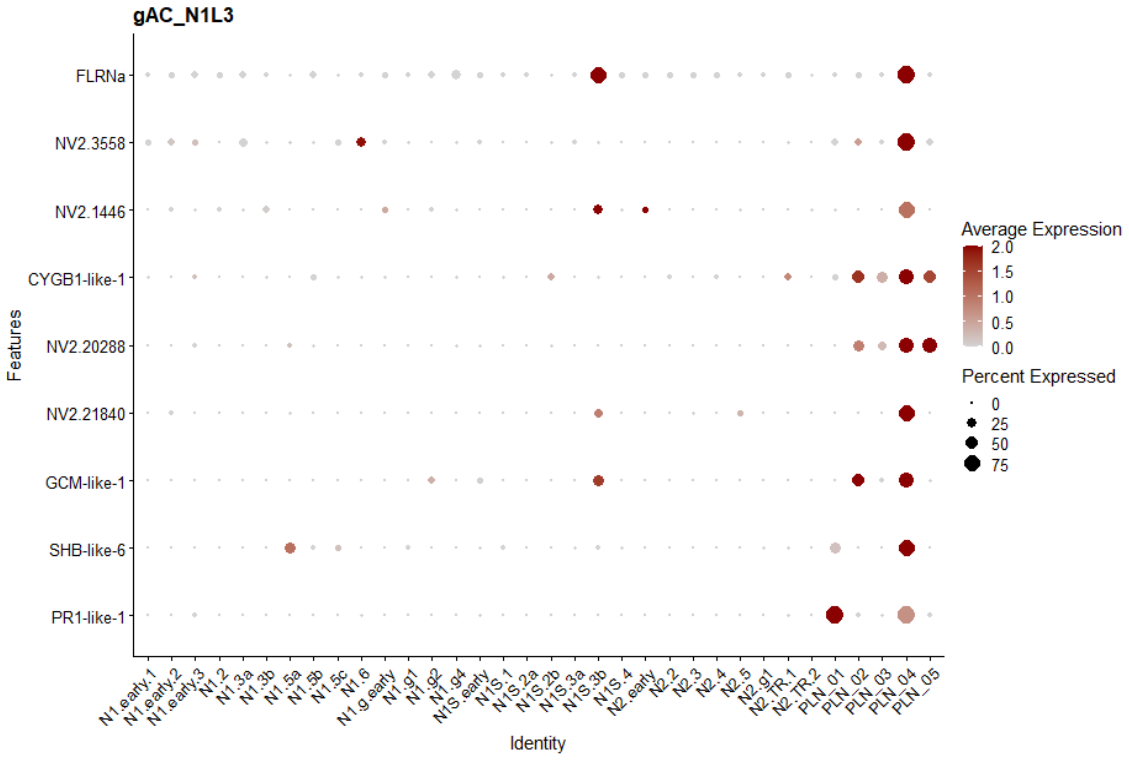

Figure 16. Dot plot representation of expression profiles of up-regulated genes of the larval neural state N1.L3. Provided by Linda Kloss.

6.3 Fluorescence live-cell imaging

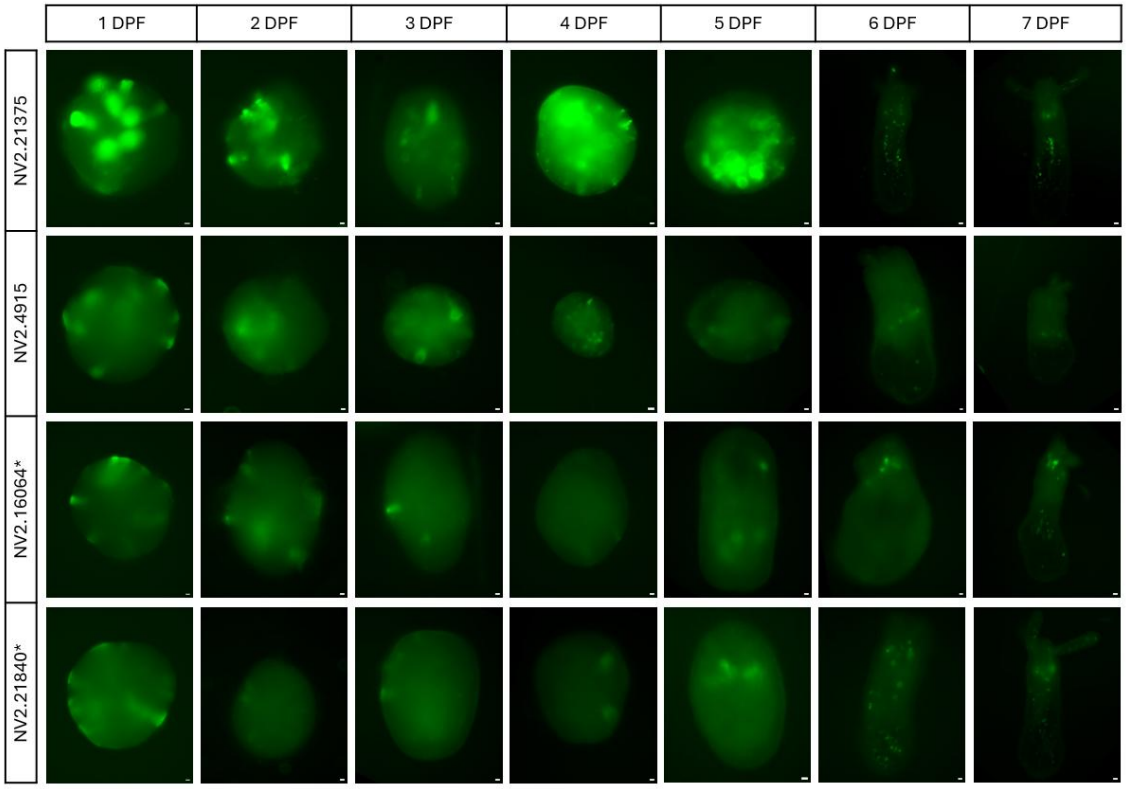

Figure 17. Fluorescence live-cell imaging of one representative gene for each larval neural and secretory state that was microinjected to generate transgenic reporter lines. Larval neurons are labelled green (GFP). For the genes *NV2.21375* and *NV2.4915* the images show the same animal respectively; the asterisk (*NV2.16064* and *NV2.21840*) indicates that the images for each time point were taken of different animals. Scalebar: 10 µm. dpf = days post fertilization. Images of primary polyps (6dpf and 7dpf) show autofluorescence and therefore were excluded from Fig. 5.

6.4 Immunostaining imaging

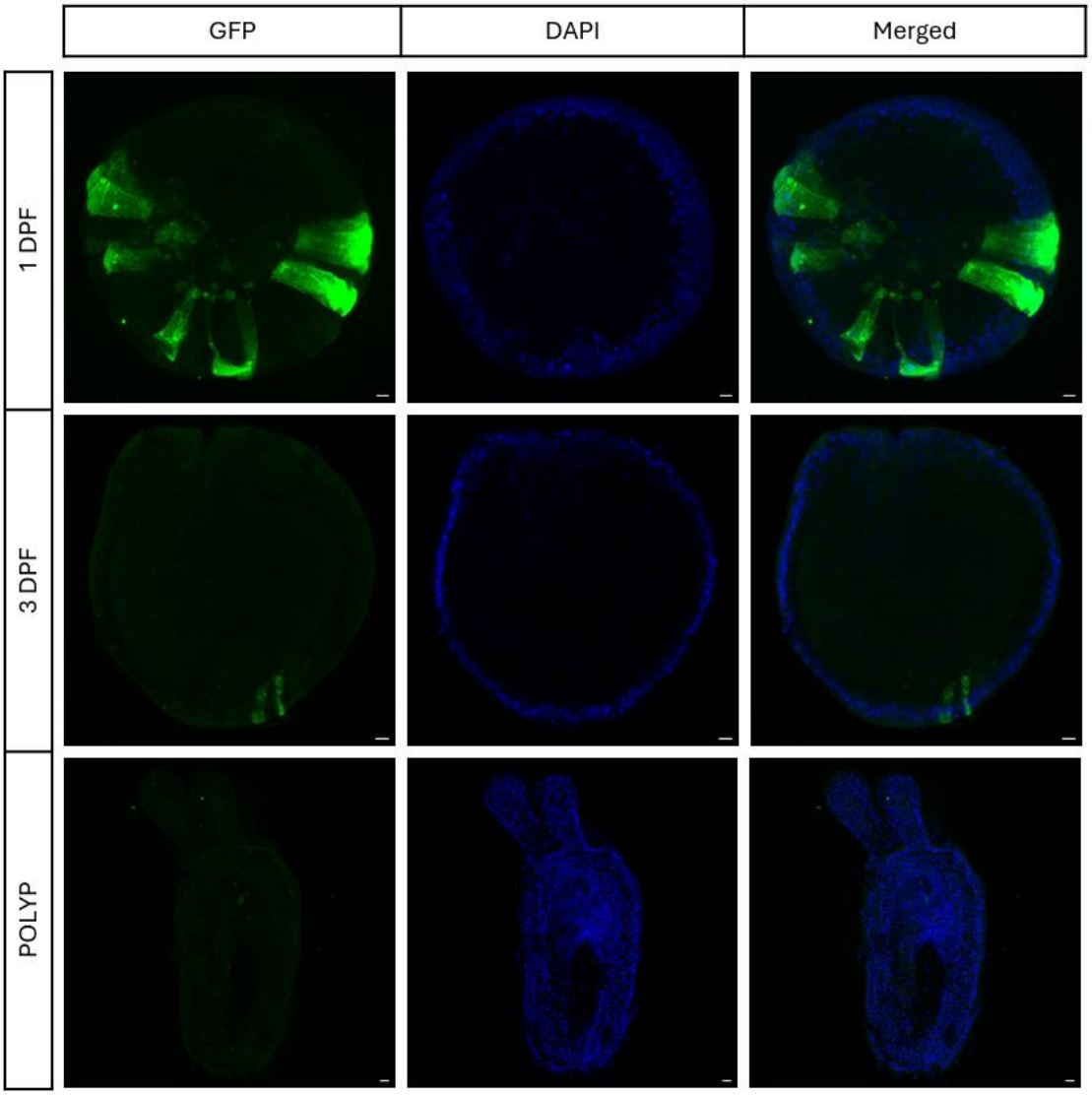

Figure 18. Immunostaining of *NV2.21375* (larval secretory state S1.L1). GFP-labelled larval specific neurons (green) and DAPI staining (blue) of planula larvae (1dpf and 3dpf) and primary polyps (8-9dpf). Scalebar: 10 µm. dpf = days post fertilization.

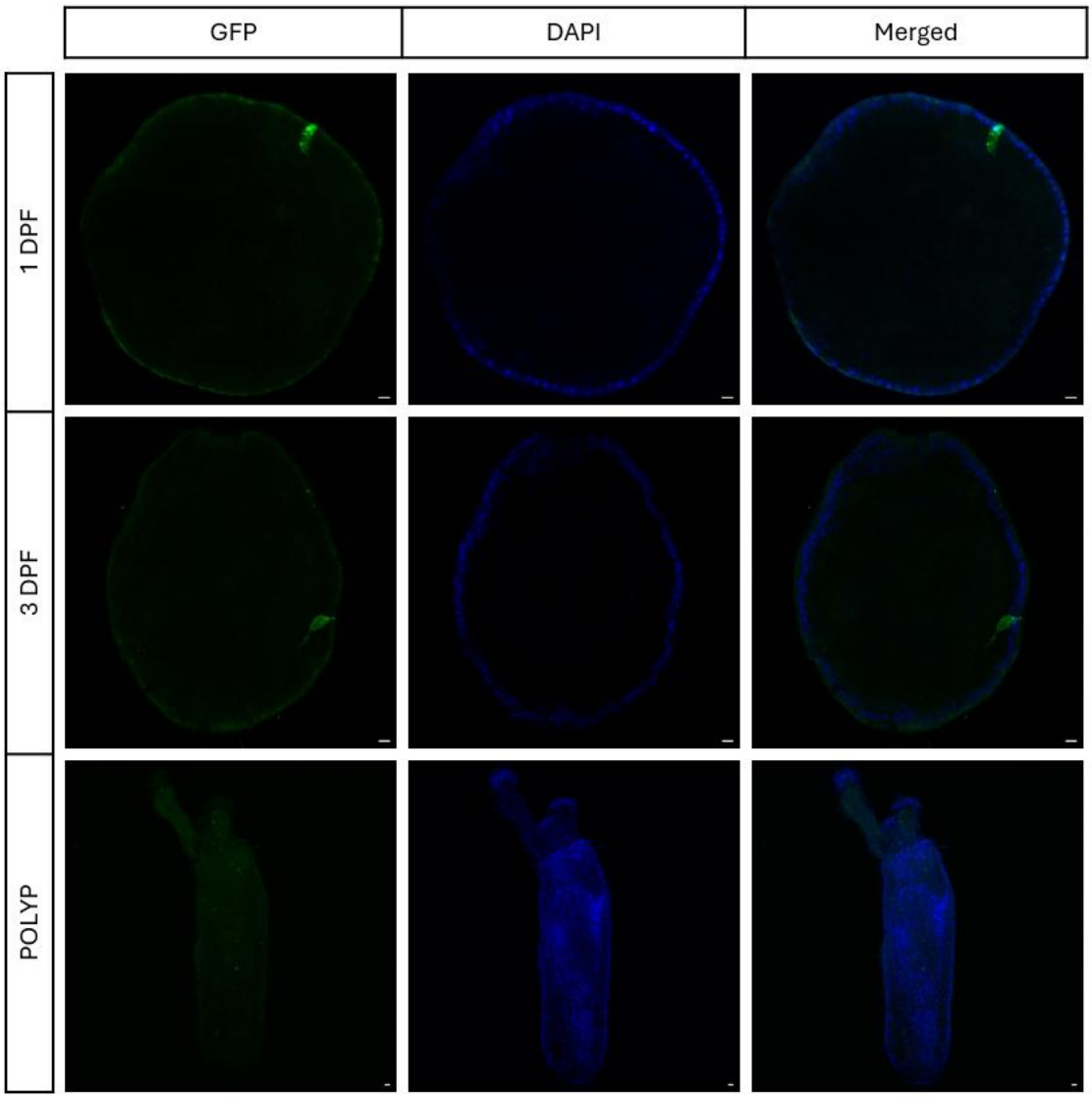

Figure 19. Immunostaining of *NV2.4915* (larval neural state N1.L1). GFP-labelled larval specific neurons (green) and DAPI staining (blue) of planula larvae (1dpf and 3dpf) and primary polyps (8-9dpf). Scalebar: 10 µm. dpf = days post fertilization.

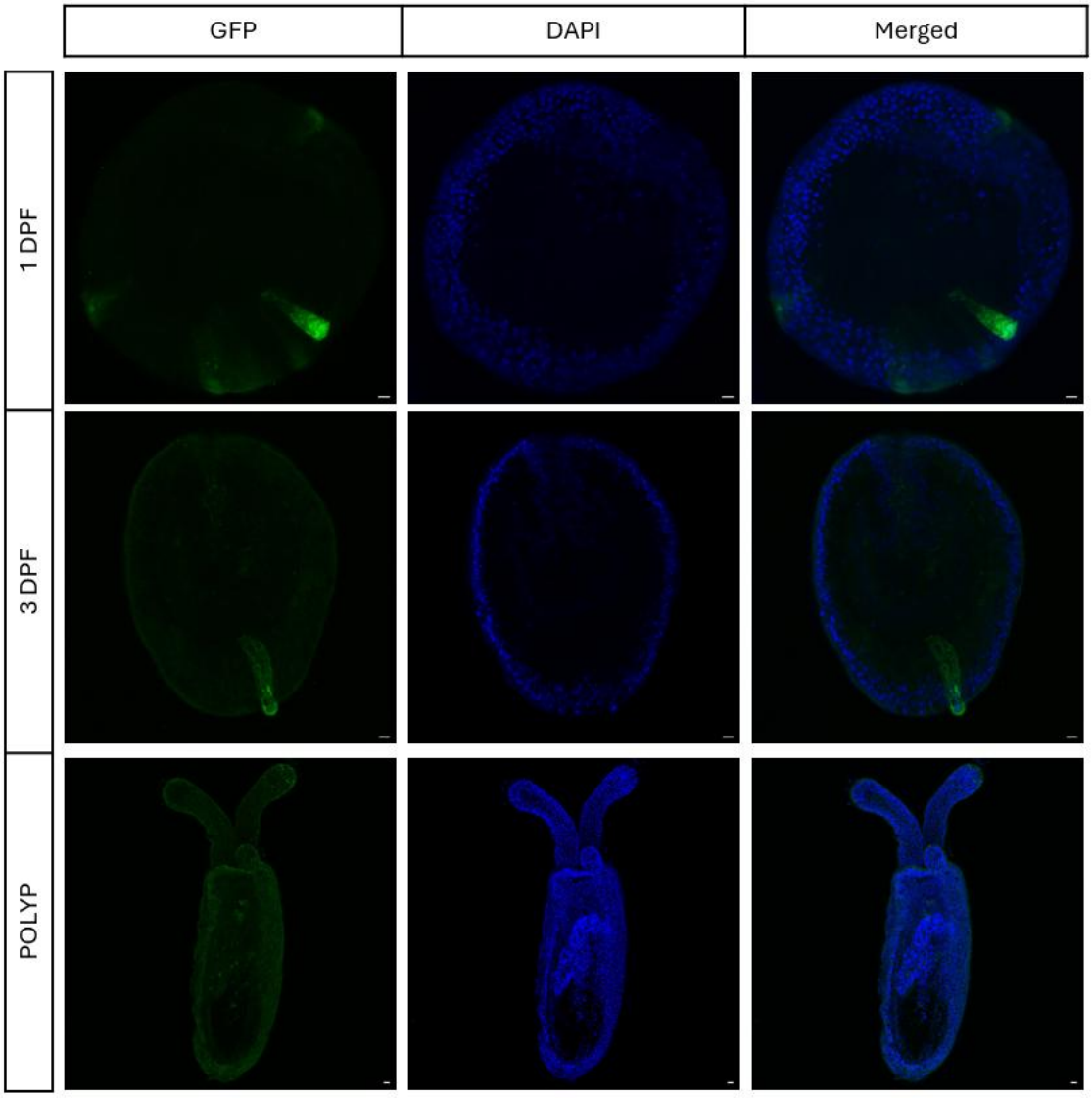

Figure 20. Immunostaining of *NV2.16064* (larval neural state N1.L2). GFP-labelled larval specific neurons (green) and DAPI staining (blue) of planula larvae (1dpf and 3dpf) and primary polyps (8-9dpf). Scalebar: 10 µm. dpf = days post fertilization.

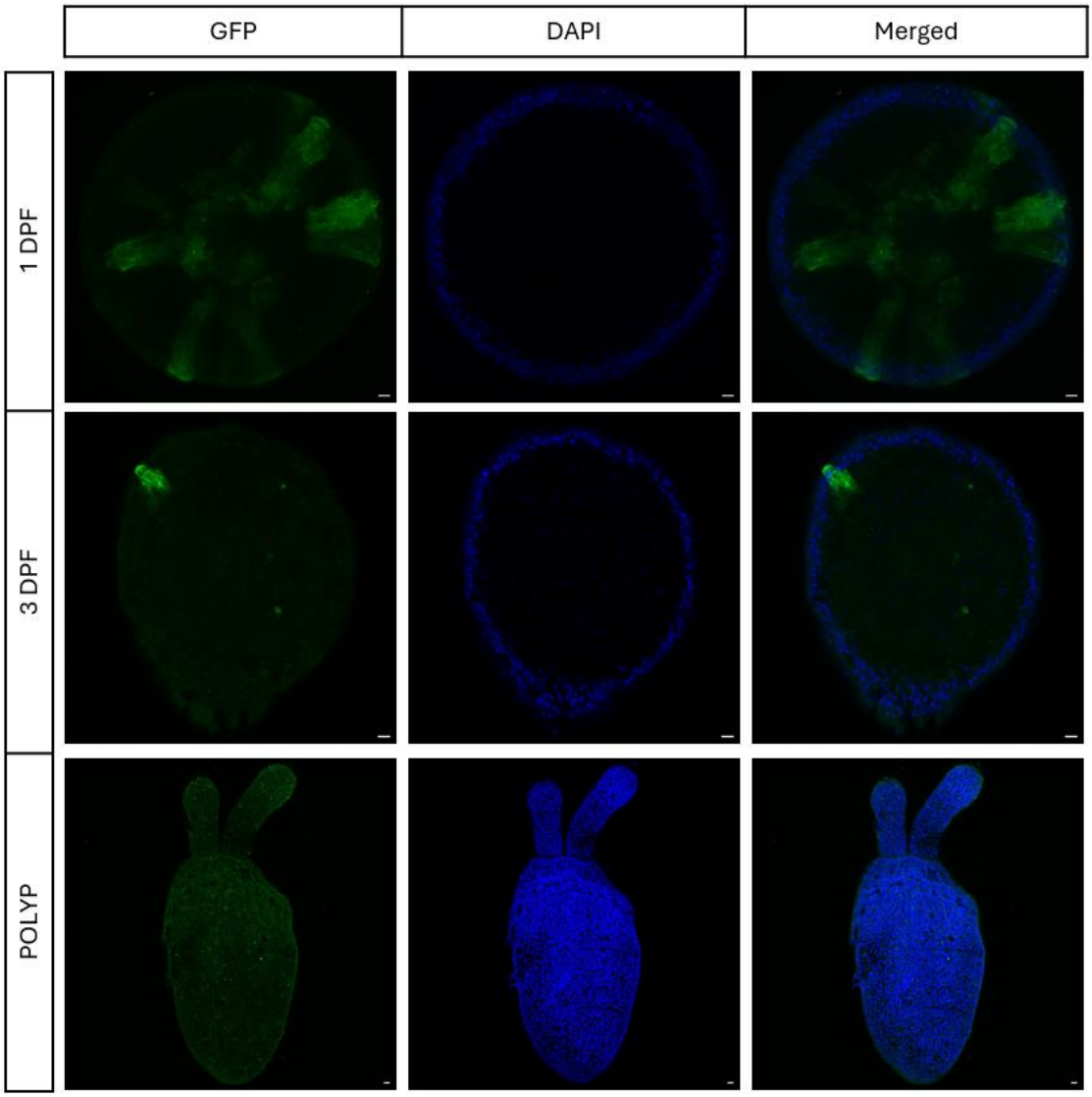

Figure 21. Immunostaining of *NV2.21840* (larval neural state N1.L3). GFP-labelled larval specific neurons (green) and DAPI staining (blue) of planula larvae (1dpf and 3dpf) and primary polyps (8-9dpf). Scalebar: 10 µm. dpf = days post fertilization.