

# **Nerve net formation in *Hydra* embryos – novel approaches and insights**

## **Dissertation**

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## Summary

The fundamental questions about evolution, emergence, functionality and development of the nervous system, as well as its significant impact on disorders and diseases, have been central to scientific research ever since the establishment of the neuron doctrine by Santiago Ramón Cajal. However, the complexity of the nervous system often poses a challenge to addressing these fundamental questions and unraveling its origin and basic functionality during evolution. Hence, model organisms such as *Hydra*, a cnidarian with its comparatively low complexity and self-renewing nervous system, have become emerging models for studying neural evolution and development due to its evolutionary position and accessibility to transgenic lines. In this study various aspects of the *de novo* nerve net formation in *Hydra* embryos during late embryogenesis and in early hatchlings have been explored.

Moreover, characteristics of specific developmental timepoints to gain insights into the nervous system of *Hydra vulgaris* are investigated. Dissociation of whole embryos and hatchlings revealed the absence of neurons in early stages, followed by a significant increase in morphological differentiated neurons during early development, correlating with the emergence of the first contraction behavior. Using antibodies against specific neuropeptides expressed in six out of seven neural populations, the earliest accessible stages of development reveal already existing and network-like connected cells. Electron-microscopic images provide unique insights into cell-cell communication between neuronal and non-neuronal cells via gap junctions and a vesicle-mediated communication. These mechanisms support and drive a rapid assembly of the nerve net during specific developmental stages.

This work employs advanced techniques in handling and maintaining transgenic lines to induce sexual reproduction in *Hydra*. Using calcium indicators and immunohistochemistry, the functionality and topology of the emerging nerve net during the early development of embryos and hatchlings are compared and examined. A developed recording pipeline to perform long-term  $\text{Ca}^{2+}$  activity during early nerve net assembly allows for profound insights in the fundamental processes of nerve net construction and functionality at single-cell resolution within the neuronal-specific N4 population. Furthermore distinct, unsynchronized neuronal networks in different regions of specific developmental stages, which later integrate

into a synchronized nerve net in adult polyps are observed, shedding light on fundamental principles of neural connectivity.

Furthermore, the role of environmental factors in nerve net formation during different developmental stages was investigated. External influences, such as temperature and microbiota, affect nerve net construction, highlighting a degree of adaptability during neurodevelopment. Examinations of temperature-related influences during the formation of the nerve net are shown, revealing that colder temperatures display a beneficial impact on the network assembly, whereas warmer conditions negatively affect the N4 population-specific involvement in the eating behavior, leading to deficits and a 40% mortality rate. Further this work analyzed the microbial influences during embryogenesis by examining the nerve net construction and neuronal branches in wild-type, germ-free and recolonized hatchlings. The findings suggest a pivotal role of the commensal bacteria in the number of neurons and their neuronal connectivity.

In summary, this research highlights the self-organizing and activity-driven nature of neural circuit formation in *Hydra* embryos, offering insights into the evolution and fundamental principles of nervous systems. The findings also have implications for understanding neural development in more complex organisms and the crucial role of environmental factors such as temperature and the microbiome on the nerve net assembly during early developmental stages.

## Zusammenfassung

Die grundlegenden Fragen zur Evolution, der Entstehung, der Funktionalität und der Entwicklung des Nervensystems sowie dessen erhebliche Auswirkungen auf Störungen und Krankheiten stehen seit der Formulierung der Neuronentheorie durch Santiago Ramón Cajal im Fokus wissenschaftlicher Forschung. Die Komplexität des Nervensystems stellt jedoch oft eine Herausforderung dafür dar, um diese fundamentalen Fragen zu beantworten und seinen Ursprung sowie seine grundlegende evolutionäre Funktionalität zu entschlüsseln. Modellorganismen wie *Hydra*, ein Vertreter der Cnidaria, mit vergleichsweise geringer Komplexität und einem sich beständig erneuernden Nervensystem, haben sich aufgrund ihrer evolutionären Position und der Erzeugung transgener Linien als aufstrebende Modellorganismen für die Untersuchung der neuronalen Evolution und Entwicklung etabliert.

In dieser Arbeit werden verschiedene Aspekte der *de novo*-Bildung des Nervennetzes in *Hydra*-Embryos während der späten Embryogenese und bei frühen Schlüpflingen untersucht. Außerdem werden Merkmale spezifischer Entwicklungszeitpunkte analysiert, um Einblicke in das sich entwickelnde Nervensystem zu gewinnen. Die Dissoziation von ganzen Embryos und Schlüpflingen zeigt das Fehlen von Neuronen in frühen Stadien, gefolgt von einem signifikanten Anstieg der Anzahl morphologisch differenzierter Nervenzellen während der frühen Entwicklung, in Korrelation mit dem Auftreten des ersten Kontraktionsverhaltens. Mit Hilfe von Antikörpern gegen spezifische Neuropeptide, die in sechs von sieben neuronalen Populationen exprimiert werden, zeigen die frühesten zugänglichen Entwicklungsstadien bereits existierende und miteinander netzwerkartig verbundene Zellen. Elektronenmikroskopische Aufnahmen liefern einzigartige Einblicke in die Zell-Zell-Kommunikation zwischen neuronalen und nicht-neuronalen Zellen durch Gap Junctions und eine vesikelgesteuerte Kommunikation. Diese Mechanismen sind in der Lage einen schnellen Aufbau des Nervennetzes in spezifischen Entwicklungsstadien zu unterstützen und zu fördern.

Für diese Arbeit werden fortschrittliche Techniken im Umgang mit und der Pflege von transgenen Linien eingesetzt, um die sexuelle Reproduktion bei *Hydra* zu induzieren. Mithilfe von Kalzium-Indikatoren und Immunohistochemie werden die Funktionalität und Topologie des entstehenden Nervennetzes während der frühen Entwicklungsphasen von Embryos und Schlüpflingen untersucht und verglichen. Eine speziell entwickelte Aufnahmemethode, die

langfristige Kalziumaktivitäten während der frühen Assemblierung des Nervennetzes ermöglicht, liefert detaillierte Einblicke in die grundlegenden Prozesse von dessen Aufbau und Funktionalität mit Einzelzellauflösung innerhalb der nervenzellspezifischen N4-Population. Es gelang die Beobachtung voneinander abgegrenzter, nicht synchronisierter neuronaler Netzwerke in verschiedenen Regionen einzelner Entwicklungsstadien, welche sich später zu einem synchronisierten Nervennetz in adulten Polypen zusammenfügen und damit die Exploration grundlegender Prinzipien der neuronalen Konnektivität.

Darüber hinaus wird die Rolle von Umweltfaktoren bei der Bildung des Nervennetzes in verschiedenen Entwicklungsstadien untersucht. Externe Einflüsse wie Temperatur und das Mikrobiom beeinflussen den Aufbau des Nervennetzes und deuten auf ein gewisses Maß an Anpassungsfähigkeit während der neuronalen Entwicklung hin. Bei der Analyse temperaturbedingter Einflüsse auf die Nervennetzassemblierung konnte festgestellt werden, dass geringere Temperaturen einen positiven Einfluss auf den Netzwerkaufbau haben. Höhere Temperaturen hingegen zeigen einen negativen Einfluss durch Defizite im Fressverhalten, genauer im Verschlucken der Nahrung und einer daraus resultierenden Sterblichkeitsrate von 40 %. Im weiteren Verlauf werden die mikrobiellen Einflüsse während der Embryogenese analysiert, indem die Netzwerkstruktur und die neuronalen Verbindungen bei Wildtyp-, keimfreien und rekolonisierten Schlüpflingen untersucht werden. Die Ergebnisse weisen hier auf eine entscheidende Rolle der kommensalen Bakterien bei der Anzahl der Neuronen und deren neuronalen Verbindungen zu anderen Zellen hin.

Zusammenfassend zeigt diese Forschungsarbeit die selbstorganisierende und aktivitätsgetriebene Bildung neuronaler Schaltkreise in *Hydra*-Embryos und liefert Einblicke in die Entstehung und grundlegenden Prinzipien von Nervensystemen. Die Ergebnisse haben zudem Implikationen für das Verständnis der neuronalen Entwicklung in komplexeren Organismen und zeigen die entscheidende Rolle von Umweltfaktoren, hier aufgezeigt für Temperatur und Mikrobiom, bei der Assemblierung des Nervennetzes in frühen Entwicklungsstadien.



## Introduction

### Evolution of the nervous system: From basic neural networks to complex information processing

Through evolution organisms are able to adapt to all conceivable habitats, ranging from the tiny *Nanoarchaeum equitans* in the inhospitable appearing deep sea (Jahn et al., 2008) or Bactrian camels living in arid deserts facing extreme temperature fluctuations (Gebreyohanes and Assen, 2017; Li et al., 2019). Looking into the phylogenetic tree of life, evolution has not only shaped the vast diversity of life but has also produced fundamental mechanisms to ensure survival and reproduction in an ever-changing environment.

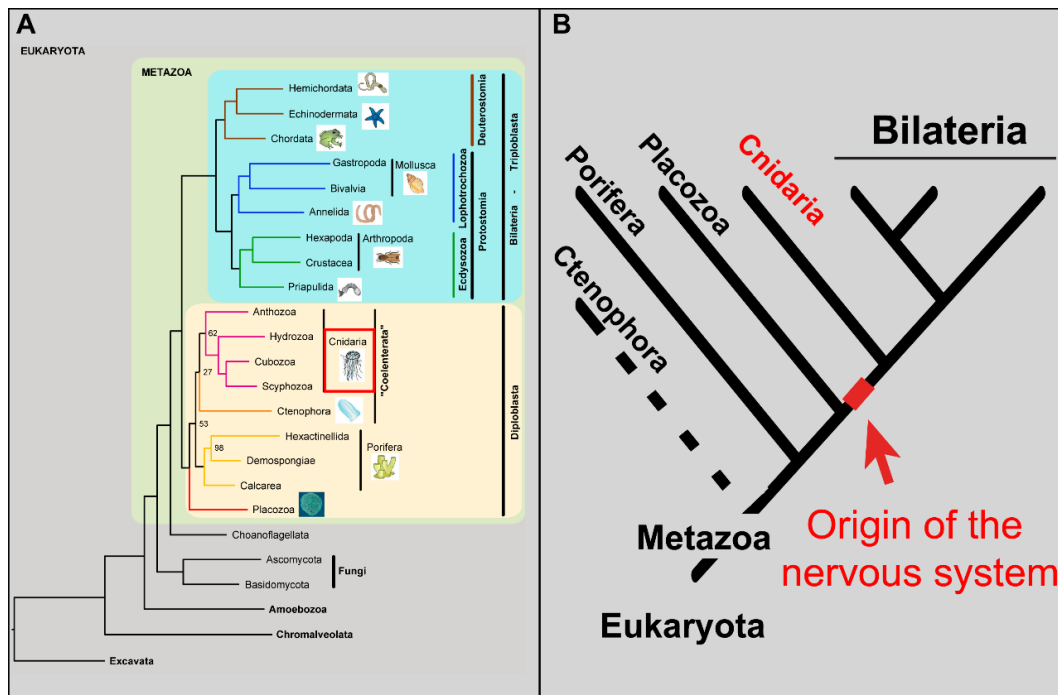
Fundamentally, evolution is based on natural selection, whereby individuals with advantageous traits display a higher probability of passing their genes on to the next generation. Mutations and, in sexually reproducing species, recombinative processes generate the genetic variation that allows natural selection to favor advantageous traits under specific and changing environmental conditions (Barton, 2010; Vogt, 2023). This process is not only crucial for the adaption to environmental changes or conquer new niches but also confirms the emergence of innovative and adapted biological structures. One of the most remarkable innovations in the history of evolution is the development of nervous systems in metazoans. The emergence of metazoans marks a pivotal moment in evolutionary history, as these organisms represent a shift from unicellular to highly organized multicellular life (Tikhonenkov et al., 2020). This transition required the emergence of new mechanisms for communication, coordination and specialization of cellular clusters, enabling metazoans to function as integrated units rather than simple aggregations of cells. Early metazoans displayed relatively basic body plans and mechanisms for interacting with the ambient environment. Compared to organisms that solely rely on chemical or mechanical reactions to environmental stimuli, the emergence of nervous systems enabled for complex behaviors (Arendt, 2021), a rapid signal transmission (Ovsepiyan et al., 2020) or a coordinated response to external stimuli. Basal metazoans such as sponges (Porifera) rely on passive mechanisms or basic cellular coordination for an environmental interaction. In contrast, the appearance of an early and diffuse nervous system in other metazoans like cnidarians, enabled for active, dynamic



responses and the ability to detect and react to environmental stimuli (Ludeman et al., 2014; Nickel, 2010).

During the diversification of metazoans, nervous systems evolved and supported increasingly complex body plans and behaviors. Bilaterians, a group that includes most modern and complex animals, developed centralized nervous systems, characterized by an aggregation of neurons into structures such as nerve cords and brains (Arendt et al., 2016). This advantage enabled and increased the efficiency for information processing and behavioral complexity, facilitating the ability to conquer various ecological niches and driving the adaptive metazoan success across terrestrial and aquatic environments (Kelava et al., 2015). Studying organisms with basic nervous systems can provide insights into the basic functional principles of the evolution of more complex nervous systems. This approach can highlight the interplay between genetic variety, environmental pressures and anatomical adaptations that shaped the tree of life to the current degree.

As Leon C. Megginson paraphrased Charles Darwin's *On the Origins of Species*: "It is not the strongest of the species that survives, nor the most intelligent; but the one most adaptable to change". Darwin himself further stated: "Embryology rises greatly in interest, when we thus look at the embryo as a picture, more or less obscured, of the common parent-form of each great class of animals". These statements are also applicable for the evolution of the nervous systems and forms the foundation of the questions explored in this dissertation.



**Figure 1 Phylogenetic position of *Hydra*.** a, b. Phylogenetic tree highlighting the position of cnidarians and the origin of the nervous system (Jékely et al., 2015; McCarthy et al., 2023; Whelan et al., 2017) (modified from (Bosch et al., 2017; Schierwater et al., 2009)).

## Milestones in neural evolution

The evolution of centralized nervous systems in bilaterians represents a major step towards an increase of neural complexity. Organisms such as planarians (Platyhelminthes) and annelids possess well-defined central nervous systems (CNS), enabling enhanced behavioral capabilities such as a target-directed movement, spatial navigation and associative learning (Inoue et al., 2015; Perry et al., 2013). These advancements demonstrate the gradual refinement of neural structures, highlighting the selective pressures that supported the improvement of signal processing and behavioral adaptability.

## The emergence of neurons: From ancestral signaling cells to specialized units

During the late 1800s, Camillo Golgi and Santiago Ramón Cajal defined the neurons as the basic functional unit of the nervous system (Luo, 2021; Yuste, 2015a). Ever since, research gained and refined the knowledge in the neurobiological field, developed a range of tools such as gene editing, transgenic models, imaging tools (Calcium indicators, Optogenetics), sequencing techniques or computational and modelling approaches allowing scientists to explore neural processes at unprecedented depth and precision (Yuste et al., 2024).

Additionally, neuronal ensembles serve as essential building blocks at the core of all nervous systems and form the fundamental unit of neural function. Neurons are cells specialized for rapid electrical and chemical signaling, forming interconnected networks for processing and transmitting information (Lovinger, 2008). Neurons are evolutionary derived from ancestral secretory and sensory cells, which initially functioned in basic stimulus detection and paracrine-like signaling (Moroz, 2021). These early cells likely resembled peptidergic signaling cells found in metazoans lacking neurons (Porifera, Placozoa), releasing neurotransmitter-like molecules to coordinate physiological responses (Varoqueaux et al., 2018). For the transition to dedicated neurons specific key innovations were required, including membrane excitability, voltage-gate ion channels and the development and implementation of synapses. The evolution of axons and dendrites enabled a rapid and efficient signal propagation, while synaptic vesicles and neurotransmitter systems facilitated precise intercellular communication (Arendt, 2020). These developments enabled the formation of complex neural circuits, supporting increasingly sophisticated behaviors and adaptability in bilaterians, thereby accentuate their achievements and success throughout evolution.

## **Mechanisms to shape neural evolution**

The refinement and structural organization of nervous systems were driven by various interacting mechanisms, including activity-dependent processes, molecular gradients, genetic programs and biomechanical forces. These factors ensured the patterning, connectivity, assembly and differentiation of neural networks, supporting a level of adaptability and functional optimization.

### **Activity-guided neural development**

Activity-dependent mechanisms influenced the enhancement of nervous system architecture, assuring that synaptic connections and neural circuits were shaped by functional demands. This principle remains conserved in modern neurodevelopment, where neural activity fine-tunes synaptic strength and connectivity, optimizing network efficiency. Activity-dependent neural development and synaptic refinement are fundamental aspects of neural development and are observable across a wide range of organisms, including both invertebrates and vertebrates. In *Drosophila melanogaster* neural activity influences the synaptic connectivity

and plasticity (Tessier and Broadie, 2009). In complex organisms such as cephalopods, activity-dependent mechanisms such as Hebbian plasticity and synaptic pruning are crucial for the development of adaptive and efficient neural networks and enable learning, memory and precise sensory-motor control (Lazari et al., 2022; Shomrat et al., 2015). Besides the strengthening or elimination of connections in neural architecture, more elements have to be considered:

- **Cell-cell interactions** as a crucial role in regulating neuronal fate and connectivity (Jiao et al., 2017). The **Notch-signaling** which influences neurogenesis by determining whether progenitor cells differentiate into neurons or remain as stem cell-like precursors (Lathia et al., 2008).
- **Stochastic gene expression** introduced a degree of randomness in neuronal differentiation and connectivity, particularly in systems where diversity in synaptic connectivity enhances network function (Raj and van Oudenaarden, 2008). Exemplarily, the random expression of specific receptor types on olfactory neurons generates a broad sensory repertoire to detect a wide range of odorants (Monahan and Lomvardas, 2015).
- **Temporal regulation through gene expression waves** coordinate neuronal differentiation and circuit formation over time, allowing a precise patterning of neural structures in bilaterians. This involves sequential waves of gene expression that guide neural progenitors through various stages of differentiation to induce the production of specific neuronal subtypes and the formation of complex neural circuits (Kohwi and Doe, 2013).

Through these mechanisms, neural development progressively evolved to become more adaptive and self-organizing, allowing the nervous system to shape its structure and function in response to both intrinsic neural activity and external environmental stimuli. This dynamic process enables the assembly of nerve nets and complex nervous systems to optimize their connectivity, enhance learning efficiency and refine sensory and motor responses over time (Dresp-Langley, 2020; Krüppel and Tetzlaff, 2020; Marzola et al., 2023).

## Molecular gradients and structural patterning of the nervous system

Along with activity-guided assembly, the emergence of nervous systems was further shaped by molecular gradients that directed neuronal differentiation, positioning and connectivity. These gradients provided spatial cues that orchestrated neural tube formation, axon guidance and synaptic targeting:

- **Hox genes and cell polarity regulators** control the regionalization of the developing nervous system and are responsible for the distinct anterior-posterior patterning in bilaterians (Smith and Kratsios, 2024).
- **Morphogen gradients** such as Wnt, BMP and Sonic Hedgehog establish neural identity and guide axon trajectories by defining attraction and repulsion zones (Wilson and Stoeckli, 2012).
- **Chemotactic signals** like Netrins and extracellular matrix (ECM) components guide neuronal migration and axon extension, leading to synaptic connections being formed at precise locations (Franco and Müller, 2011; Ly et al., 2005).
- **Mechanical forces** play an essential role in neural tube formation, where coordinated cell movements and cytoskeletal rearrangements drive the folding and closure of the developing CNS (Abuwarda and Pathak, 2020).

These molecular and biomechanical mechanisms provided a framework for the nervous system formation, ensuring that nerve nets were assembled with neurons that were not only correctly specified but also properly connected within functional networks.

The emergence of the nervous system was not a singular event but rather a gradual process shaped by evolutionary pressures, integrating activity-dependent assembly, molecular gradients, genetic programs and biomechanical forces. The interplay between these mechanisms enables the transition from diffuse neural networks to highly specialized and centralized nervous systems. Understanding these fundamental principles and the evolutionary roots of neural complexity and adaptability can provide insights into the evolution and development of complex nervous systems.

## Effect of environmental factors on the development and function of the nervous system

The emergence of the nervous system is a milestone during evolution of complex life forms. However, its development is a complex process that is genetically programmed and can further be influenced by environmental factors. While the degree of genetic control differs between organisms, some fundamental processes apply universally. Considering the neurogenesis, there is a genetic frame which is regulated by specific transcription factors, signal molecules and cell adhesion molecules, that controls the differentiation and migration of cells. Signal pathways such as Notch (regulation of neuronal differentiation), Wnt (neurogenesis, axis formation), Hedgehog (cell migration, pattern formation) BMP (regulation of the dorsoventral axis) (Borggreffe et al., 2016) or Hox-genes determine the spatial identity of neurons along the body axis. In zebrafish (*Danio rerio*), temperature can have an influence on neurogenesis (Irion and Nusslein-Volhard, 2022) whereas the neuronal development in *Caenorhabditis elegans* is subject to a strict genetic control with minimal external influences (Chiu et al., 2011). The genetic code provides the framework for the development of the nervous system, whereas the influence of the environment can vary between organisms.

External factors such as nutrition, stress, toxins, physical activity, temperature and the microbiome can either be beneficial or cause neurodegenerative disorders and also play a crucial role during early development. Prenatal and early postnatal periods appear to be sensitive to influences such as maternal nutrition (Marques et al., 2013), exposure to stress (Doi et al., 2022) and environmental toxins (Padula et al., 2020).

**Nutrition** is a fundamental factor for the development und functionality of the nervous system. Nutrients can influence synaptic plasticity, synthesis of neurotransmitters and cognitive performances. Omega-3 fatty acids as nutritional components support synaptogenesis and can reduce neuroinflammation, potentially lowering the risk of neurodegenerative diseases (Gomez-Pinilla, 2008). Vitamins and minerals, particularly vitamin B12, vitamin D and iron are crucial for brain development. Deficiencies have been linked to cognitive deficits and neurodevelopmental disorders like autism spectrum disorder (ASD) and attention-deficit hyperactive disorder (ADHD) (Wang et al., 2022, 2017; Zhang et al., 2016). Antioxidants, which are abundant in fruit and vegetables can protect neurons from oxidative

stress, which is reported as one of the key factors in aging and neurodegeneration (Mattson, 2012). Additionally, different forms of diets have shown to be critical factors in modulating the gut microbiota and hence influencing behavior, mental health and cognitive function through metabolites produced by microbes (Cryan et al., 2019).

Chronic **stress**, significantly alters the brain structure and functionality through dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis. Prenatal stress can impact fetal brain development, thereby leading to a modified connectivity and increased risk for anxiety and depression in later stages of life (McEwen, 2012). Constant levels of stress increase the cortisol levels which can affect the hippocampal neurogenesis and contribute to neurodevelopmental disorders (Lupien et al., 2018). Particularly during early life stages, stress exposure lead to epigenetic changes, affecting emotional regulation or cognitive flexibility. Social and psychological stressors like isolation or trauma can increase the susceptibility to neurological disorders such as post-traumatic stress disorder or schizophrenia (Grandjean and Landrigan, 2014).

Environmental **toxins** have been shown to negatively impact neuronal health and function especially during early development. Heavy metals like lead and mercury interfere with synaptic signaling and may lead to neurodevelopmental disorders and cognitive impairment (Grandjean and Landrigan, 2014). Further, an altered brain development and cognitive function can be a result of industrial chemicals and pesticides caused by a disruption of the endocrine signaling (Gore et al., 2015).

Beside the previously mentioned external factors, regular **physical activity** can evidently enhance brain function and increase resilience against neurological disorders. Exercising promotes neurogenesis and synaptic plasticity by increasing levels of brain-derived neurotrophic factor (BDNF) which supports learning and mermory (Cotman et al., 2007). More criteria to underpin the positve effects of physical activity are the regulation of immune responses, the reduction of neuroinflammation (Radak et al., 2008) and the mitigation of syndromes of anxiety and depression (Kandola et al., 2019; Schuch et al., 2018). Furthermore, physical activity during early life has been linked to an improved cognitive output, a positive emotional regulation and a reduction of risk for neurodevelopmental disorders (Belcher et al., 2021).

## Temperature as environmental factor

As previously described, external factors can have a crucial role on nervous system development in organisms. Temperature is a key factor as all organisms face its attendance during development and further throughout all life stages. Bio-chemical reactions have a temperature coefficient, which directly refers to the rate of change within these reactions and influence physiological processes. Enzymatic reactions, sex determination, hibernation or even animal migration are all aspects influenced by temperature (Valenzuela et al., 2019; Xiao and Xu, 2021). In humans for instance, an elevated maternal body temperature during pregnancy due to illness or high ambient temperatures can affect fetal development and neurodevelopment, specifically by causing neurodevelopmental disorders and an elevated rate of stillbirths (Antoun et al., 2021; Gustavson et al., 2019; Lakhoo et al., 2024).

Temperature has profound effects on neuronal activity, cognitive function and neuroprotection. Heat stress can lead to increased neuroinflammation, reduced cognitive performance and a higher risk to heat-related neurological conditions such as stroke (Epstein and Yanovich, 2019; Huang et al., 2022). In contrast, studies on *Caenorhabditis elegans* indicated a beneficial impact of low temperature on the activation of longevity pathways involving the TRPA-1 channel (Xiao et al., 2013). The temperature-related impact on neurodevelopment appears to be critical during early developmental stages and can lead to alterations in structure and function. In *Drosophila*, a slower development at lower temperatures results in higher brain connectivity and leads to an increased specificity of neuronal connections in the olfactory pathway (Züfle et al., 2025). In zebrafish embryos, an elevated temperature has been associated with an accelerated neuronal development, however, may lead to abnormalities in brain structure and function (Toni et al., 2023). Hence, zebrafish larvae have the ability to perform non-directional and directional movements towards ambient temperature close to its physiological setpoint (Palieri et al., 2024). Furthermore, studies in Zebrafish have shown that both hypo- and hyperthermic conditions have an influence on adult stages by altering protein expression in the brain, increase anxiety-like behaviors and exploratory patterns (Angiulli et al., 2020; Maffioli et al., 2023).

Collectively, this underscores the profound temperature-related impact on neurodevelopment and neural function during later developmental stages across various species. Both, elevated



and reduced temperatures can impact genetic and cellular pathways involved in neuronal development, emphasizing the imperative for a precise thermal regulation during critical developmental phases. Although many organisms invest in maintaining a specific body temperature, aquatic living early metazoans are governed by seasonal fluctuations without the ability to maintain their optimal temperature. Temperature-changes can trigger specific life-cycle phases such as migration, dormancy, embryogenic and larval development, egg hatching and timing (Stocker et al., 2024). Ectothermic animals such as macroinvertebrates exhibit an increased microbial metabolic activity that affects development and performance of biotic communities (Bonacina et al., 2023), showing the pivotal role of temperature, especially regarding overall climatic changes (Doney et al., 2012). Thermal stress can further impact symbiotic microbial communities and induce coral bleaching by destabilizing the nutrient cycling in the corals holobiont, which includes bacteria, archaea, fungi and viruses (Rädecker et al., 2021).

### **The microbial impact as environmental factor**

The microbiome, defined as the entirety of all microorganisms living in and on a (specific part or compartment of a) host, has attracted considerable research interests as a fundamental factor in health, disease and physiological processes. It is comprised of bacteria, archaea, fungi, viruses and protozoa (Berg et al., 2020) and forms a dynamic ecosystem interacting with and influencing its host by affecting digestion, metabolic processes, immune regulation and neurological functions (Cryan et al., 2019; Dinan and Cryan, 2017; Hou et al., 2023). Distinct factors such as genetics, lifestyle, environment and diet can contribute to its composition and diversity (Johnson, 2020). Novel methods such as advanced sequencing technologies and metagenomics provide in-depth insight into the complexity of the microbiome and its contribution in health and disease. Furthermore, the microbiome's contribution to basic neurodegenerative processes such as formation of the blood-brain-barrier, neurogenesis or microglia maturation underscores its relevance for neurodevelopmental processes (Cryan et al., 2019; Sharon et al., 2016).

## The impact of the microbiome on neural development

Microbes are ubiquitous companions throughout evolution, nothing in nature seems to have emerged or evolved under sterile conditions, scientists even assume our last universal common ancestor (LUCA) as a part of an established ecosystem (Moody et al., 2024). From early development on, the microbiome exerts an influence on the nervous system, shaping neurogenesis, synaptic formation and neuronal plasticity. Germ-free (gf) mice, which were raised in sterile environments provide evidence for the importance of microbial signals during neurodevelopment. The results in gf mice revealed significant deficits in neural function, influencing critical processes such as resilience and social behavior (Heijtz et al., 2011; Luczynski et al., 2016a). Furthermore, studies revealed that the absence of a maternal gut microbiota directly affects fetal brain development with regard to the number of neuronal connections (Vuong et al., 2020) and that neurodevelopmental alterations (microglial labelling, altered neuronal cell death) persist even if the offspring of gf mothers are conventionally colonized immediately after birth, implying the significance of the maternal microbiome (Castillo-Ruiz et al., 2023).

Similarly, studies in *Danio rerio* larvae demonstrate the importance of microbial signals as essential for activating neural circuits and the establishment of functional neural networks during development. In a germ-free environment, zebrafish exhibit malfunctions, characterized by an affected neural communication and abnormal behaviors, thereby emphasizing the importance of external signals to ensure an unhindered neurodevelopment (Bruckner et al., 2022; Davis et al., 2016).

Likewise, studies suggest that interactions between microbiome and neurons may have deeper evolutionary origins notable in neurodevelopment highlighted by ancient evolutionarily microbial interactions with neural systems in different invertebrate phyla. In sponges (Porifera), despite their lack of a nervous system, microbial communities regulate larval settlement and metamorphosis (McFall-Ngai et al., 2013). These findings emphasize the importance of microbial signals in influencing early animal development even before the emergence of neurons. Furthermore, the interplay between microbiome and neural development is detectable in invertebrates such as *Drosophila*. The relatively compact but highly organized nervous system is influenced by symbiotic microbial communities regarding

its neural development and function. In *Drosophila*, the gut microbiome has been shown to affect neurotransmitter levels, which in turn can modulate behaviors like feeding and locomotion (Schretter et al., 2018). Further, a microbial presence during larval development plays a crucial role in shaping sensory neural circuits. Montanari et al. (2024) demonstrated that specific bacterial species present during larval stages can modify gustatory capacities in adult flies. They found that recolonizing germ-free larvae with *Lactobacillus brevis* restored their ability to respond to peptidoglycan, highlighting the influence of the microbiome on the maturation of taste-related neural circuits (Montanari et al., 2024). Another study showed that the gut microbiota of *Drosophila* affects the olfactory neural development, as flies raised on microbe-enriched diets exhibited changes in olfactory behavior (Qiao et al., 2019).

While the evidence from various models strongly supports the role of the microbiome in neural development, aspects such as molecular and biochemical pathways remain crucial. Their influence, mediated by microbe-derived metabolites and neurotransmitters, shapes synaptic plasticity, neuronal connectivity and behavioral outcomes. These biochemical signals also play a fundamental role in early neurodevelopment.

Short-chain fatty acids (SCFAs) as microbial metabolites have been shown to influence neuronal function by modulating synaptic activity and neurogenesis (Silva et al., 2020). In germ-free mice the offering of SCFAs has been demonstrated to restore microglial function, suggesting that microbial metabolites are essential for normal brain development and immune function within the central nervous system (Luczynski et al., 2016b). Furthermore, certain bacteria synthesize neurotransmitters or their derivatives such as gamma-aminobutyric acid (GABA) or serotonin, directly influencing neuronal signaling or brain development and behavior (O'Mahony et al., 2015; Strandwitz, 2018).

The microbiome plays a crucial role in neurodevelopment, shaping key processes such as neurogenesis, synaptic formation and neuronal plasticity. Various model organisms including gf mice, zebrafish, and *Drosophila* underscore the need for microbial signals in establishing functional neural circuits. These findings suggest that microbial-neural interactions are strongly conserved across evolution and influence neural development.

## The need for an advanced understanding – Early metazoans as an opportunity

There is a debate in the current scientific literature on evolution about multiple theories on the evolution and emergence of neurons and nervous systems. In an evolutionary context the development of a complex structure like the brain still remains unresolved. The evolution of the nervous system is a highly complex process, which started from early, simple modes of communication between cells and resulted in highly specific and complex structures like the human brain. The question of whether the emergence of the neuron as fundamental unit of the nervous system was a unique event throughout evolution is still a matter of scientific debates. On the one hand, neurons have the ability to communicate by receiving, transmitting and processing signals via precise electrical and chemical transduction. This level of complexity, achieved through a high number of evolutionary events, renders an independent emergence of similar structures potentially improbable. On the other hand, a convergent evolution of complex structures such as the retina, which has developed several times throughout evolution (Koenig and Gross, 2020), demonstrates that the independent evolution of neurons is not impossible.

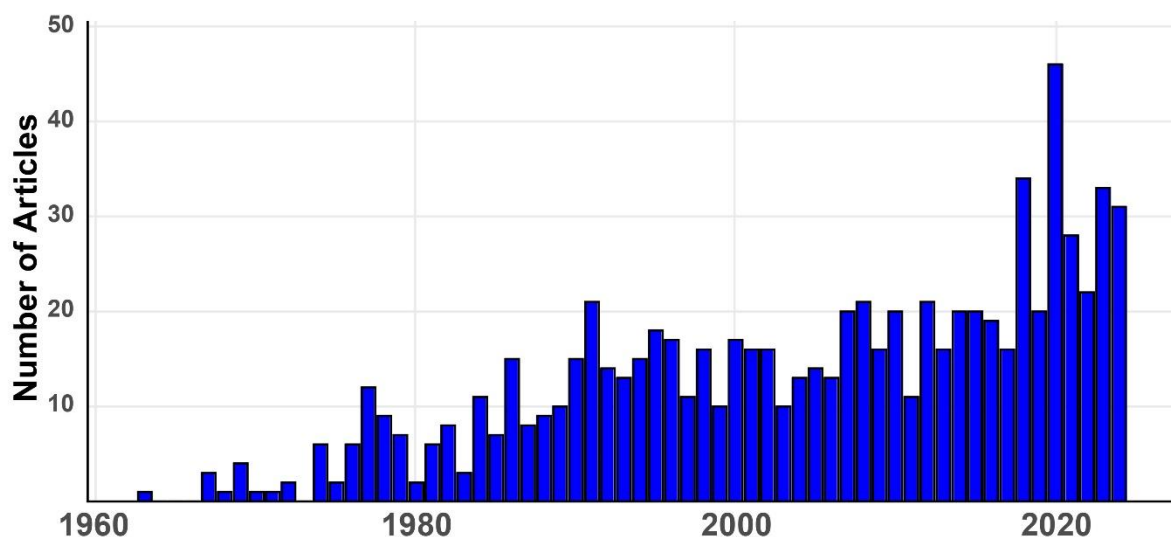
The evolution of neuronal centralization indicates multiple origins of central nervous systems (Moroz, 2012). Still, scientific research focusses on the development of nervous systems starting with diffuse nerve nets towards further complex structures like nerve rings, nerve cords towards brains, or the examinations of various types of neurons to understand their origin and diversification (Arendt et al., 2019, 2016). A focus on distinct perspectives regarding the origin of the nervous system has led to different hypotheses. Former studies concentrated on the cellular level and considered vertical sensory-motor reflex arcs as the first nervous systems, while later work emphasized the value of the nervous system at the tissue level (Arendt, 2021). The combination of comprehensive analyses, genetic studies, modern sequencing technologies and modelling approaches will provide deeper insights in the understanding of mechanisms underlying the evolution of the nervous system.

Understanding the fundamental functionality and evolution of nervous systems often requires strategies to reduce complexity. The growing availability of diverse model organisms coupled with advanced technologies and methods allow for studying less complex nerve nets close to the origin of the nervous system. Especially cnidarians provide the opportunity to unravel

fundamental insights into basic neurodevelopmental questions. In this work, *Hydra vulgaris*, as one of those emerging model organisms, was employed to extend the understanding of early nerve nets.

## ***Hydra* – A multifaceted model organism**

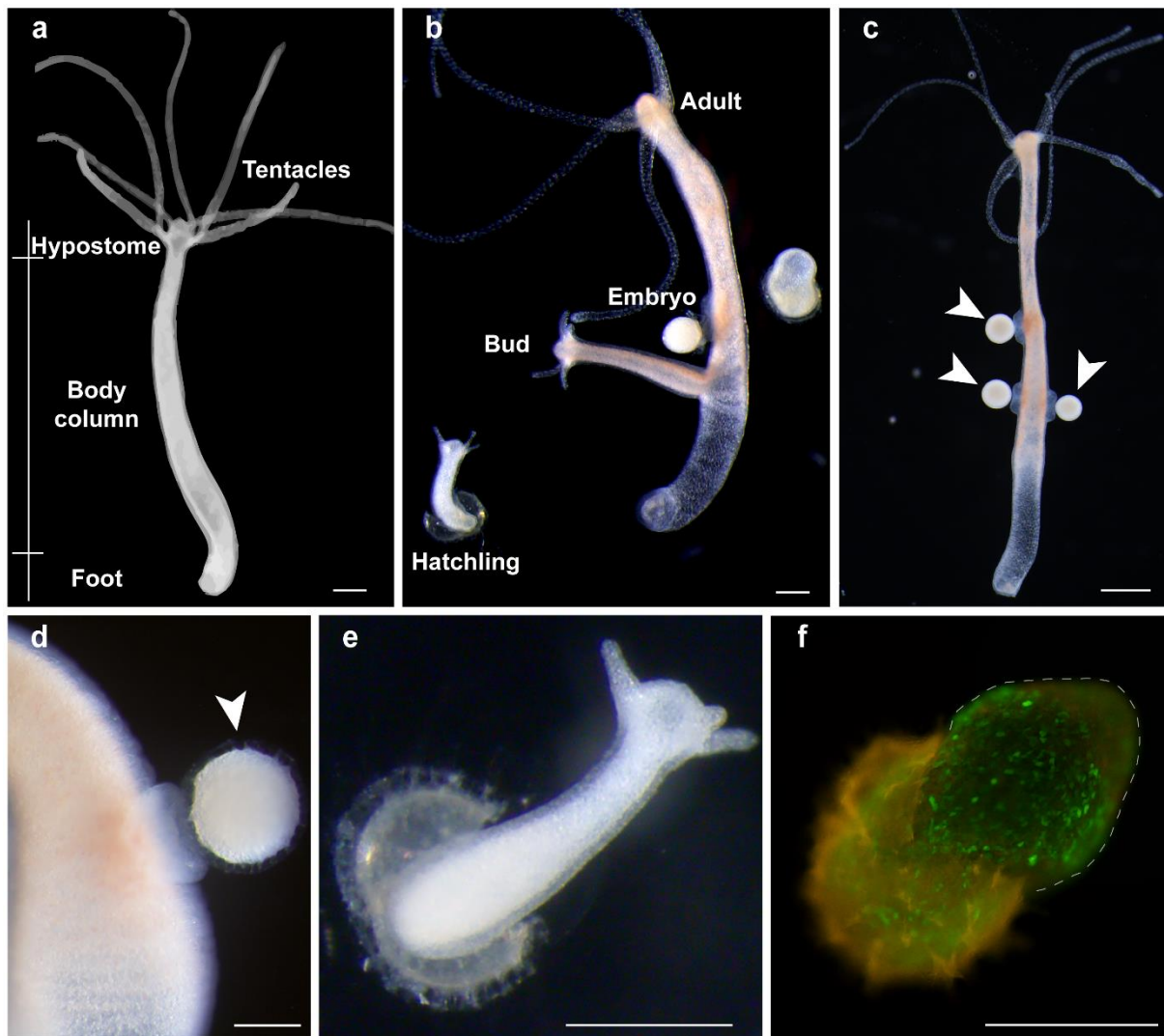
The freshwater polyp *Hydra* is a versatile and accessible model organism with tremendous qualities in various research sectors which has aroused scientific interest for more than 300 years. In 1702 *Hydra* was first described by Antoni Van Leeuwenhoek and in 1744 Abraham Trembley began to investigate this freshwater polyp and discovered its regeneration capacities, its light-directed movement (Phototaxis) and grafting (Koid, 1940; Lenhoff and Lenhoff, 1989; Trembley, 1744). In addition to the low efforts required to keep even numerous and large-sized cultures, *Hydra* is an established research object in the fields of regeneration (Livshits et al., 2022; Vogg et al., 2019), evolution & development (Hemmrich et al., 2007; Meinhardt, 2012), stem cells (Bosch, 2009a; Bosch and David, 1986), ageing and longevity (Boehm et al., 2013; Martins et al., 2016), microbiome (Bosch, 2013; Fraune et al., 2015) and neuroscience (Dupre and Yuste, 2017; Giez et al., 2023). Even the fields of ecotoxicology and environmental research utilize *Hydra* as bioindicator for water quality or to examine the effects of micro plastics and nanoparticles (Murugadas et al., 2019), suggesting the increasing interest in *Hydra* as model organism.



**Figure2. Increasing research interest in the model organism *Hydra*.** In recent years the number of scientific articles has increased. The displayed data represent studies on *Hydra vulgaris*, *H. oligactis*, *H. viridissima*, *H. magnipapilata* and *H. attenuate*. In the past 20 years, 44% of these studies focused on *H. vulgaris*.

*Hydra* has a simple body plan, consisting of two epithelial layers referred to as ectoderm and endoderm, separated by the mesoglea (Sarras Jr et al., 1993). Both layers, though separated, are forming processes throughout the mesoglea which connect via gap junctions (Giez et al., 2024; Noack et al., 2025, in final revision). The polyps are radially symmetric and organized in a single oral-aboral axis. At the apical end *Hydra* has a structure called hypostome with the mouth opening, which serves for both ingesting prey and discharging of undigested food debris. Polyps use the basal disk, termed foot, to stick to the surface or release gas bubbles, secreted by ectodermal cells to rise and float on the surface and be carried passively by water current. Between head and foot, the ecto- and endoderm form the body column, also termed as gastric cavity where the food is digested (**Figure 3a**). Furthermore, the body column is that part of the polyp which is responsible for the regeneration capacity due to the located multipotent stem cells. All cells in *Hydra* originate from three specific stem cell populations – ectodermal epithelial stem cells, endodermal epithelial stem cells and the interstitial stem cells (i-cells). Epithelial cells continuously divide and move towards their respective destinations along the body axis, before they get sloughed off and are replaced (Reddy et al., 2019). The interstitial stem cells on the other hand give rise to multiple cell types such as germ cells, nematocytes, gland cells and neurons.

All cells in *Hydra* are subject to a continuous renewal process, with various replacement times for each cell type (David and Campbell, 1972; David and Gierer, 1974; Reddy et al., 2019). Due to its ability of continued self-renewal, *Hydra* is often called a perpetual or everlasting embryo (Barve et al., 2021; Ghaskadbi, 2020; Martínez and Bridge, 2012). The ability of fully dissociated adult *Hydra* to regenerate polyps from aggregates as well as the asexual reproduction through budding offers further insights into embryogenic development (Gierer et al., 1972). However, comparisons of gene expression levels revealed differences between adult polyps and embryos (Genikhovich et al., 2006), demonstrating the limitations of this correlation and strengthen the need to investigate the development during embryogenesis to understand the underlying and fundamental *de novo* mechanisms.



**Figure 3. Body plan and reproduction strategies of *Hydra*.** **a.** Structural segmentation of an adult *Hydra vulgaris* polyp. **b.** Lifecycle of *Hydra* with both asexual and sexual reproduction. Further an early hatchling (mid right) and a hatching polyp with tentacles (lower left corner). **c.** Female polyp carrying embryos along the body column. **d.** *Hydra* embryo and egg holder on female polyp at higher magnification. **e.** Juvenile polyp with tentacles, still attached to the cuticle several hours after hatching. **f.** Early transgenic hatchling (i-cells) during development without tentacles. Scale bars 1mm.

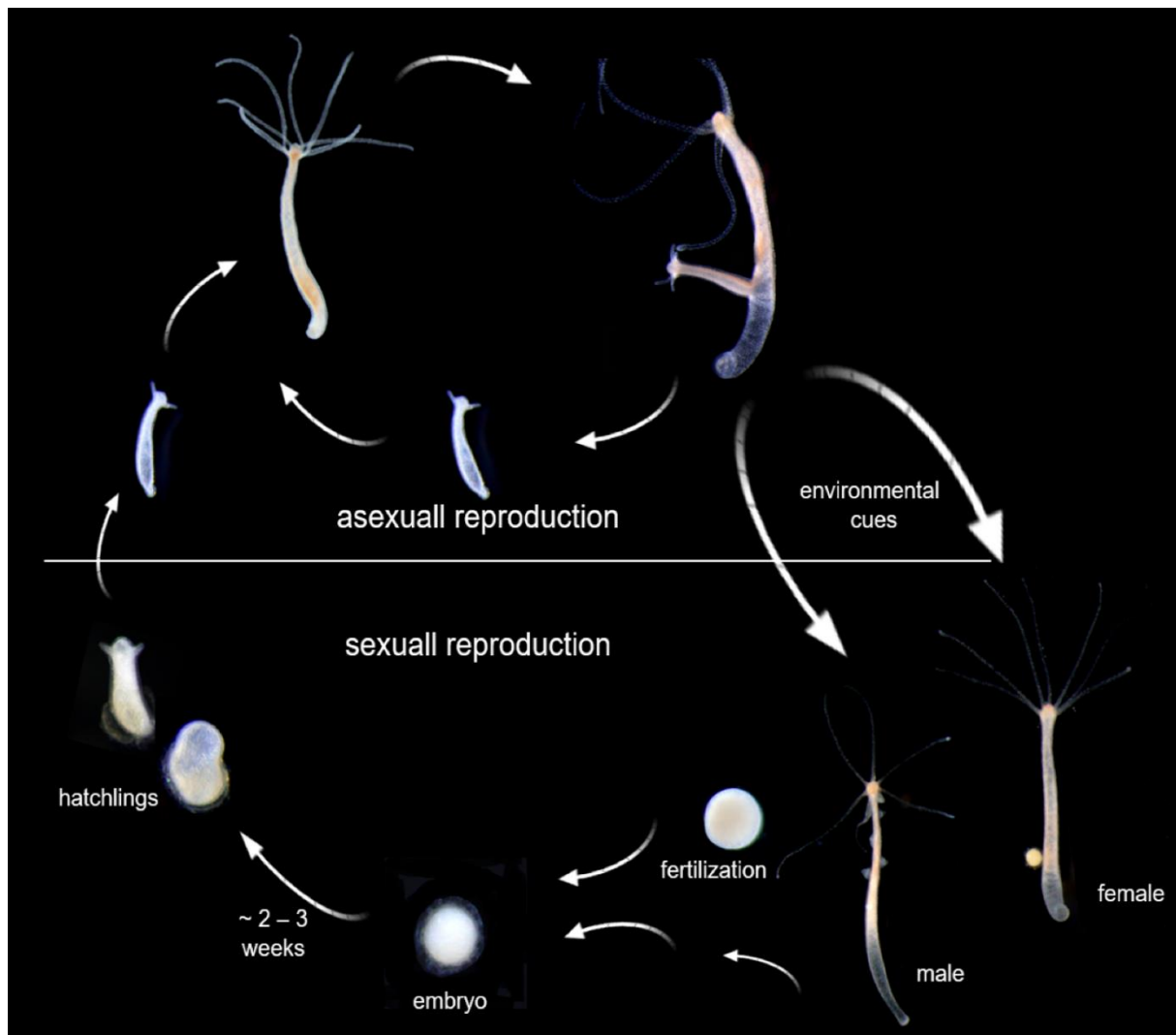
### Reproduction strategies of *Hydra*

*Hydra vulgaris* exhibits both asexual and sexual reproduction, enabling it to react to environmental changes like temperature or nutrition (**Figure 3b**). During optimal conditions polyps prefer to asexually reproduce, forming a genetically identical clone within 72-84 hours (Otto and Campbell, 1977). This allows for a rapid duplication and growth of populations if ideal conditions are prevalent. However, if environmental cues change, *Hydra* shifts to a sexual reproduction and develops female and male characteristics (**Figure 4**). In this process, female polyps develop an embryo which allow for a phase of dormancy to overcome non-ideal

environmental periods (**Figure 3c,d**). Prior to the formation of an embryo a characteristic egg patch develops in the ectoderm, whereby roughly 4.000 germline cells contribute to a growing oocyte. This cluster of germline cells differentiates into one single oocyte and numerous nurse cells. This formation process is divided into seven stages and takes approximately six days. In the final stage, the oocyte breaks through the ectoderm and is ready for fertilization (Alexandrova et al., 2005). The embryogenesis begins with the external fertilization by sperms, released from testes of male polyps into the surrounding environment. The fertilized egg undergoes holoblastic cleavage, a process during which the entire embryo divides into smaller blastomeres, similar to other cnidarians. The early cleavage stages progress rapidly, after approximately 20h hours post fertilization (hpf) the embryo initiates the formation of the cuticle. For this, cells in the outer layer start to extend filopodia and cuticular material gets deposited continually around the apical end of the filopodia until a multi-layered structure is built and a spike-like structure becomes visible. At the end of this stage (40-48h hpf) the filopodia are retracted and the formation of the cuticle (or theca) is completed (**Figure 3d**). Following this stage, the embryo enters a prolonged and partly unpredictable period of dormancy during which morphological changes are not apparent. Therein, the embryo remains encased in the cuticle, which serves as a barrier against mechanical damage and further environmental impacts throughout embryogenesis (Martin et al., 1997). This structure poses a major challenge for researchers as it increases the refractive index and impedes microscopic observations, requiring advanced imaging techniques and approaches. Unlike many other hydrozoans, *Hydra* does not form a typical larval stage, but instead undergoes a direct development, transitioning from the gastrula to a polyp without an intermediate swimming planula stage. This adaption is unique among hydrozoans and is likely an evolutionary trait related to its freshwater habitat (Martin et al., 1997; Steele et al., 2019).

Despite the ability of both strategies to reproduce, research on embryos remains challenging but is essential for understanding *de novo* concepts that are even hidden from examining adult polyps. A major concern of embryo-based studies is the small output, as sexual reproduction occurs under specific environmental conditions, often requiring artificial induction in laboratory settings. The hatching rate is low and the duration of embryogenesis is unpredictable, ranging between 2 – 52 weeks, making experimental planning and





**Figure 4.** The life cycle of *Hydra* in respect to the different reproduction strategies. Optimal environmental conditions result in an asexual reproduction. If environmental cues change, adult polyps initiate oogenesis and the formation of gonads. After fertilization, embryos develop a protective cuticle within the first 24 hours. After around 2 – 3 weeks the hatching process is initiated and the juvenile polyp starts to develop.

reproducibility difficult (Martin et al., 1997; Steele et al., 2019). In addition, a high mortality rate (Sugiyama and Fujisawa, 1977), a strict and precise maintenance and the formation of a thick cuticle after fertilization also increase the expenses (Martin et al., 1997). Despite these difficulties, *Hydra* embryos remain a valuable model for studying fundamental biological processes and mechanisms, including developmental plasticity, stem cell dynamics and evolutionary regeneration (Bosch, 2009b; Juliano et al., 2010). The phylogenetic position of *Hydra* and the understanding of processes during its embryogenesis may provide deep insights into further evolutionary or developmental mechanisms such as the neurogenesis and the fundamental basics of a *de novo* network assembly in an early metazoan.

## *Hydra* as model for neurobiology

Cnidarians, including jellyfish, corals and sea anemones represent some of the phylogenetically most ancient metazoans that developed nervous systems. Their diffuse nerve nets demonstrate the initial steps toward more complex nervous system architectures. These nerve nets enable basic behaviors such as locomotion, feeding, contractions and responses to environmental stimuli, offering insights into how early nervous systems evolved to meet the demands of survival and reproduction in an aquatic ecosystem. Studying evolutionary ancient metazoans such as the cnidarian *Hydra vulgaris* allows for tracing the evolutionary trajectory from simple neural circuits to the highly specialized nervous systems found in further developed metazoan groups (Watanabe et al., 2009).

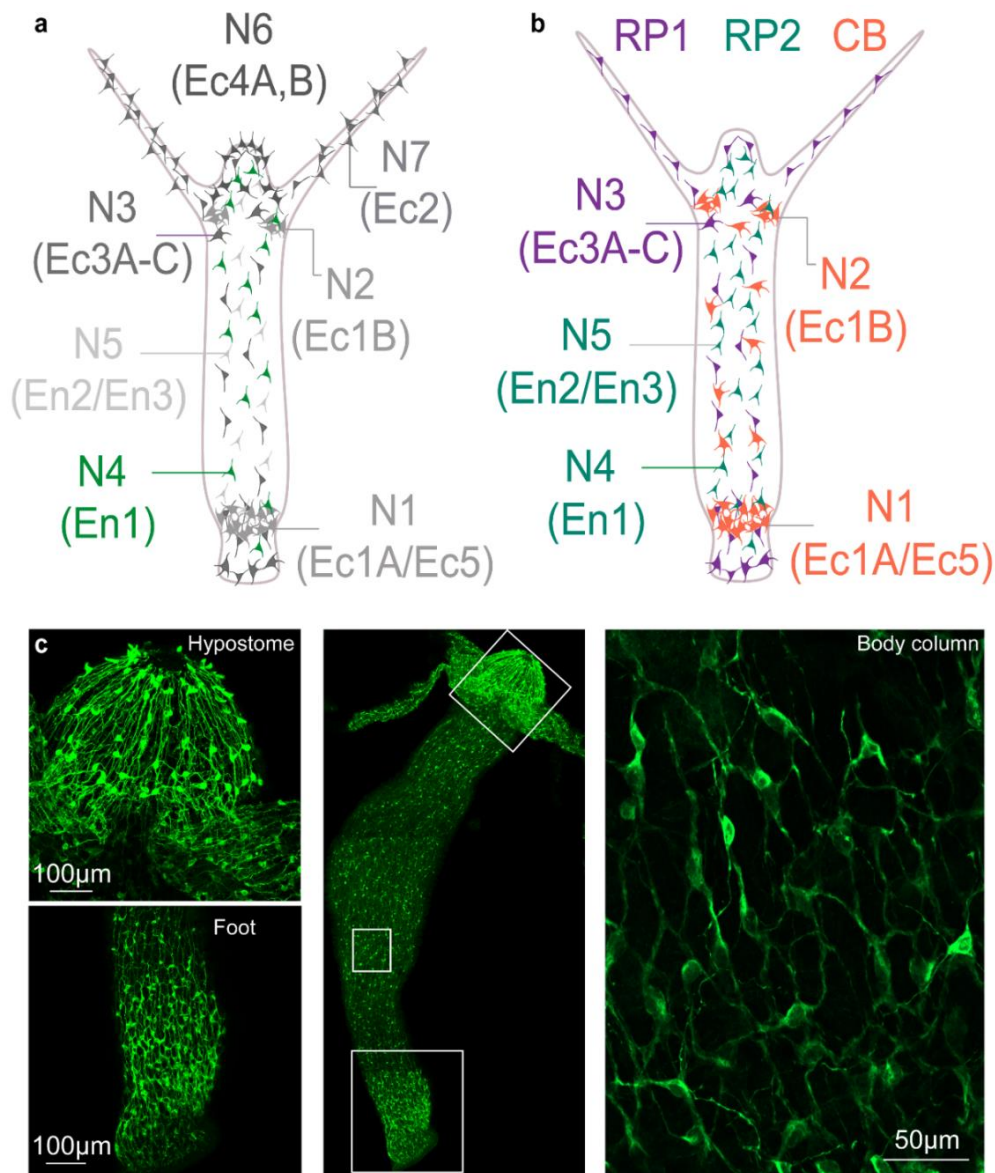
In recent years, *Hydra* has gained attention as a model organism for neurobiology due to its less complex, yet sophisticated nervous system. New technologies and methods enable deep insights into its gene expression patterns or functionality by using transgenic lines for  $\text{Ca}^{2+}$  imaging and ablation systems (Giez et al., 2024, 2023; Lovas and Yuste, 2021) and therefore provide opportunities to answer basic questions concerning the relation between neuronal activity and behavior (Yuste, 2024). To unravel those fundamental concepts a nervous system of lower complexity is needed. Despite its diffuse, non-centralized nerve net *Hydra* exhibits a rich repertoire of behaviors like contractions, feeding or complex locomotion such as somersaults and inch-worming (Yuste, 2024).

Despite being studied for more than 300 years, the first detailed description of the nervous system in *Hydra* was given by Jovan Hadzi (1909). In this work the structure and function of the nerve net was characterized by using different staining techniques to visualize neurons, their distribution and morphologic characteristics (Hadzi, 1909). In 1964, the first neuronal populations, among those sensory and ganglion cells, and their distribution within the network have been analyzed and the authors found first evidence for interstitial stem cells to be the origin of neurons (Burnett and Diehl, 1964). Today, advanced sequencing technologies provide information about the identity of neurons based on specific expression levels, the existence of eleven neuronal populations in *Hydra* (**Figure 5a**), including their subdivision into distinct subpopulations (Cazet et al., 2023; Klimovich et al., 2020; Siebert et al., 2019). This was in concordance with previous findings and proved early observations which proposed eleven

neuronal populations based on morphology by utilizing dissociated cells (Epp and Tardent, 1978).

One of the most distinctive features of *Hydra* is its capacity for whole-body regeneration, including the nervous system in well-fed animals (Campbell, 1967; David and Campbell, 1972; David and Gierer, 1974; Keramidioti et al., 2024), which allows for unique insights into neural development under homeostatic conditions. The *Hydra* genome has been extensively studied, revealing a dynamic genetic structure with transposable elements and horizontal gene transfer events that influence neuromuscular function. Moreover, the presence of conserved genes related to synaptic function and neuromodulation suggests an evolutionary link between cnidarian and bilaterian nervous systems (Chapman et al., 2010). At the functional level, studies have shown that *Hydra* utilizes neuronal ensembles to generate behavior (Yuste, 2024; Yuste et al., 2024). Observations showed that during regeneration after complete dissociation, neural activity is initially uncoordinated but gradually synchronizes over time, forming functional ensembles that ultimately control the animal's behavior (Lovas and Yuste, 2021; Yuste, 2024). The diffuse nerve net of *Hydra* consists of multiple, anatomically non-overlapping circuits that govern distinct behavioral patterns (Dupre and Yuste, 2017). These circuits include three main networks spanning the body of the polyp which are selectively activated during longitudinal and radial contractions as well as in response to light stimuli (**Figure 5b**). Particularly, neuronal populations are clustered into rhythmic potentials circuit 1 (RP1 – elongation as light response), rhythmic potentials circuit 2 (RP2 – radial contractions) and the contraction burst circuit (Cb – longitudinal contraction). A fourth localized network near the hypostome was associated with head movements such as nodding (STN – sub tentacle network). Moreover, recent studies on *Hydra* behaviors could gain deep insights into the interaction between the nervous system and behavioral output using transgenic lines for individual or combinations of specific neuronal populations to track activity and ablation systems. Thus, the neuronal subpopulations N3, N4 and N6 coordinate the eating behavior in *Hydra* and further suggest close signaling between non-overlapping populations in ecto- and endoderm (Giez et al., 2023). In this behavior, the N4 population appears to be involved in the engulfment and the expulsion of digested debris, whereas N3 performs nervous system-like functions, including the inhibition of the feeding response, contractions, somersaults and phototaxis (Giez et al., 2024). This further elucidates the role of neuronal populations and

aligns with the findings of a previous study that attributed a pacemaker function to the N2 population, controlling the spontaneous contraction patterns (Klimovich et al., 2020). In summary, the hypothesis of a non-overlapping organization challenges the traditional view of a fully diffuse nerve net and suggests a higher degree of functional compartmentalization in the nervous system of *Hydra* polyps. Such a structure allows for the parallel processing of sensory and motor information, contributing to the remarkable behavioral flexibility observed in this “simple” organism (Badhiwala et al., 2021; Dupre and Yuste, 2017; Giez et al., 2024, 2023; Keramidioti et al., 2024; Klimovich and Bosch, 2018).



**Figure 5. The nerve net of *Hydra vulgaris*.** a,b. Schematic overview of distinct neuronal populations categorized through RNA-Sequencing. c. Immunohistochemical analysis of a pan-neuronal Ca<sup>2+</sup> activity line (P46) generated by crossbreeding of various lines for specific neuronal GCaMP6s lines. Close magnification on foot and hypostome (left), total view of a whole polyp (middle) and neurons forming a dense network in the body column (right).

## Cell-cell connections and the role of epithelial cells in *Hydra*

Not only is the nervous system of *Hydra* characterized by different neuronal subpopulations but also by the presence of chemical and electrical synaptic connections, allowing multifunctional neural interactions to support behavior. Previous studies investigated the nervous system on an ultrastructural level and revealed synaptic connections, linking neurons to each other and to epitheliomuscular cells (Westfall and Kinnamon, 1984; Westfall et al., 1980). Chemical synapses are polarized, containing dense core vesicles filled with neuropeptides to facilitate neurotransmission (Koizumi et al., 1989; Westfall and Kinnamon, 1984). These synaptic structures are found between various neuronal populations in *Hydra*, including sensory, ganglion and sensory-ganglion connections (Westfall and Kinnamon, 1984). Additionally, chemical synapses are also formed between neurons and epitheliomuscular cells as well as between neurons and nematocytes (Westfall et al., 1980). Electrical synapses rely on gap junctions, which are formed by innexins and allow for a direct electrical coupling between neuronal and epithelial cells, thereby supporting the view that innexin-based gap junctions are an ancestral eumetazoan feature (Chapman et al., 2010). In *Hydra* most neuronal populations express these innexins, except for N6 and N7, while genes associated with chemical synapses are more widely distributed within the neuronal subpopulations (Klimovich et al., 2020). Electrical connections provide a mechanism for a rapid communication between cells, often co-existing with chemical synapses in the same neuron (Westfall et al., 1980). Further, the nervous system of *Hydra* exhibits complex synaptic pathways such as two-cell pathways or three-cell pathways. In the former, sensory neurons are directly connected to epitheliomuscular cells or nematocytes, whereas in the latter, ganglion cells serve as interposed structures between sensory and epitheliomuscular cells (Westfall and Kinnamon, 1984). Additionally, ganglion cells can form axo-axo-epitheliomuscular synapses, further highlighting the elaborated connectivity encountered in *Hydra*. Immunostaining to visualize the nervous system revealed a high neuronal density in the hypostome and foot in adult polyps (**Figure 5c**). In the head region, ultrastructural analysis revealed that neurons often form clusters, with an average of five up to a maximum of eleven neurons per cluster (Kinnamon and Westfall, 1981). Further, ciliary complexes in up to 96% of the neurons in the head and foot region, potentially demonstrate a contribution to a mechanosensation in *Hydra*.

The epitheliomuscular cells play a central role in *Hydra*'s neuromuscular interactions, as they can receive synaptic input from neurons via both chemical and electrical synapses, suggesting a neural role in coordinating muscle activity (Westfall and Kinnamon, 1984; Westfall et al., 1980). The multifunctional capacity of cells in *Hydra* also applies to epitheliomuscular cells where they acquire neurosecretory functions in nerve-free mutants and are capable of controlling patterning and regeneration (Holstein, 2023; Marcum et al., 1977). Also, neurons are multifunctional, as sensory and ganglion cells have been described as sensory-motor-interneurons which exhibit features of all three types of neurons (Kinnamon and Westfall, 1981; Westfall et al., 1991). Ganglion cells, in particular, possess cilia (indication of sensory function), form connections with epitheliomuscular cells (motor function) and connect to other neurons (interneuron) (Westfall and Kinnamon, 1978). Especially sensory neurons exhibit complex synaptic interactions in the head region where one single epithelial cell can interact with up to four sensory neurons (Westfall and Kinnamon, 1984). This structural complexity in the nervous system including epitheliomuscular cells suggests a dynamic and adaptable neural network where neurons and epithelial cells can assume multiple roles. Furthermore, ultrastructural analysis revealed no direct connections between endodermal and ectodermal neurons, but they remain in close contact with the mesoglea, suggesting the possibility of a vesicle-mediated communication between both networks. In addition, endodermal and ectodermal epithelial cells are in contact across the mesoglea, facilitating direct information processing via gap junctions (Giez et al., 2024) and could support a rapid exchange of information and a neuro-epithelial signal transduction.

The nervous system of *Hydra* provides a unique model for understanding basic neural circuits, where chemical and electrical synapses coexist and neurons, as well as epithelial cells exhibit multifunctionality. The interaction between neurons and epitheliomuscular cells suggests a direct link between neural activity and behavior, mediated by gap junctions and synaptic vesicles. While many structural aspects of *Hydra*'s neural connectivity are well-described, the functional significance of these arrangements remain unclear. Further research integrating molecular, electrophysiological and functional approaches will be essential to fully unravel the role of these connections in behavior and signal transduction.

## Neurogenesis in *Hydra* embryos

Even though there is an increasing interest in the nervous system of *Hydra* polyps, the insights into the *de novo* nerve net formation during embryogenesis and early developmental stages are sparse. Since sexual reproduction is a response to adverse environmental conditions, the formation of an embryo requires an external induction. However, the maintenance of large cultures, and deleterious cues under lab conditions to produce a sufficient number of embryos, remains challenging in comparison to adult polyps or bud development. Most studies on *Hydra* embryos tried to improve the understanding of oogenesis, structural elements, gene expression and general embryogenesis, the hatching behavior and microbial impacts (Alexandrova et al., 2005; Fraune et al., 2010; Fröbius et al., 2003; Genikhovich et al., 2006; Martin et al., 1997; Miller et al., 2000; Steele et al., 2019; Technau et al., 2003), but the data on the neuronal landscape in *Hydra* embryos is still limited. In contrast to *Nematostella vectensis*, in which first neurons appear during the gastrulation stage regulated by the MAPK pathway (Layden et al., 2016; Nakanishi et al., 2012), the first emergence of neurons or neuron-specific transcripts during embryogenesis in *Hydra* is still unknown. However, a previous study based on macerated samples showed the absence of neurons during embryogenesis, with the first emerging neurons between 0h and 5h post hatching and increasing in later stages (Martin et al., 1997). In 2002, Brumwell and Martin identified first neurons during late embryogenesis by using antibodies against specific neuropeptides (RFamide and JD-1). Their study further revealed that these neuropeptides are expressed in the anterior and posterior regions of early hatchlings, suggesting an early developmental patterning (Brumwell and Martin, 2002). However, the identity of these neurons remained unknown, and it took 17 years until the first single-cell transcriptome atlas characterized the neuronal populations in *Hydra*. RFamide is still in use, and its expression has been assigned to the populations N1, N6 and N7 (Klimovich et al., 2020), indicating that these populations are already present in late embryos and early hatchlings. Additionally, further missing pieces include the network composition in embryos and the functionality of emerging neurons, as previous studies examined only fixed samples and provided no evidence for neuronal activity. Although, first studies could quantify the number of neurons during specific developmental stages and an early patterning in hatchlings, the (*de novo*) neurogenesis, nerve net assembly and neuronal identities during embryogenesis still remain unknown. The knowledge about the

nerve net in *Hydra* has nearly exclusively been derived from adult polyps, bud development or reaggregation and regeneration experiments. The approach to examine fundamental evolutionary processes in adult polyps may be challenging due to the permanent ongoing neurogenesis or their ability to re-establish the nerve net during regeneration, as a previous study already revealed general differences in gene expression levels between adult polyps and embryos (Genikhovich et al., 2006). In contrast, the *de novo* nerve net formation in *Hydra* embryos could reveal new fundamental features to the understanding of the basic architecture in complex nervous systems of higher metazoans.



## Aims of the study

This study sets out to investigate the fundamental mechanisms underlying the emergence and assembly of functional neuronal circuits at the base of metazoan evolution. The insights gained from this study aim to contribute to a deeper understanding of nervous system evolution hidden from more complex systems. Moreover, this study highlights the pivotal role of *Hydra* embryos as a model for uncovering *de novo* mechanisms close to the evolutionary origin of the animal nervous system.

1. Characterization of the neuronal landscape in *Hydra* embryos and early hatchlings and the emergence of early behaviors.
2. Establishment of transgenic *Hydra* embryos to investigate the emergence and assembly of a functional neuronal circuit in embryos using  $\text{Ca}^{2+}$  imaging and bioinformatic approaches.
3. Understanding the role of environmental factors such as temperature and microbial influences on the *de novo* nerve net formation in *Hydra* embryos and early hatchlings.

## Methods and Materials

### Cultivation

*Hydra* polyps (*Hydra vulgaris* AEP) were cultivated in standard *Hydra* medium (CaCl<sub>2</sub> 0.042g/l; MgSO<sub>4</sub> x 7H<sub>2</sub>O 0.081g/l; NaHCO<sub>3</sub> 0.042g/l; K<sub>2</sub>CO<sub>3</sub> 0.011g/g in dH<sub>2</sub>O) according to previous established protocols (Bode et al., 1973). The animals were cultured in either 20 x 20 x 6cm culture dishes or 250ml glass beakers at 18°C depending on culture size. Polyps were held at a 12/12h light cycle and strictly fed three times a week to induce sexual reproduction, utilizing *Artemia nauplii*. Animals were washed after a digestion period of 8-14h. Adult polyps were not fed 48h prior to any experiments. For long-term calcium recordings hatchlings were fed at day 2 and day 8 after removing the cuticle.

### Embryos

#### Collecting

*Hydra* cultures were frequently observed and embryos were collected in batches of 25 and stored in 6-well plates (Eppendorf cat#0030720113) at 18°C and 12/12h light conditions in *Hydra* medium. Embryos were observed daily (from day 10 on) with a dissecting microscope (Wild M.3) throughout their development to determine the hatching rate. *Hydra* embryos were cut off from the female polyp 24 hpf during the cuticle stage and collected in 6-well plates.

#### Determination of time points

To distinguish between important changes in morphology, up to three late non-hatched embryos (day 10+) were recorded using a dissecting stereomicroscope (Olympus SZX16) equipped with a camera (Olympus DP74) at one frame per minute (Olympus cellSense Standard 1.18) in a microscope slide with a depth of 5mm and covered with a cover glass to avoid evaporation of the *Hydra* media.

## Preparation

Embryos and hatchlings were collected at specific developmental stages, the cuticle of embryos in the Bilayer stage was removed with a scalpel or a preparation needle prior to fixation. The embryos were relaxed with ice-cold 2% Urethane in *Hydra* media and fixed in Zamboni for 2h (RT) or overnight (4°C). The samples were then washed three times for 15min in PBS with 0.1% Tween (PBST) before storing them for further analysis in PBST + NaN<sub>3</sub> at 4°C to avoid fungi formation.

## Maceration and determination of total cells

To investigate the specific structural composition of *Hydra* embryos and early hatchlings regarding the number of neurons and neural precursor cells as well as the determination of total cells in each of the developed stages, the established maceration technique (Charles N. David, 1973) was modified. Here, one single embryo or early hatchling was transferred to a Durham test tube (Carl Roth cat#TX27.1). The supernatant of HM was completely removed before adding 25µl maceration solution (1:1:13; Glycerol, Acetic acid, HM). The test tube was sealed with a lid and each individual sample was incubated for 30min at 32°C. Following this step, 25 µl 8% PFA was added and the sample was dissociated by gently pipetting the media up and down with specialized hydrophobic pipette tips (Nerbe Plus cat#06-642-5300). The entire mixture was transferred to a field of 2x2cm on a gelatin-coated slide with the addition of 10µl of 0.5% Tween. The solution was distributed using the previous used hydrophobic pipette tip to avoid the loss of cells. The slide was left to dry at room temperature protected from dust. For analysis, the number of neurons and precursor cells for 10 fields of vision were counted and extrapolated to the entire field of 2x2cm after calculating the area for the field of vision. Cells on the right side, which were not fully visible in the complete field of vision were taken into account for the calculation of the total number, while cells partially visible on the left site were omitted.

In order to identify the number of neurons and precursor cells, the previous described method was used to determine the total amount cells for the defined developmental stages. Therefore Neubauer improved counting chambers (Carl Roth, cat#PK36.1) were used an 10µl of the cell suspension transferred into the counting chamber. The number of cells was determined and used to calculate the total cells for 60µl in the cell solution.

## Generating germ-free embryos

Embryos in the cuticle stage (24hpf) were separated from the female and treated with an antibiotic cocktail containing Ampicillin (50mg/ml), Neomycin (50mg/ml), Rifampicin (50mg/ml in DMSO), Streptomycin (50mg/ml) and Spectinomycin (60mg/ml) as described previously (Franzenburg et al., 2013b). Throughout the 6-day treatment, the antibiotics solution was exchanged once after three days under sterile conditions and the embryos were incubated at 18°C in the dark on an orbital shaker (IKA, Rocker 3D digital cat#0004001000) at 12 rpm. After the treatment, embryos were rinsed with sterile HM to avoid transfer of antibiotic residue and placed in sterile media afterwards. To ensure the absence of bacteria, two-step sterility tests were performed at 2- and 8-days post-antibiotic treatment. As a first step, two embryos from each treatment were homogenized in 100µl sterile media and plated on R2A agar plates and incubated at 18°C. The second step to ensure the germ-free status was via PCR by isolating whole DNA from single embryos (Qiagen cat#69504) using universal 16S rRNA Primers (Weisburg et al., 1991). Absent amplification products and bacterial colonies after 1 week of incubation on the R2A agar confirmed the germ-free status.

## Recolonization experiments

Germ-free embryos were generated as described above to perform recolonization experiments. Two days post-antibiotics treatment, the sterile embryos were transferred to a glass beaker (Carl Roth cat#C095.1) with HM along with 70 wild-type adult polyps to restore and establish a natural microbiome. Cultures with adult polyps and embryos were fed two times a week and washed 12-14h after feeding. Embryos were observed two times a day after 10d post-cuticle formation.

## Behavioral assays

### Contractions and somersaults

To investigate first emerging behaviors during specific developmental stages in early wild-type *Hydra* hatchlings, first full body contractions and somersaults were documented. Recordings were made using M3C Wild Heerbrugg binocular microscopes and Axiocam 208 color (Zeiss) placed in an insulated climate chamber at a constant temperature of 18°C. Up to 15 embryos

of similar developmental age were collected and placed into custom-made chambers of 7 mm height and a volume of 1.5-2ml which allowed the juvenile polyps to move freely after hatching. During recordings, the medium in the chambers was kept at a sufficient level by adding HM as needed. The embryos in each chamber were recorded at once for up to 10 days with a frame rate of 0.5 frames per second. The recordings were blinded, and both behaviors were manually annotated. In total, a time window of 10h after hatching was analyzed for individual samples and summarized in frequency per hour. For the contraction behavior, all full body contractions were scored as previously established (Klimovich et al., 2020). These contractions were characterized by a reduction of the full body length of the juvenile polyps to a minimum of 50% to a ball-like structure in addition to contracted tentacles. For the somersaults only the first full behavior was analyzed for each of the hatched polyps from the recordings. As somersaults display a more complex behavior compared to contractions the following behavioral sequences were defined and only the full set was counted as a somersault event: detachment of the foot, attachment of head to the surface, contraction, bending and re-attachment of the foot in accordance with previously published work (Frost, 2023; Yamamoto and Yuste, 2023). To analyze the recordings for first emerging contractions and somersaults Fiji (Schindelin et al., 2012) was used.

### Tentacle movement and mouth opening induced by Glutathione and *Artemia*

To analyze the tentacle movement (TM) and the mouth opening (MO) as part of the general eating behavior in *Hydra* a diverging behavioral assay was conducted. TM was defined as active, non-directed movement of the tentacles and head of the early-staged hatchlings, whereas the MO behavior was defined as a constant opening of the mouth. A short opening and direct closing were not scored as MO. To analyze both behaviors in early hatchlings, three different treatment groups were defined: reduced Glutathione (Roth, cat#6382.1) as artificial food stimulus, shred *Artemia nauplii*, and intact *Artemia nauplii*. To conduct the experiment, early hatchlings were collected in previously defined determination time points, starting with the Cigar stage as earliest morphological stage. For each of the samples the residual cuticle was removed and up to 5 samples were placed in a petri dish (Eppendorf, cat#0030702018) and observed with a stereomicroscope (Olympus SZX16). First, for the Glutathione (GSH) assays a final concentration of 10  $\mu$ M (CG23,24) for each of the different developmental stages

was used. The hatchlings were placed in a petri dish (Eppendorf cat#0030701011) filled up with GSH solution, the TM and MO were scored over 5 min and were annotated manually. Hatchlings, which did not display TM or MO during the experimental time were scored as “negative”. As a second treatment group shredded *Artemia nauplii* was used. Therefore, 1 ml freshly prepared shrimps were filled into a 1.5 ml Eppendorf cap (Sarstedt cat#72.690.001) and grounded with a pestle. A drop of the resulted protrusion was transferred to the sorted hatchlings, the TM and MO behaviors were scored as previously described for the GSH assay. As third treatment group *Artemia nauplii* as living prey was used to examine the time when early hatchlings are able to fulfil the complex eating behavior including the catch of the prey, a directed movement to the hypostome and the swallowing of the prey to actively incorporate *Artemia* as energy source. To quantify the results, freshly prepared *Artemia* were added in surplus to guarantee possibility of catching. As previously described, the results were annotated manually after 5min. A further quantification was made after 15 min to eliminate the possibility of a time delay. For all experiments the polyps were used only for one treatment group, no second treatment was conducted and no polyp was followed during development individually.

## Transgenic lines

The transgenic line N4 (GCaMP6s) used to generate embryos has been published in another study (Giez et al., 2023). Here, animals in the F1 generation were used to obtain transgenic embryos, thus ensuring an incorporation of the construct. To generate a pan-neuronal cross-breeding line for  $\text{Ca}^{2+}$  activity (GCaMP6s) we only used animals from F1-generations of previously published lines (Giez et al., 2024, 2023) (N4, N6, alpha tubulin). For cross-breeding, alpha tubulin females were incubated with N4 males. Positive embryos were cultured and sexual reproduction was induced. Next, these animals were used to cross-breed females with N6 males to generate the P46 line.

## **Histology**

### **Immunohistochemistry**

Previously fixed embryos were washed 3 x 15min with PBST, permeabilized for 30min in PBS + 0.5% Triton X-100 followed by a blocking step (PBST + 1% bovine serum albumin, BSA) for 1h. Animals were then incubated overnight at 4°C with a primary antibody in PBST + 1% BSA. Primary antibodies used in this study were: anti-FMFR (BMA Biomedicals, cat# T-4322, 1:1000), anti-GLWamide (KPNAYKGKLPGLW-amide, 1:2000, Takahashi 2003) and anti-GFP (Biozol, cat# GFP-1010, 1:1000). After incubation with the primary antibody, samples were washed four times each for 15min with PBST + 1% BSA before adding the secondary antibody. Here, the following secondary antibodies were used: goat anti-chicken IgY Alexa Fluor 488 (Invitrogen, cat# A11039, 1:1000) and goat anti-rabbit IgG (Invitrogen, cat# A11034). Samples were incubated for 2h (RT) without light in the antibody solution followed by another four washing steps in PBST + 1%BSA (with 0.5% Tween). Samples were either stored at 4°C for a short period or mounted in Mowiol on a coverslip (Carl Roth, cat# 1871.2) covered by a cover glass (Carl Roth, cat# 1870.2) separated by an imaging spacer (Grace Bio-Labs, cat#SS1X20) and stored in a humid chamber at 4°C until imaging.

### **Histology sections**

Samples were embedded in epoxy resin as described for TEM ultrastructural analysis. Semi-thin (1 µm) epoxy resin sections were cut on a Leica UC7 ultramicrotome using a histo diamond knife. The sections were deposited on Superfrost® Plus adhesive slides, stained with Richardson's solution (alkaline solution of azure II (50%) and methylene blue (50%)), embedded between a glass slide and a cover slip using a CV Mount medium (Leica) and imaged using a Zeiss LSM900 microscope equipped with a 20x Plan-Apochromat objective (NA 0.8), an AxioCam 305 color camera and a ZEN 3.2 system including automated tiling function.

### **Ultrastructural analysis by transmission electron microscopy**

Hydra embryos and hatchlings were relaxed with 2% urethane prepared in Hydra medium for 1 min and transferred to 10mM cacodylate buffer + 1% glutaraldehyde for fixation. Polyps were post-fixed with 1% osmium tetroxide prepared in 1.5% potassium ferricyanide/dH<sub>2</sub>O for 1 h on

ice, and contrasted en-bloc with 2% aqueous uranyl acetate for 1 h at room temperature. Dehydration was performed at room temperature with ascending ethanol series (each step 15 min), followed by acetone (2 × 30 min). After progressive infiltration with epoxy resin (Epon\_812 substitute, Sigma), samples were heat polymerized. Thin, 80-nm sections were prepared using Leica UC7 ultramicrotome and a Diatome diamond knife, and contrasted with saturated uranyl acetate and Reynolds lead citrate. Sections were imaged with a Tecnai G2 Spirit BioTWIN transmission electron microscope (FEI), operated at 80 kV, and equipped with a LaB6 filament, a CCD side-mounted MegaView III G2 camera, and iTEM v.5 software (both Olympus Soft Imaging solutions) as described previously (Giez et al., 2024).

## Imaging and analysis

The fixed and stained embryos and hatchlings were imaged with a confocal laser-scanning microscope (LSM900, Zeiss) using the Airyscan mode and a Plan-Apochromat 20x / NA 0.8 objective, or the Axio Vert.A1 (Zeiss) using Colibri 7 (Zeiss) as a light source. Images were further processed with Zen Blue 3.4 software (Zeiss) or Fiji. For determination of total neuron numbers, all RFamide-positive neurons were counted only in the head area of the embryos and hatchlings with a minimum of five samples for each stage and treatment. In addition, to identify neuronal connections, neurites originated from a neuron soma for up to 10 cells per sample with a minimum amount of 5 samples for each stage were analyzed. To examine neuronal numbers and neurites, composites of stacks (maximum projections) were used, whereas brightness and contrast were modified to increase visibility. The analysis was carried out using R (2023.12.0+369) (RC Team, 2020) over RStudio IDE. To visualize the plugins “ggplot” (v3.5.1), “tidyr” (v1.3.1), “ggpubr” (v0.6.0) and “dplyr” (v1.1.4) were used.

## In vivo single-cell Ca<sup>2+</sup> imaging

To analyze the neuronal activity on single-cell resolution, a self-made chamber which allows for examining the whole embryo throughout long-term recordings at following temperatures was developed: standard: 18°C; cold: 13°C; warm: 23°C. To adjust the temperature, an air-conditioned room was used, which was set to the requested temperature 3 days prior to recordings. Embryos in the Bilayer stage were placed into an imaging spacer affixed to a 24x50mm cover slip (Carl Roth cat#1871.2) filled with *Hydra* media and closed with a small



22x22mm cover slip (Carl Roth, cat#H874.2) with two openings on both sides of the imaging spacer to fill and refill media. To reopen the chamber, the upper plastic liner remained on the spacer which constituted a standard height of 0.24mm during recordings. Hatchlings and adult polyps were placed into commercially available channel slides that were 0.1 or 0.2mm in height (ibidi cat#80661, ibidi cat#80161). Once the samples were placed into the chambers or channel slides, animals were allowed to adapt for 10min to the chamber and additional 2min to the laser on the microscope stage prior to recordings. For these recordings a 14% laser intensity was used and animals during the first 7.5h were recorded for 30min with 25fps. After recording, the samples rested for 60min within the self-made chamber, which was filled with an additional amount of 400µl *Hydra* media to slightly lift the cover glass to increase the space between top and bottom. After a resting time of 60min, the previously added media was removed and recorded as previously described. Animals after 1 or 10 days were recorded for 10min with the same setup. Imaging was performed using the Axio Vert.A1 (Zeiss) using Colibri 7 (Zeiss) as light source equipped with a 38HE filter (Zeiss), the AxioCam 705 mono (Zeiss) and a 10x Plan Apo objective. The recordings were implemented to Zen Blue 3.4 software (Zeiss) for image export, further processed in ImageJ (Schindelin et al., 2012) to 8-bit and in total 2000 frames were selected from each dataset which was analyzed with ICY software (Lagache et al., 2020). Tracked neurons were automatically traced using the protocol “Detection (with cluster un-mixing) and Tracking of neurons with emc2” whereas parameters were constituent adjusted depending on individual samples. Accuracy was manually perused for each tracked neuron, missing links connected and merged. Non-specific and false tracks were removed.

## Community Detection

Community detection results were obtained using the Leiden algorithm (Traag et al., 2019) that has been shown to outperform the previous state-of-the-art Louvain algorithm. The resolution parameter for the constant Potts model (CPM) used as the quality measure of the Leiden algorithm was set to  $\gamma = 0.96$ . Connectivity data to be processed by the algorithm were given by correlation matrices estimated from the measurement and simulation data. Since the CPM mode of the Leiden algorithm failed to process the complete correlation matrices, only non-negative values were used. To increase the accuracy of the correlation analysis, the measurement was processed with the spike inference method MLspike (Deneux et al., 2016).

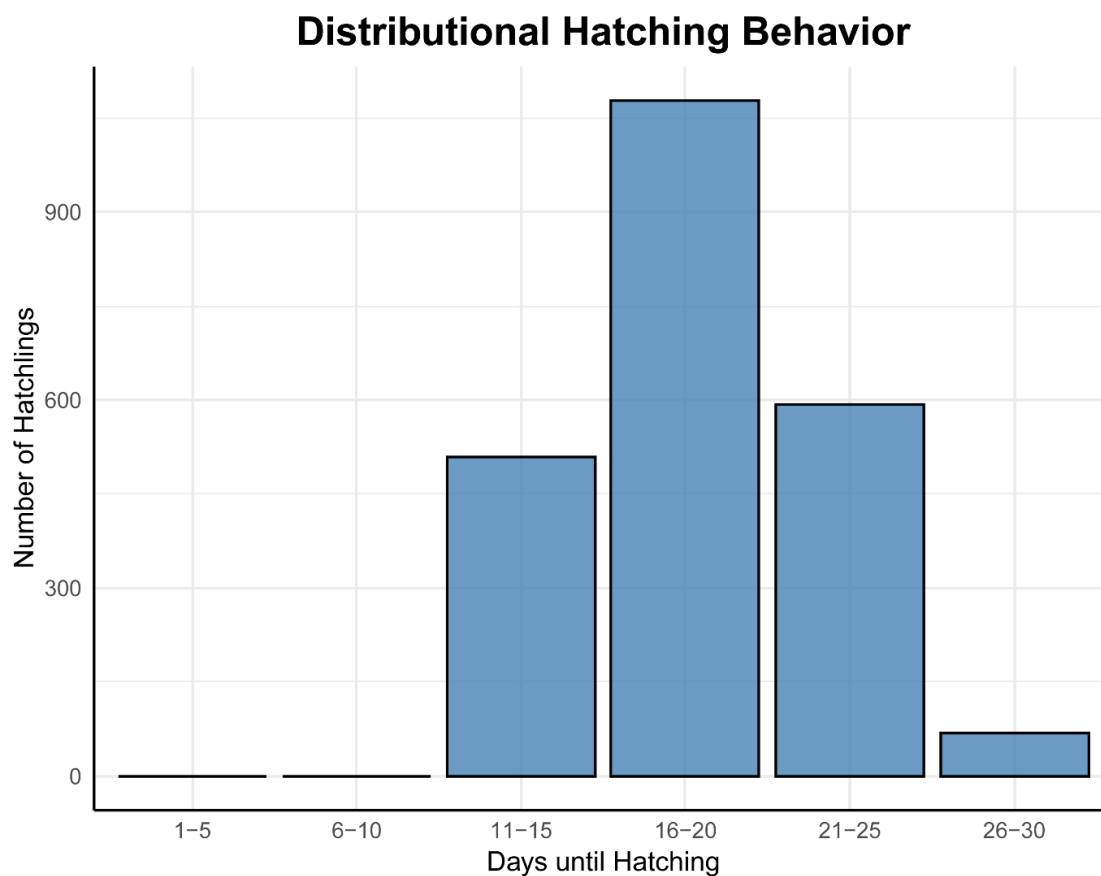
This allowed the removal of the drifting baseline and the noise present in the measured fluorescence trace and therefore normalized recorded tracks to improve the comparison.

## Results

### The development of *Hydra* embryos and hatchlings with respect to the formation and maturation of the nerve net

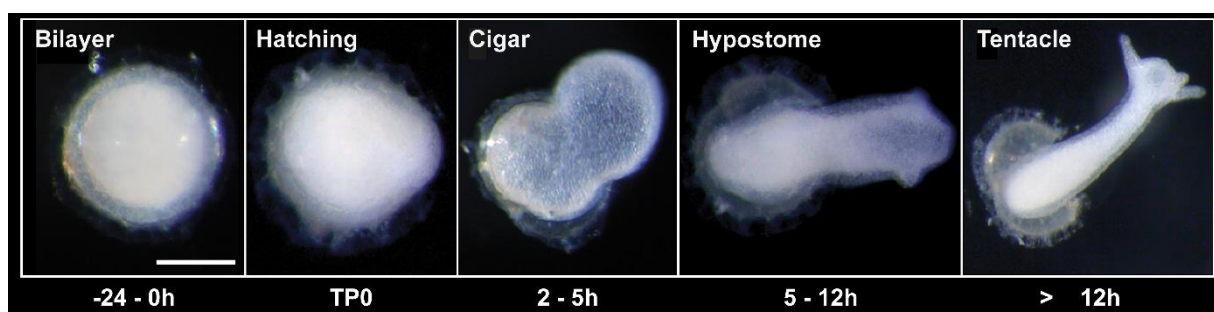
#### First characterization of *Hydra vulgaris* AEP embryos and hatchlings

In the initial step of investigating the developmental properties of *Hydra* embryos the general hatching behavior was observed. To gain a deeper insight into structural and environmental characteristics related to neuronal properties, the developmental timespan until hatching was monitored. Here, hatching was defined as the rupture of the cuticle. Over a period of 12 months 2.250 embryos were collected in 86 batches (ranging between 5 and 76 embryos per batch) to observe and analyze the hatching behavior. The results showed that most of the embryos did not hatch earlier than 16-20 days post fertilization, although the first hatchlings were detected after 13 days (**Figure 6**) and an overall hatching rate of 53% was identified.



**Figure 6.** *Hydra vulgaris* AEP embryos were monitored and displayed the highest probability of hatching between 16- and 20-days post fertilization (dpf). Furthermore, no hatching of embryos was observed before 10 days and after 30 days. (n = 2.250)

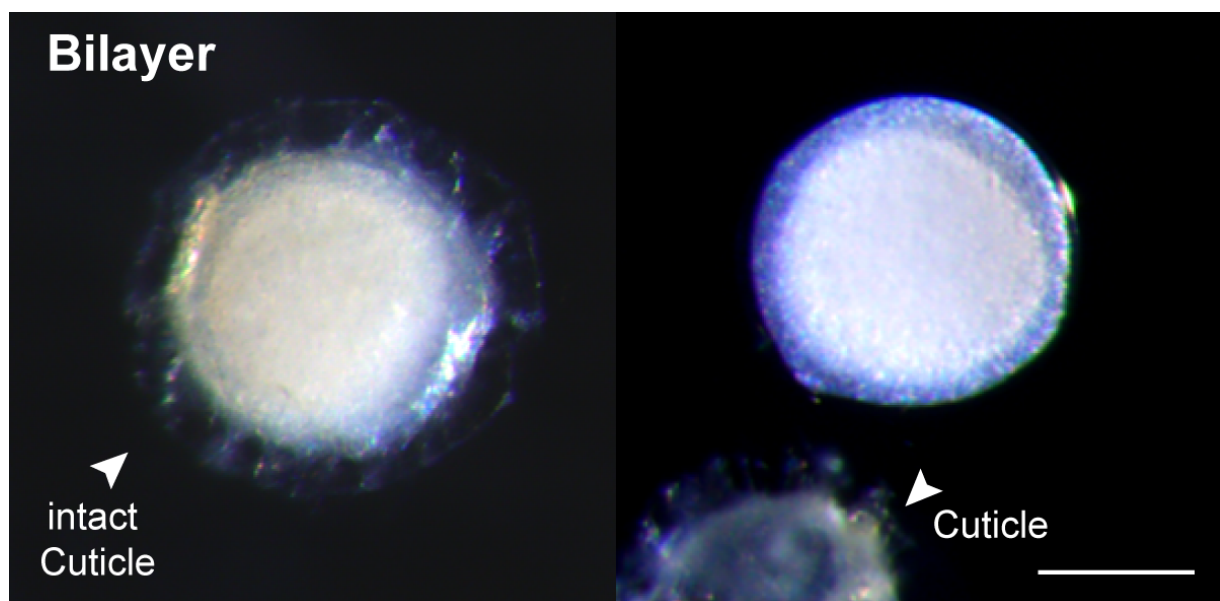
This information was used to define specific stages based on morphological changes to increase comparability as well as a quantitative and qualitative investigation of structural nerve net characteristics in late-staged embryos and early hatchlings. To ensure the recording of the whole hatching process, embryos (12 dpf) were recorded at one frame per minute (fpm) for 3 days (Appendix Video S1). Analyzing these recordings revealed five different developmental timepoints subsequently termed as Bilayer, Hatching, Cigar, Hypostome and Tentacle stage (**Figure 7**). In this work, the Bilayer stage represents the final phase of embryogenesis and the earliest stage accessible for experiments. At this stage, the formation of the ectoderm is completed, however, the embryos remain covered by a thick protective cuticle which was removed before experiments as it is functioning as a robust barrier to protect the embryo from external influences. Embryos prior to this stage were not accessible due to the inability to survive the removal of the cuticle. The Hatching stage is characterized by the rupture of the cuticle, allowing the spherical embryo to elongate by incorporating surrounding media, transitioning into a cylindrical shape (Cigar stage). The initial contractile behavior induces a significant morphological transformation, referred to as collapsing event, during which two small tentacles and the hypostome become visible (Hypostome stage). In the subsequent hours, the early hatchling begins to exhibit a set of behaviors including periodic contractions or bending similar to adult polyps. These behaviors are followed by the extension of the tentacles and an inducible mouth opening (Tentacle stage). The stages outlined above served as central references for further experiments conducted throughout this work.



**Figure 7.** Developmental timepoints of *Hydra* hatchlings, determined based on morphological changes during development. In the Bilayer stage, the ectodermal layer is formed, marking the latest embryogenic stage and thereby displays the earliest accessible stage for further investigations. Scale bars, 500  $\mu$ m.

## Cuticle removal to access late embryogenesis

During their embryogenic development, *Hydra* embryos remain covered by a thick protective structure referred to as cuticle. This cuticle protects the embryo from environmental influences or even antibody staining, even after fixation. Furthermore, the cuticle increases the refractive index and make the observation of internal developmental processes in living embryos particularly challenging. Before entering the Bilayer stage, external access was limited, as the disruption of the cuticle caused embryogenic death. However, during the final embryogenic stage, the thickening of the ectodermal layer made it possible to carefully remove the cuticle. Using a scalpel, the cuticle was carefully removed at the Bilayer stage, maintaining embryogenic integrity without causing developmental disadvantages. In fact, the removal of the cuticle induced the hatching process, prompting the embryo to the intake of surrounding media and surging of the polyp before obtaining the characteristic Cigar stage-specific shape a couple of hours after the treatment.

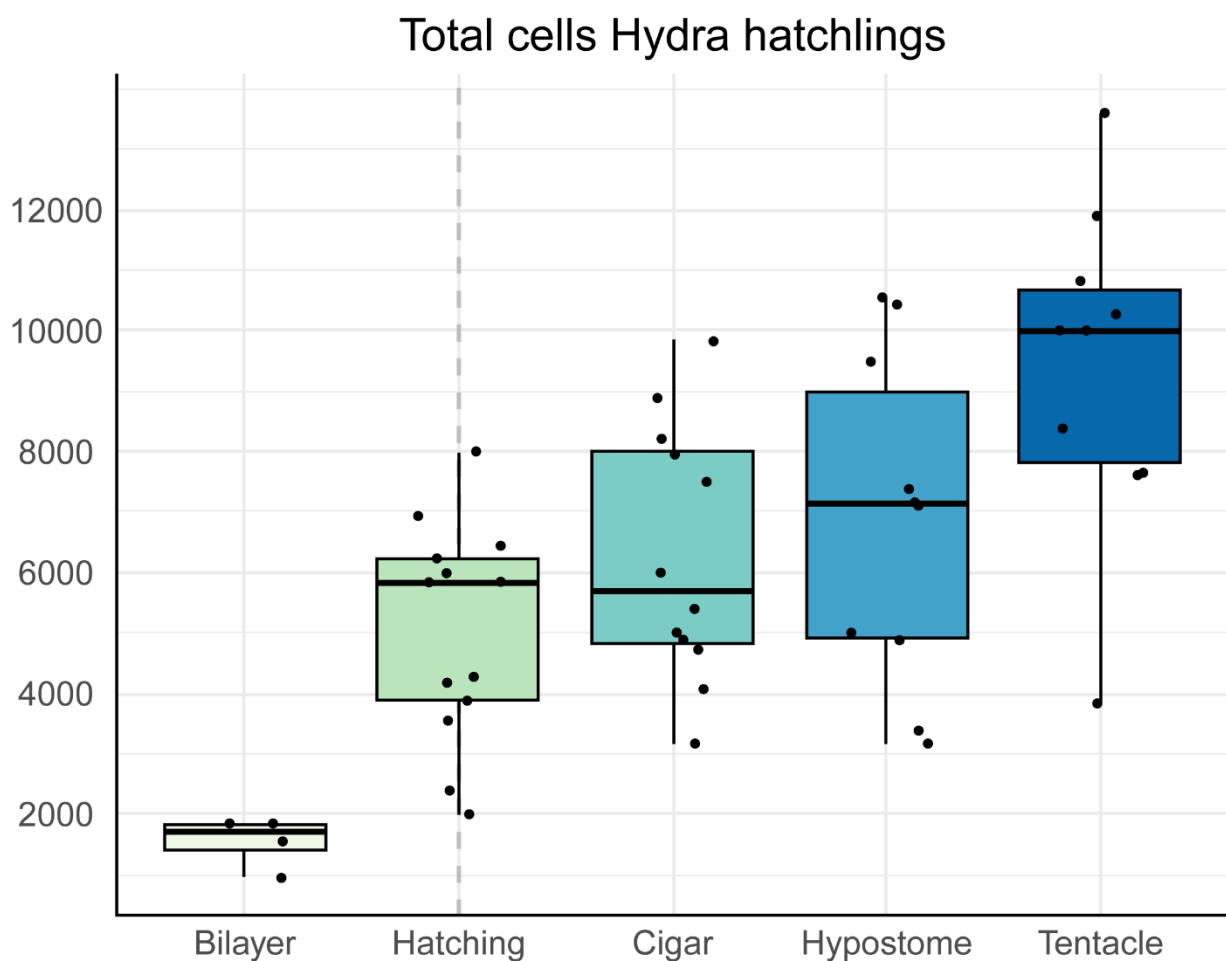


**Figure 8.** Intact bilayer-staged embryo with intact cuticle during the swelling of the ectoderm (left). The thickening of the ectodermal layer during the Bilayer stage allows the removal of the cuticle without a loss of integrity (right). Scale bar 500µm

## Cell-structural properties of embryos and early hatchlings

To determine the structural composition in terms of total number of cells, maceration techniques were employed in combination with a counting chamber (Neubauer improved). Embryos and hatchlings from the previously described stages were dissolved using a modified maceration protocol. Each sample was dissolved individually and counted manually. Analysis

of early hatchlings following the initiation of the hatching process revealed a consistent increase in total cell numbers, starting at approximately 5.000 cells in the Hatching stage (**Figure 9**). During the rapid transition from early to later-staged hatchlings, a doubling of the cell numbers was observed. Notably, hatchlings in the Tentacle stage already contained 10% of the total cells observed in adult polyps. Interestingly, embryos in the Bilayer stage exhibited only one-third of the cells traceable in the Hatching stage, suggesting that the number of 4.000 – 6.000 cells may play a crucial role in facilitating the rupture of the cuticle and serving as an indicator or trigger for the initiation of the hatching process.



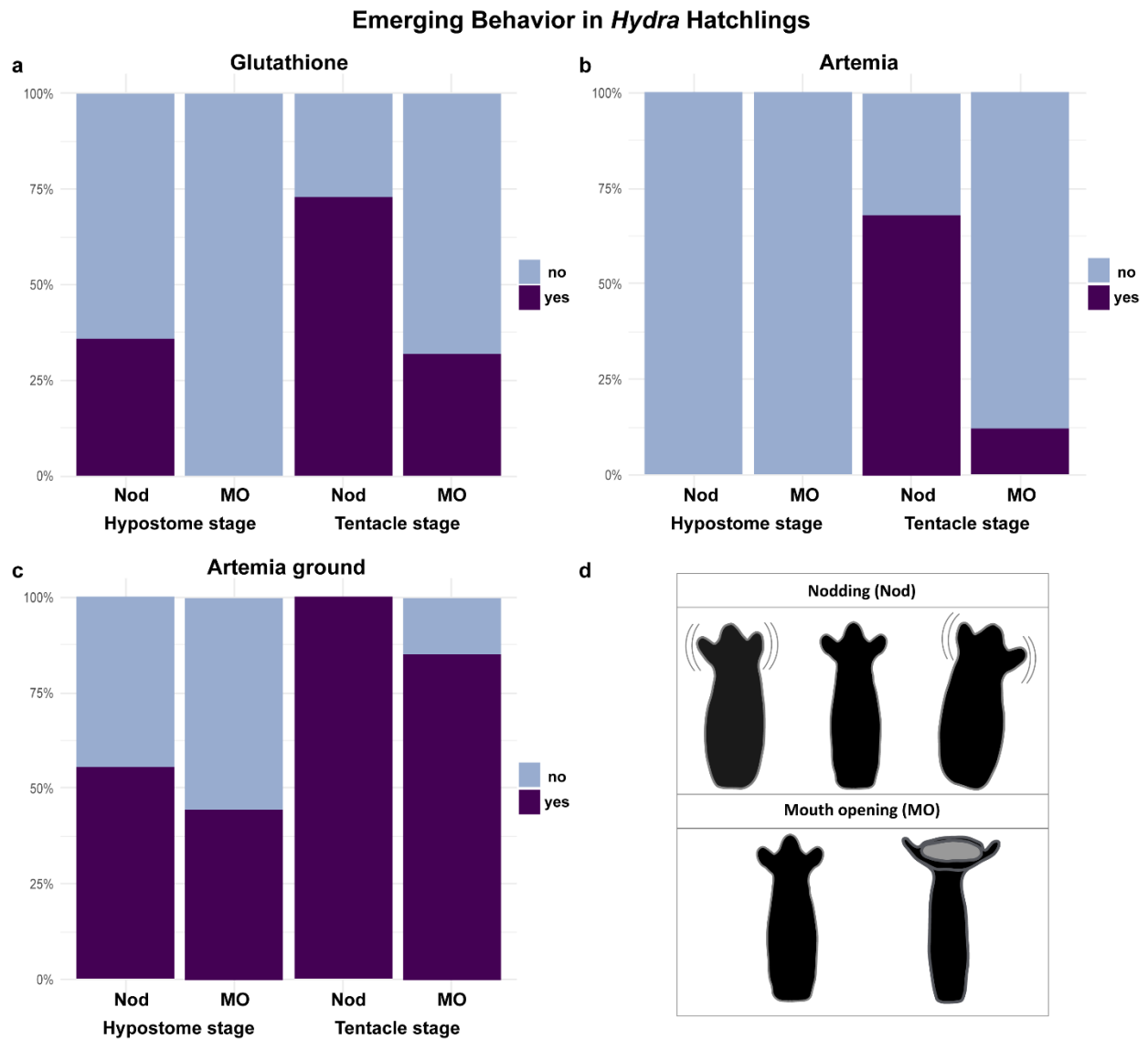
**Figure 9.** Total cell count in embryos and early hatchlings determined through macerations and manual counting using hemocytometers, displayed a twofold increase when comparing the Hatching and the Tentacle stage. Mean: Bilayer 1541; Hatching 5042; Cigar 6300; Hypostome 6861; Tentacle 9408. (n = 4-13). Each dot represents one individual sample and the dashed line marks the initiation of the hatching process.

## First emerging behaviors in *Hydra* hatchlings

Numerous studies on *Hydra* have analyzed and aimed to understand specific behaviors (Dupre and Yuste, 2017; Giez et al., 2024, 2023; Kim et al., 2024) to determine the involvement and role of neurons and specific neural populations. To further characterize the development during early hours after hatching, various assays were conducted to match emerging behaviors with different developmental stages. As an initial approach, recordings of late-staged embryos were performed to examine the first behaviors in the life of *Hydra*. Up to 15 bilayer-staged embryos were placed in a custom-made chamber and recorded at two fpm for a maximum of 10 days, ensuring that the full set of behaviors could be captured for each hatchling. These recordings focused on the emergence of initial contractions and somersaults during the early developmental stages. The analysis of the recordings revealed contractions as the first observable behavior in the life of *Hydra*. On average, these contractions occurred 8 hours after hatching, resulting in a significant morphologic transformation, including the formation of the hypostome and the initial appearance of small tentacles. Following this first behavior, the small polyp enters a novel developmental stage referred to as Hypostome stage, characterized by the emergence of a visible hypostome. Interestingly, the timing of the first contractions varied among hatchlings, ranging from three to 14 hours after cuticle rupture ( $n = 17$ ), indicating considerable individual variability. Additionally, the recordings facilitated the analysis of first emerging somersaults after hatching. The results revealed even greater variability in the occurrence of this complex behavior. On average, polyps performed their first somersaults after 33 hours post-hatching, although the spectrum ranged from 12 to 59 hours ( $n = 12$ ), highlighting the complexity of this behavior (Appendix Figure S1). In a next attempt, the eating behavior in early hatchlings was observed, an essential progress for maintaining energy intake for subsequent developmental processes such as growth of the juvenile polyp. Three separate examinations were conducted during the early developmental stages after hatching, each split into two conditions – nodding (Nod) and mouth opening (MO), both measured up to 5min after the stimulus. On the one hand, *Artemia nauplii* were used as living prey, as well as cell-extracts, since the presence of specific cells, such as nematocytes, responsible for prey capture, could not be verified in all examined samples. On the other hand, GSH was used as artificial food stimulus to induce the eating behavior in early hatchlings. In contrast to the previously conducted behavioral examinations, no timeline recordings were performed.

Instead, the hatched polyps were classified into specific determination time points, as described earlier (**Figure 7**). Here, the Bilayer, Hatching and Cigar stages were excluded from analysis, as no behavior related to Nod and MO were observed during the Cigar stage (n = 15), and the first tentacles became visible as early as in the Hypostome stage. Starting with the GSH assay, 35,7% of the observed hatchlings during the Hypostome stage responded positively in terms of Nod to the GSH stimulus (n = 14), while 72,7% of Tentacle-staged samples (n = 22) displayed nodding. Deviating from this observation, no MO was observed for the Hypostome-staged hatchlings (n = 14) and only 31,8% of the polyps in the Tentacle stage exhibited a MO after GSH induction (n = 22). Interestingly, the MO was only observed in polyps that had previously exhibited nodding. Samples without displaying nodding after GSH stimulus did not display a MO, suggesting nodding to occur earlier during development (n = 36) (**Figure 10a**). Furthermore, the detection of GSH and the emergence of a behavioral response during the Hypostome stage marked the first possible external signal detection and interaction with the surrounding environment. In a next attempt, *Artemia nauplii* were used as a food stimulus. Here, only the Hypostome and Tentacle stage were considered, as developed tentacles were prerequisite to catch prey (**Figure 10b**). In the Hypostome stage, neither Nod nor MO was observed in any of the examined samples (n = 10). However, 68% of the Tentacle-staged hatchlings exhibited a nodding behavior, although only 12% of the samples displayed the ability to open the mouth. Interestingly, all polyps that exhibited the MO were further capable of catching and engulf prey (n = 25). This suggests an absence or incomplete development of all involved structural elements, such as cnidocytes for prey capture, receptors for detection or the nerve net to coordinate the full behavioral repertoire. To further examine the Nod and MO in early hatchlings, ground *Artemia nauplii* were used to induce both behaviors. Similar to the GSH stimulus, no response was observed in cigar-staged hatchlings during the examination (n = 17). Interestingly, during the Hypostome stage, the number of positive responders increased compared to both the GSH stimulus and living prey. Here, 55,5 % of the hatchlings exhibited a nodding behavior, and exclusively for this treatment, 44,4% displayed a MO (n = 18) (**Figure 10c**). Considering the Tentacle stage, all observed hatchlings responded with nodding and 85,2% exhibited a MO (n = 27).





**Figure 10.** The response to specific food stimuli during the Hypostome and Tentacle stage depends on the stimulus itself. While both Glutathione (a) and especially ground *Artemia* (c) induced an increased response, even in hypostome-staged hatchlings, a low response to living prey (b) was observed. d. Schematic illustration of nodding (Nod) and mouth opening (MO) in early *Hydra* hatchlings. (Data displayed in percentage: *Artemia* n = 35; GSH n = 36; ground *Artemia* n = 45)

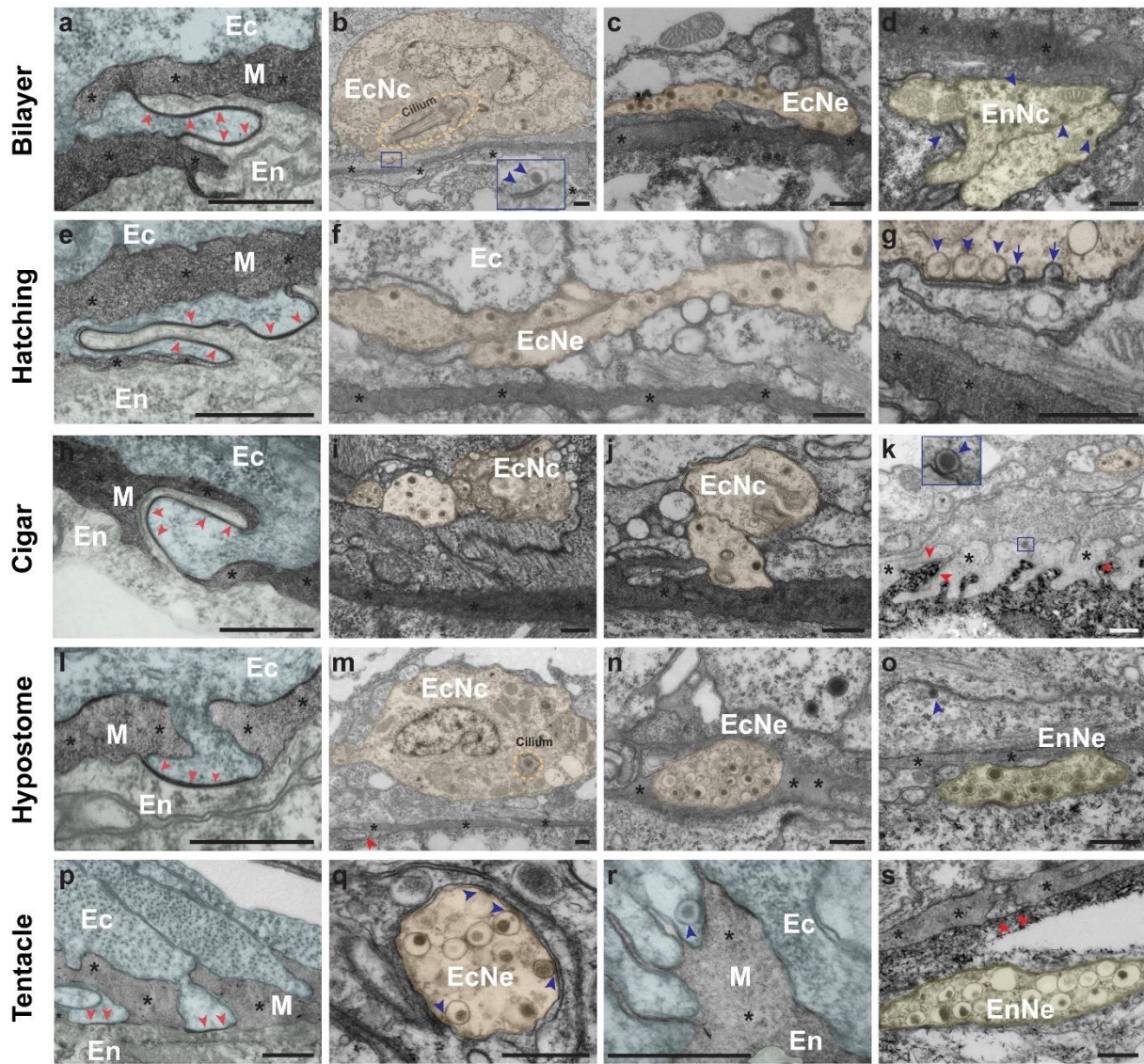
The results of the behavioral analysis on the eating behavior revealed the rapid developmental capabilities of early hatchlings. Within a couple of hours after hatching *Hydra* hatchlings displayed the ability to sense their environment and performed basic behaviors. The full set of the eating behavior, including nodding, a directed movement, tentacle movement, the mouth opening and the prey engulfment initially emerged during the Tentacle stage.

## Ultrastructural analysis of neurons in *Hydra* embryos at specific developmental stages

To investigate structural characteristics in late-stage embryos and early *Hydra* hatchlings, the previously defined specific developmental stages based on morphological changes occurring before and after hatching were analyzed (**Figure 7**).

Following these stages, a transmission electron microscopy (TEM) analysis was performed by imaging longitudinal resin sections to gain insights into the nerve net structure and the connectivity during the growth of *Hydra* hatchlings (**Figure 11a-s**). Structures characteristic for the adult nerve net were observed as early as in the Bilayer stage and increased during development. Gap junctions (red arrowheads) were found interrupting the mesoglea and were characterized by closely apposed plasma membranes of epithelial cells separated by narrow electron-dense layer (**Figure 11a,e,k,l,p**, Appendix Figure S2a-d), which is consistent with the appearance of gap junctions in adult *Hydra* (Chapman et al., 2010; Hand and Gobel, 1972). Additionally, neuron-like cells were identified based on distinguishing features such as cilia, a compact nucleus (**Figure 11b,m**) and dense-cored vesicles (DVs) (**Figure 11g**, Appendix Figure S2e-h). Cell-bodies of neuron-like cells found in the ectoderm (**Figure 11c,f,i,j**) and endoderm (**Figure 11d,o,s**) were separated from the mesoglea by muscular processes. Occasionally, neurites were observed in contact with the mesoglea, with some exhibiting a high density of DVs (**Figure 11c,j,n,o**). Furthermore, DVs within ectodermal epithelia cells (**Figure 11k,o,r**) attached to the plasma membrane were identified (**Figure 11g**, Appendix Figure S2e-h). In the polar regions of later staged samples some of these DVs were exposed directly to the mesoglea (**Figure 11k,r**). During early development DVs were particularly prominent in neuronal compartments and were frequently observed in close proximity to the plasma membrane of cells likely to be non-neuronal (**Figure 11b,g,q,s**), suggesting a potential role in facilitating cell-cell communication. DVs attached to the plasma membrane in neuronal cells (**Figure 11g**, Appendix Figure S2e-h) were previously hypothesized to be synaptic vesicles (Kinnamon & Westfall, 1982; Takaku et al., 2014). Two DVs were even captured in the process of exocytosis as suggested by the absence of the clathrin coat on the vesicle membrane. As maturation progresses, the polar region, which indicate the early formation of a head or foot could be identified by numerous extensions of ecto- and endodermal cells crossing the mesoglea in polyps of the Cigar (**Figure 11k**) or Tentacle stage (**Figure 11r**). Additionally, the extensions of

the ecto- and endodermal muscular processes appeared to interact within the mesoglea via gap junctions (Appendix Figure S3). Analyzing the later staged sections, the analysis revealed signs of neuronal maturation, supported by maceration (**Figure 12b-d**). This was evidenced by an increase in heterochromatin (**Figure 11b,m**) and the accumulation of dense-cored vesicles in neurites (**Figure 11n,o,q,s**) indicating a transition to a more differentiated neuronal state (Noack et al., 2025, in final revision).

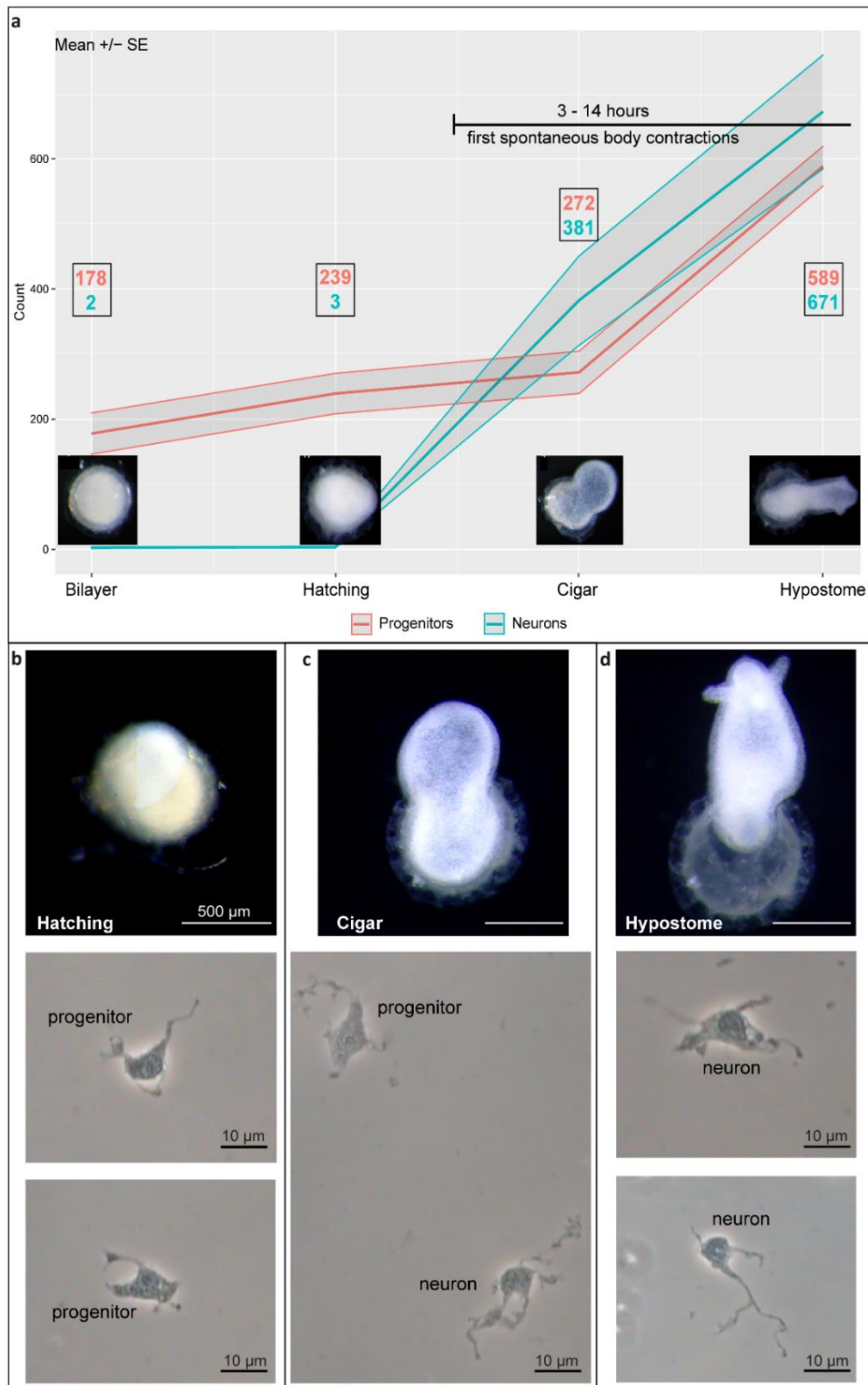


**Figure 11.** Longitudinal resin sections of early hatchlings analyzed with TEM. Images orientated to display the ectoderm above the mesoglea, below the endoderm. Structures like ectodermal and endodermal neurons and neurites characteristic for the adult nervous system were observed as early as the Bilayer stage and increased in frequency during development. Gap junctions (red arrows) between epithelial cells and electron dense vesicles (blue arrows) in nerve net and non-neuronal compartments as potential way to communicate between ecto- and endoderm. Scale bars, 500nm. Ec, ectoderm; En, endoderm; M, mesoglea (\*); EcNc, ectodermal neuron; EnNc, endodermal neuron; EcNe, ectodermal neurites; EnNe, endodermal neurites; red arrows, gap junctions; blue arrows, dense-cored vesicles (DVVs). **(Conducted and analyzed by Urska Repnik)** (Noack et al., 2025, in final revision)

Collectively, these results provide novel insights into connectivity and potential signaling mechanisms in *Hydra* embryos. The rapid assembly of a functional nerve net capable of facilitating complex behaviors may be driven by gap junctions as well as vesicle-mediated communication between nerve cells, transmitting signal molecules throughout the mesoglea and non-neuronal cells, indicating their pivotal role in nervous system development. While no evidence for direct connections between the endodermal and ectodermal nerve nets could be found, epithelial cells were observed to remain in close contact across the mesoglea as described previously (Giez et al., 2024). Ectodermal and endodermal epithelial cell processes can enter and even traverse the mesoglea. Furthermore, nerve cells within the ecto- and endodermal layer are in contact with mesoglea and epithelial cells as well as other neurons within each layer. Interestingly, ectodermal and endodermal neurons appear not to communicate directly (Giez et al., 2024).

### Morphological determination of early neurons during development

In a next experiment a modified maceration protocol was used to gain deeper insights into the neuronal distribution of late-staged embryos and early hatchlings. Additionally, the distribution between neural progenitor cells and already differentiated neurons was examined by dispersing fully dissociated samples onto a 2x2cm gel-coated slide. Ten precisely defined fields of view were counted and the number of neurons and non-differentiated cells were extrapolated to estimate the total cell distribution across the entire slide. Interestingly, almost no differentiated ganglion or sensory neurons were observed in the Bilayer and Hatching stage. In contrast, between two and five hours after hatching a high increase in the number of fully differentiated neurons was detected (**Figure 12a**). The differentiated cells displayed a highly compact nucleus as well as a neuron-specific cell shape and branched neurites (**Figure 12b-d**) to form wide-ranging connections to further cells. During the transition from the Bilayer to the Hypostome stage, the number of neurons and progenitor cells increased from nearly undetectable levels to around 400 cells during the Cigar stage. In the Hypostome stage, approximately 670 neurons were detected, indicating a rapid developmental differentiation process of neuronal cells. Interestingly, the transition from the Cigar to the Hypostome stage was accompanied by the emergence of the first contractile behavior, supporting the hypothesis of a neural contribution in emerging behaviors during early developmental stages.

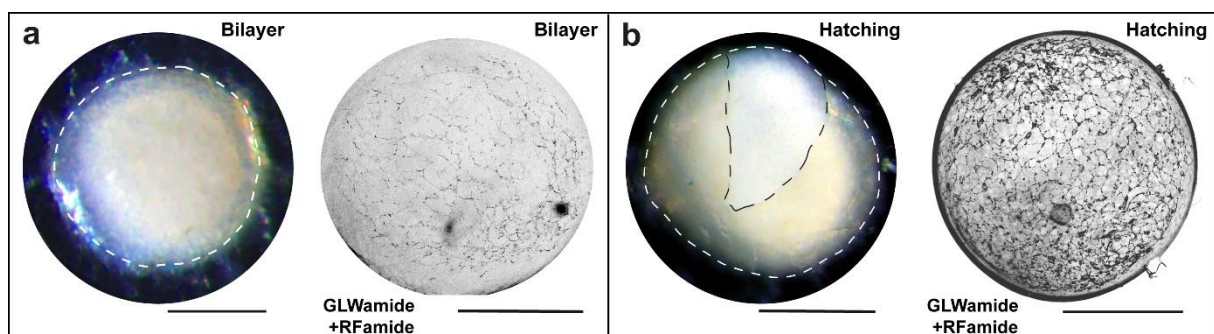


**Figure 12. a.** Morphological distinction between neurons and progenitor cells in the examination stages revealed a high increase of differentiated neurons after hatching. During the earliest stages, no neurons were observed. (Progenitors/Neurons: Bilayer  $n = 5/9$ , Hatching  $n = 7/13$ ; Cigar  $n = 7/16$ ; Hypostome  $n = 3/8$ ) **b.** Currently hatching *Hydra* polyp (top). Macerations of single individuals during Hatching stage revealed neuron-like cells with short neurites, a low number of branches and a small amount of heterochromatin (middle and bottom picture) compared to further developed individuals. **c,d.** In contrast, later-staged hatchlings exhibited extended neurites, more neuronal branches and a higher amount of heterochromatin.



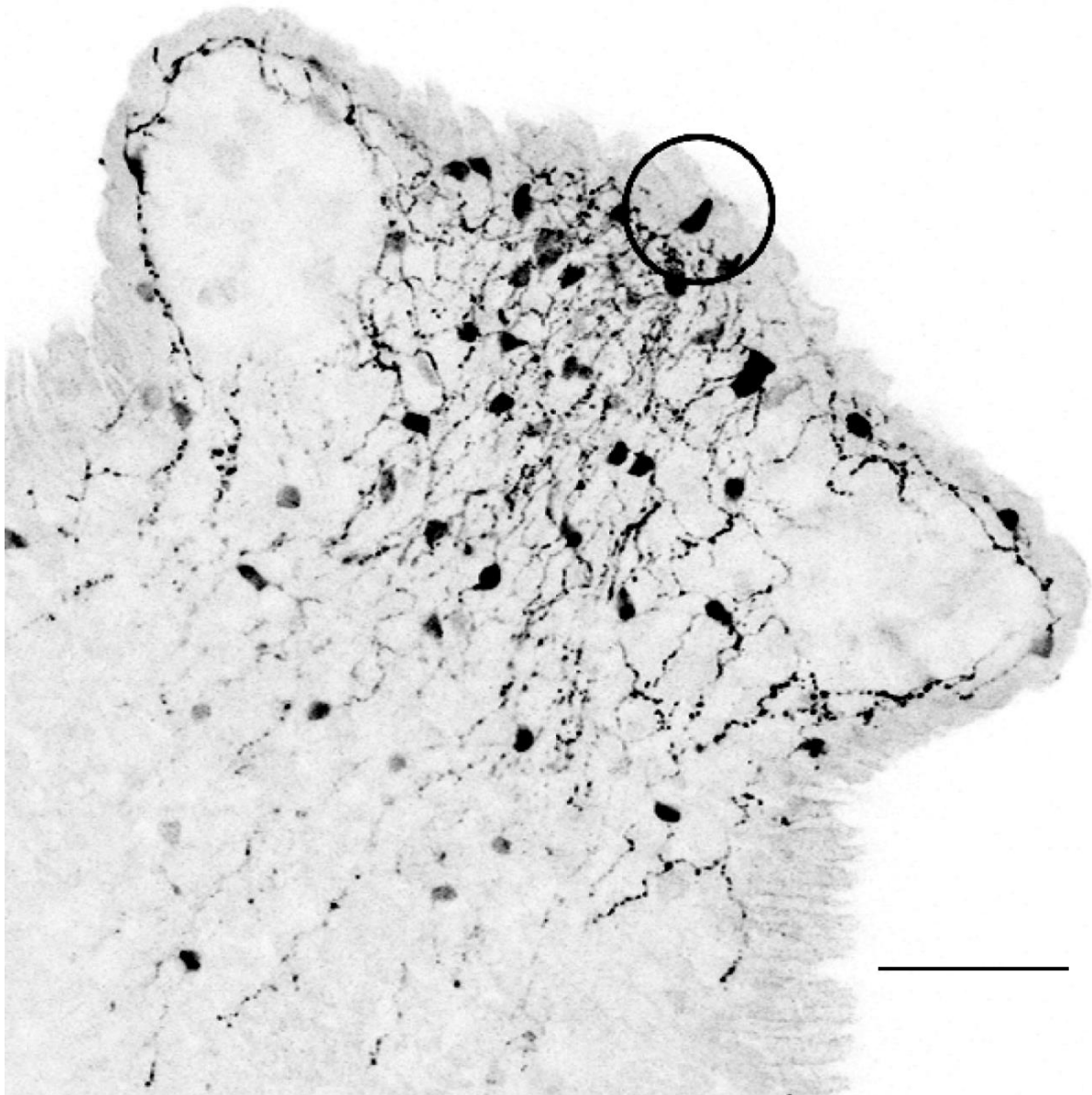
## Nerve net visualization using Immunohistochemistry

The maceration-based investigations of the different morphological stages revealed a high number of neural progenitor cells during the earliest examination stages. In contrast, a previous study reported the presence of approximately 70 neurons around the time of hatching (Martin et al., 1997). These observations prompted further experiments to get deeper insights into the nerve net development. To overcome the limitations of maceration techniques, immunohistochemical examinations were conducted. To identify specific neuronal populations and examine structural properties such as distribution, connectivity and neuronal counts, neuron-specific antibodies against RFamide and GLWamide were utilized.



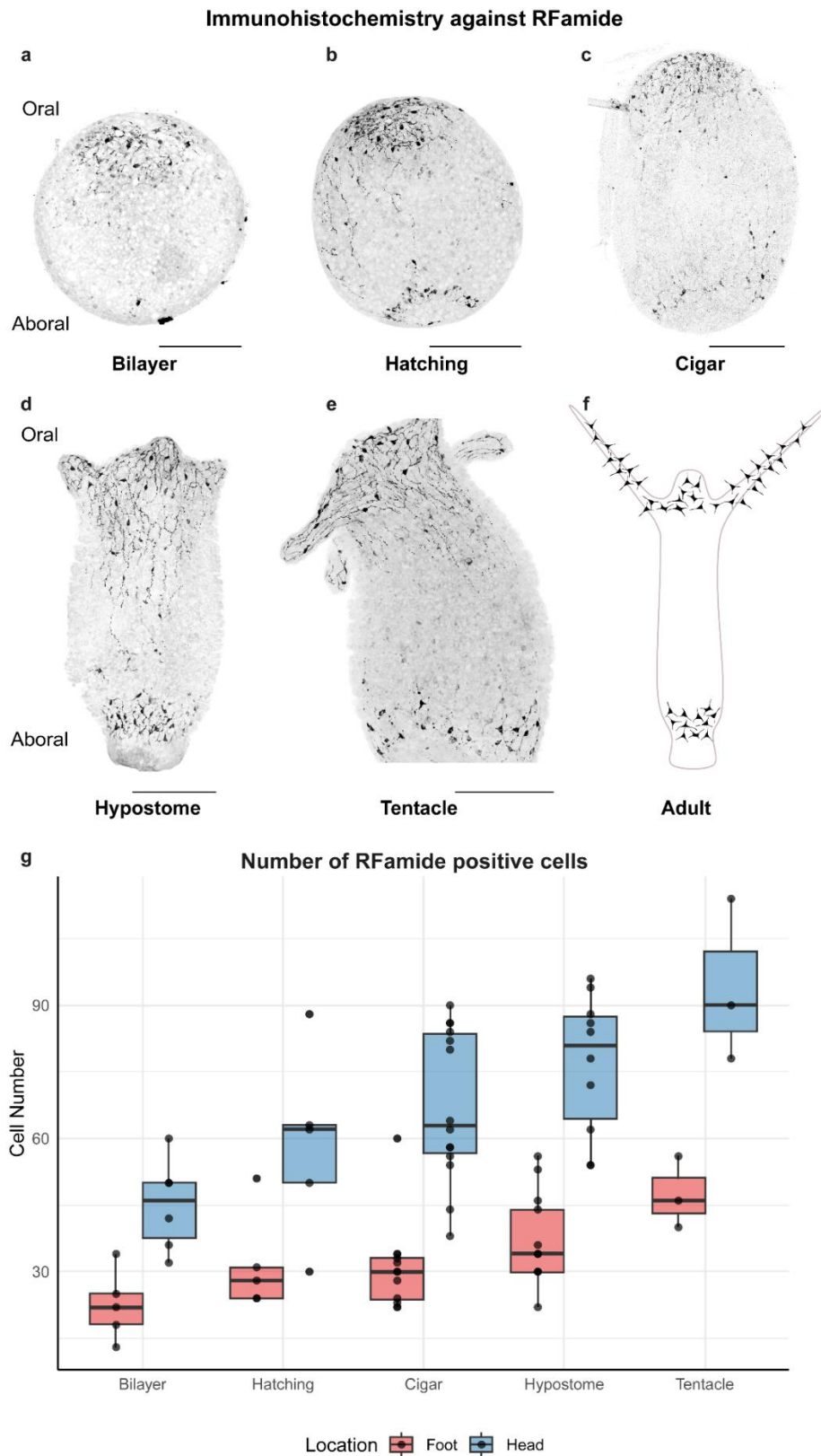
**Figure 13.** A double-staining against the specific neuropeptides GLW- and RFamide revealed an already existing and interconnected nerve net-like structure during the Bilayer (a) and Hatching stage (b). The immunostaining displayed the expression of the specific neuropeptides in 6 out of 7 neuronal populations in *Hydra vulgaris* AEP hatchlings.

These antibodies provided insights into nerve net-specific properties of the determined developmental stages (Figure 7). In the initial approach, a combination of the specific neuropeptides GLWamide (exclusive to population N2, N3, N4) and RFamide (exclusive to population N1, N6, N7) was applied to wild-type samples. The findings indicated a fully developed and interconnected nerve net-like structure throughout the hatchlings during early developmental stages (Figure 13a and b). Furthermore, the use of only the RFamide antibody identified the first emerging sensory neurons as early as in the Hypostome stage, indicating the first neural element capable of actively sense the environment (Figure 14). Additionally, a distinct oral and aboral patterning was observed by visualizing RFamide-specific neural populations, which are exclusively expressed in the hypostome and foot region of adult polyps (Klimovich et al., 2020). RFamide-positive neurons were detectable as early as in the Bilayer stage, suggesting an early patterning and the preestablished identity of specific neuronal populations during late embryogenesis (Figure 15a-e).



**Figure 14.** The immunohistochemical staining against RFamide displayed the first emerging sensory neurons during the Hypostome stage (black circle), an essential neuronal element to sense the environment. *Scale bar 100 $\mu$ m*

RFamide, as a specific neuropeptide, is exclusively expressed in neurons located in the hypostome (population N6 and N7) and the foot region (N1) of *Hydra* (**Figure 15f**). In addition, examinations in embryos and hatchlings displayed a constant increase of neurons in the hypostome- and foot-specific regions. Notably the oral area exhibited an enhanced number of neurons, highlighting a rapid and ongoing nerve net expansion during early hours of the development in *Hydra* hatchlings (**Figure 15g**).



**Figure 15.** a-e. Stage-specific staining against RFamide displayed an already existing oral and aboral patterning in early examination stages and an increase of neurons and neurites over developmental time. Scale bar, 500µm. f. Scheme of RFamide-positive neuronal populations in adult polyps. g. Determination of RFamide-expressing neurons in the head and foot area of specific developmental stages. The increase of neurons indicates an expansion of the nerve net. (head: n = 3–14; foot: n = 3–13)



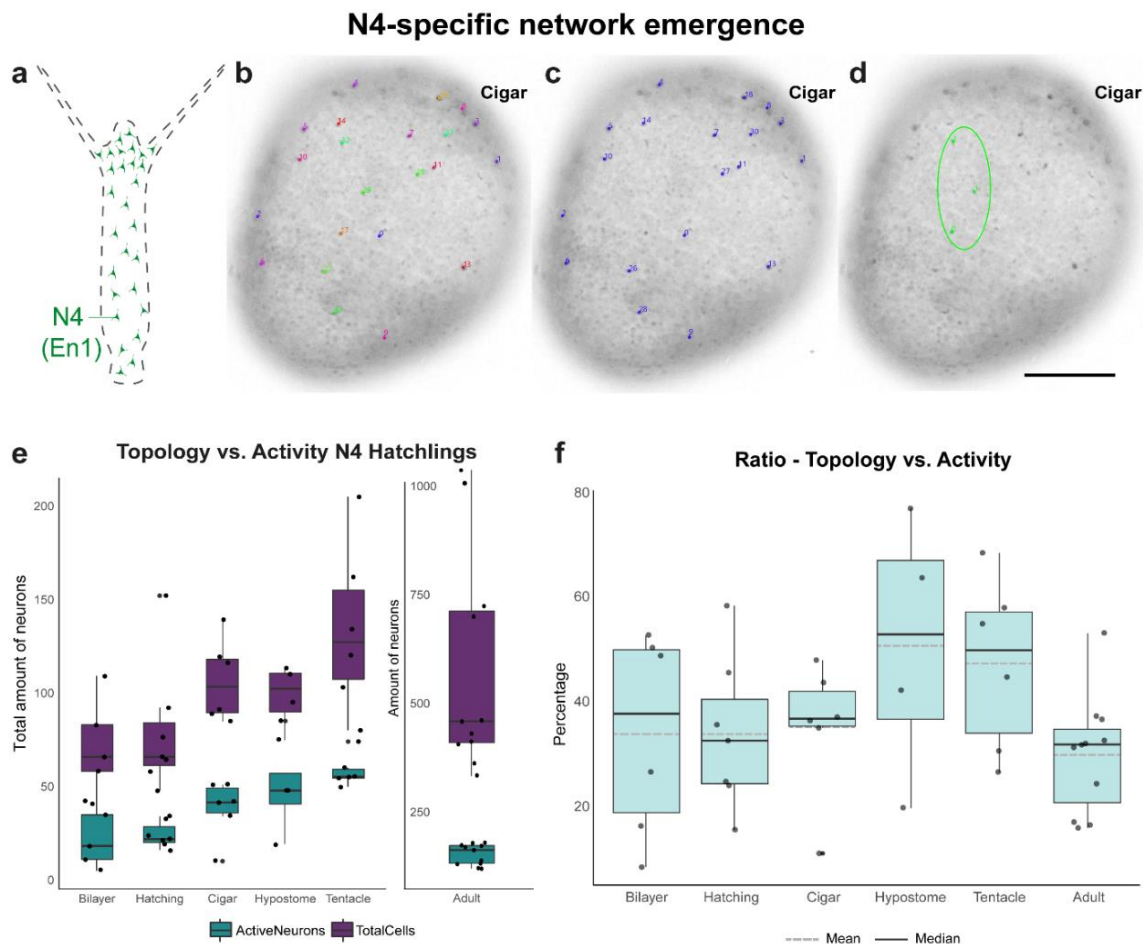
## Functionality and *de novo* nerve net formation in *Hydra*

### Network structure of Ca<sup>2+</sup> active N4 neurons

To examine the neuronal landscape in *Hydra* hatchlings and late embryos, a F<sub>1</sub> generation of an existing transgenic line targeting the neuronal population N4 was generated (Giez et al., 2023). This approach aimed to get further insights into the functionality of an early emerging neuronal population in *Hydra* embryos and hatchlings. Since immunohistochemical staining of fixed tissue does not provide any information about the functionality of neurons, early neuronal ensembles or specific neuronal populations, initial observations focused on Ca<sup>2+</sup> activity across the previously established developmental stages.

To gain a more profound understanding of the network formation within an emerging nerve net, transgenic embryos were utilized, focusing on the endodermal neuronal population N4 by expressing GCaMP6s under a N4-specific promoter (**Figure 16a**). Initially, the Ca<sup>2+</sup> activity for the previously described developmental stages (**Figure 7**) under standard conditions (18°C) for 20min with 25fps and a laser intensity of 14% were analyzed. This analysis covered the Ca<sup>2+</sup> activity between 2.000 and 2.600 frames, capturing the full spectrum of emerging Ca<sup>2+</sup> signals at single-cell resolution. Subsequently, antibody staining of the same samples against GFP were performed, a component of the GCaMP6S cassette, to compare the spatial topology of neurons and the activity of the N4-specific population. Analysis of the Ca<sup>2+</sup> signals revealed the presence of neuronal ensembles throughout all regions of the examined samples (**Figure 16b-d**, Appendix Video S2-5). These small ensembles, detectable in all areas, only varied in the number of neurons within each cluster. Comparing the results, an increase of both total and Ca<sup>2+</sup> active neurons were observed (**Figure 16e**). Notably, the number of active neurons during Ca<sup>2+</sup> imaging was lower compared to the total number of N4-positive cells identified via Immunohistochemistry. Specifically, during early stages, an active-to-total cell ratio of 1:3 was observed, which shifted to a ratio of 1:2 in later stages, before returning to the initial proportion observed in adult polyps, resembling the observations in the Bilayer stage. This finding suggests a declining pool of potentially immature neurons being progressively integrated into an expanding Ca<sup>2+</sup> active network (**Figure 16f**). A detailed comparison of this declining pool across embryos, early hatchlings and adult polyps revealed the appearance of a specific pattern. Both early and late examination stages displayed a 30/70% proportion

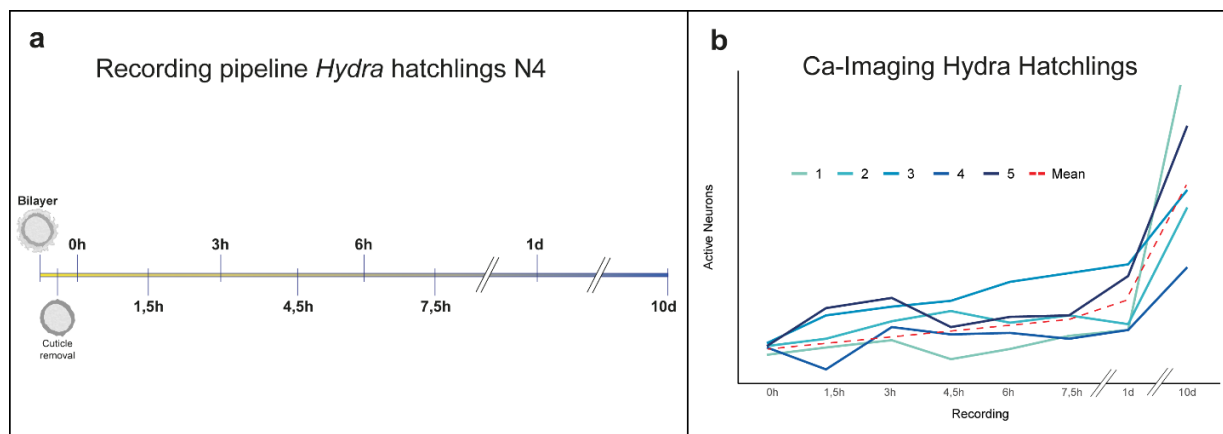
between active and non-active neurons. During the Hypostome stage about 50% of cells belong equally to either active or stained neurons, suggesting a rapid integration of cells necessary for the nerve net assembly ( $n = 4-11$ ). This observation highlights a temporally symmetric pattern of the relative neuronal activity where the ratio varies over time and the activity peak between active and non-active neurons occurred around the Hypostome stage (**Figure 16f**). The declining pool reflects a rapid increase of the nerve net functionality during early stages, which is consistent with observations from previous maceration experiments that demonstrated a high increase of differentiated neurons after the Hatching stage (**Figure 12a**).



**Figure 16.** **a.** Schematic distribution of the endodermal N4 network in adult polyps illustrating the highest density in the hypostome area. **b-d.** Example of fixed hatchling in Cigar stage showing labelled N4 neurons with anti-GFP antibody. Further, different-sized and color-coded neuronal ensembles analyzed by  $\text{Ca}^{2+}$  activity before fixation. Blue dots in **c** mark the dominant N4 community. Scale bar, 500 $\mu\text{m}$ . **e.** Quantification and comparison of active and stained N4 neurons at different developmental stages revealed lower numbers of spiking neurons compared to those expressing the N4-specific transcript, targeted by antibody staining. The number of neurons counted for the visual field in both treatments, equivalent to one half of the samples, display an overall increase during development. (for topology  $n = 6-11$ ; for activity  $n = 4-11$ ). **f.** Comparison of the ratio between topology and activity indicated a declining number of stained and  $\text{Ca}^{2+}$  active neurons. Considering the Hypostome stage about 50% of cells belong equally to either active or stained neurons suggested an integration of cells needed for the nerve net architecture facilitating a rapid assembly ( $n = 4-11$ ). Adult polyps displayed around 30% active neurons.

## Nerve net assembly during early development in *Hydra*

Recordings of  $\text{Ca}^{2+}$  activity provided initial insights into the characteristics of the early nerve net in late embryos and early hatchlings. However, the observed differences and variances within each determination time hindered a precise understanding of the assembly and emergence of the N4 nerve net structure. To address this limitation, a pipeline for a continuous recording that maintains focus on the same section of individual polyps was developed, thereby avoiding deviations in the collected data (**Figure 17a**). As previous results indicated a low number of active neurons in bilayer-staged samples (**Figure 16e**), this stage was selected as the initial reference point for the recordings. To capture early and infrequent  $\text{Ca}^{2+}$  signals, samples were recorded for 30min at 25fps with 14% laser intensity. To minimize phototoxic effects and preserve the integrity of the nerve net assembly, a 60min break between each of the recordings was selected. The single-cell measurements revealed a progressive increase in the number of active neurons over a 10-day period. Although specifically focusing on the first 7.5 hours of development to understand the N4 assembly, additionally recordings were conducted on the same polyps after 1 and 10 days to confirm the formation of a fully developed and functional N4 nerve net after the cuticle removal (**Figure 17b**).



**Figure 17.** **a.** Recording pipeline for long-term analysis of single *Hydra* samples starting in the de-cuticulated Bilayer stage. Self-devised observation chambers ensured a permanent screening throughout the whole experiment until 7.5h after starting the experiment. Recordings were conducted for 30min with 1h break. **b.**  $\text{Ca}^{2+}$  active N4 neurons analyzed for each recording in 5 individuals within 2.000 frames of 30min recordings. The mean for all measurements displayed an overall increase of neuronal activity (dashed red line).

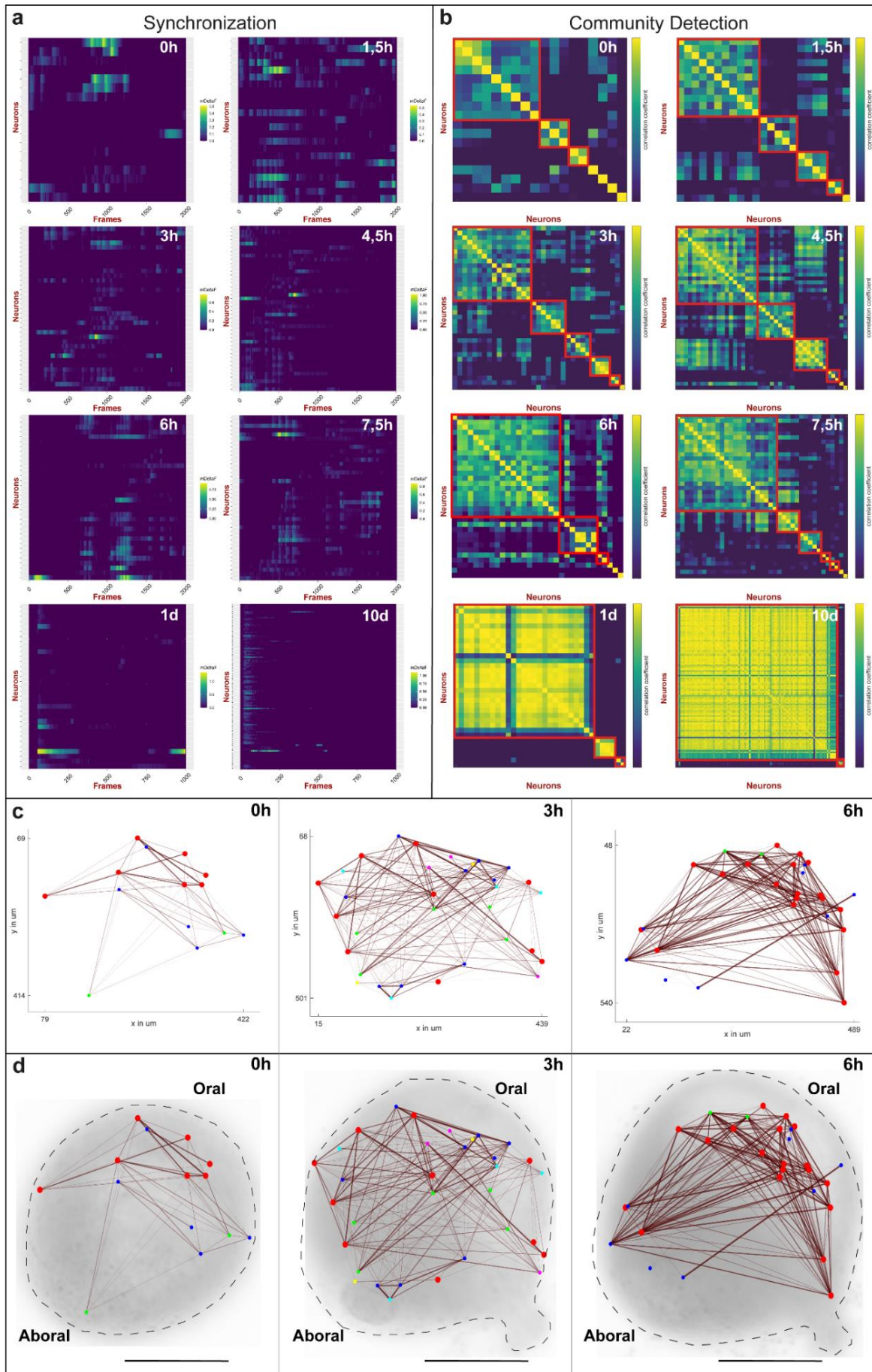
## Ca<sup>2+</sup> activity drives the synchronization and formation of the N4 network

To investigate the assembly of the evolving N4 neural circuit, neuronal activity, by tracing all detectable neurons on a single-cell basis for 2.000 frames during the initial hours after cuticle removal at the Bilayer stage, was monitored. This was followed by a detailed analysis of 1.000 frames at 1 and 10 days after extracting the embryos from the cuticle. Overall, between 12 individual neurons in early-staged embryos and 143 neurons in adult polyps were analyzed. Neuronal activity was quantified by measuring the intensity of each individual neuron and the resulting data was compared across timepoints to evaluate synchronization levels (**Figure 18a**). The findings suggest that, rather than merely an increase in the number of neurons over time, there is a progressive enhancement in synchronization among Ca<sup>2+</sup> active neurons, culminating in predominantly simultaneous activity after 1 and 10 days. Interestingly, an increase in neuronal spiking rates between 0- and 7.5-hours post-cuticle removal was observed, while enhanced synchronization was evident by a decrease in calcium spike frequency after one day (Appendix Figure S5-S8). These observations suggest that high spiking activity is crucial for the early stages of nerve net assembly, indicating a finalized network construction after 1 day. Collectively, the findings highlight a rapid incorporation of new neurons and a corresponding increase in connectivity during early developmental stages, promoting the expansion of the network. Moreover, the recordings revealed variable numbers of Ca<sup>2+</sup>-active neuronal ensembles and different levels of connectivity in distinct regions of the hatchlings (**Figure 18c**, Appendix Video S2-5). This variability implies the absence of a chemical or structural pre-patterning and instead supports an activity-driven mechanism for the assembly of the nerve net.

To further examine the network construction, a community detection approach based on the Leiden algorithm was employed (Traag et al., 2019) (**Figure 18b**). The analysis identified neuronal ensembles of varying sizes and numbers, as well as isolated neurons, particularly during early stages of development (Appendix Figure S5-S8). Notably, the correlation coefficient increased over time, with nearly all detected neurons exhibiting strong functional connectivity and integration into the spanning N4 network after 1 or 10 days. A notable feature of the analysis was the emergence of a dominant community during the study period. This neuronal ensemble, which constituted approximately 50% of the traced neurons, increased in size over time. Therefore, this large neuronal ensemble may act as a pacemaker-like structure,

overriding smaller ensembles through its activity and spiking intensity. By incorporating smaller, already connected and synchronized ensembles, it appears to facilitate the rapid expansion of the neural circuit.

To achieve a deeper understanding of the time-dependent nerve net assembly in the N4 network, a 2D network analysis was employed which uses the positional identity to reconstruct the geometric network structure. This approach provided further insights into modularity, dynamic adaptations, connectivity and integration of isolated neurons and ensembles. Building on previous findings (**Figure 18a,b**), this analysis revealed an activity-driven self-assembly of the nerve net during recordings (**Figure 18c**, Appendix Figure S5-S8). Connectivity increased not only within specific ensembles but also between diverse neuronal clusters throughout the study period. Consistent with earlier results, the dominant neuronal community exhibited growth through the incorporation of additional ensembles particularly in the upper region of the nerve net. Alongside the increase in connections, selective strengthening of certain connections was observed, particularly within the dominant community (marked by red dots), over a remarkably short period of time. Interestingly, smaller neuronal ensembles appeared to disappear after becoming locally integrated and synchronized within the main network, indicating a dynamic reorganization and expansion of the nerve net. Notably, the highest density of neurons and the establishment of the strongest connections were concentrated in the oral region of the recorded hatchlings (**Figure 18d**). This suggests that the assembly of the N4 circuit originates in the future hypostome of the hatchlings, where the N4 also exhibits its highest density also in adult polyps (Giez et al., 2024, 2023; Klimovich et al., 2020).



**Figure 18. a.** Single-cell analysis of neuronal activity during recordings indicate an increase of synchronization level and a high number of spikes in early developmental stages (0 – 7,5h) compared to later stages (1d, 10d). Each line on the y-axis corresponds to a single detected neuron, x-axis shows the spike intensity ( $\Delta F/F$ ) for each frame (25fps). **b.** Utilizing the data  $Ca^{2+}$  measurements presented in **a**, a community detection for each recording to follow the network assembly was performed. Detected communities are highlighted by red squares identified with the Leiden algorithm using the correlation coefficient to visualize the rapid ongoing network assembly in *Hydra*. Throughout the analysis, the dominant community increased in size, along with an enhancement in the correlation coefficient, while smaller communities and single neurons remained detectable in all time points. In the late stages (1d, 10d), the consistently high correlation coefficient indicated the completion of the network assembly. **c-d.** The network assembly visualized by using the positional identity of detected neurons. Colored dots represent distinct neuronal ensembles based on the correlation coefficient depicted in **b** (red = dominant community). Connections between neurons are shown by lines, the thickness reflecting connection strength. Together, these data provide evidence for a rapid assembly of the N4 neural circuit, a rise in strongly connected neurons within the dominant ensemble and an aggregation of neurons in the prospective oral region of the hatchling. More samples are demonstrated in the Appendix Figure S5-S8. Scale bar 500 $\mu$ m

## Environmental impacts on the nerve net formation in *Hydra*

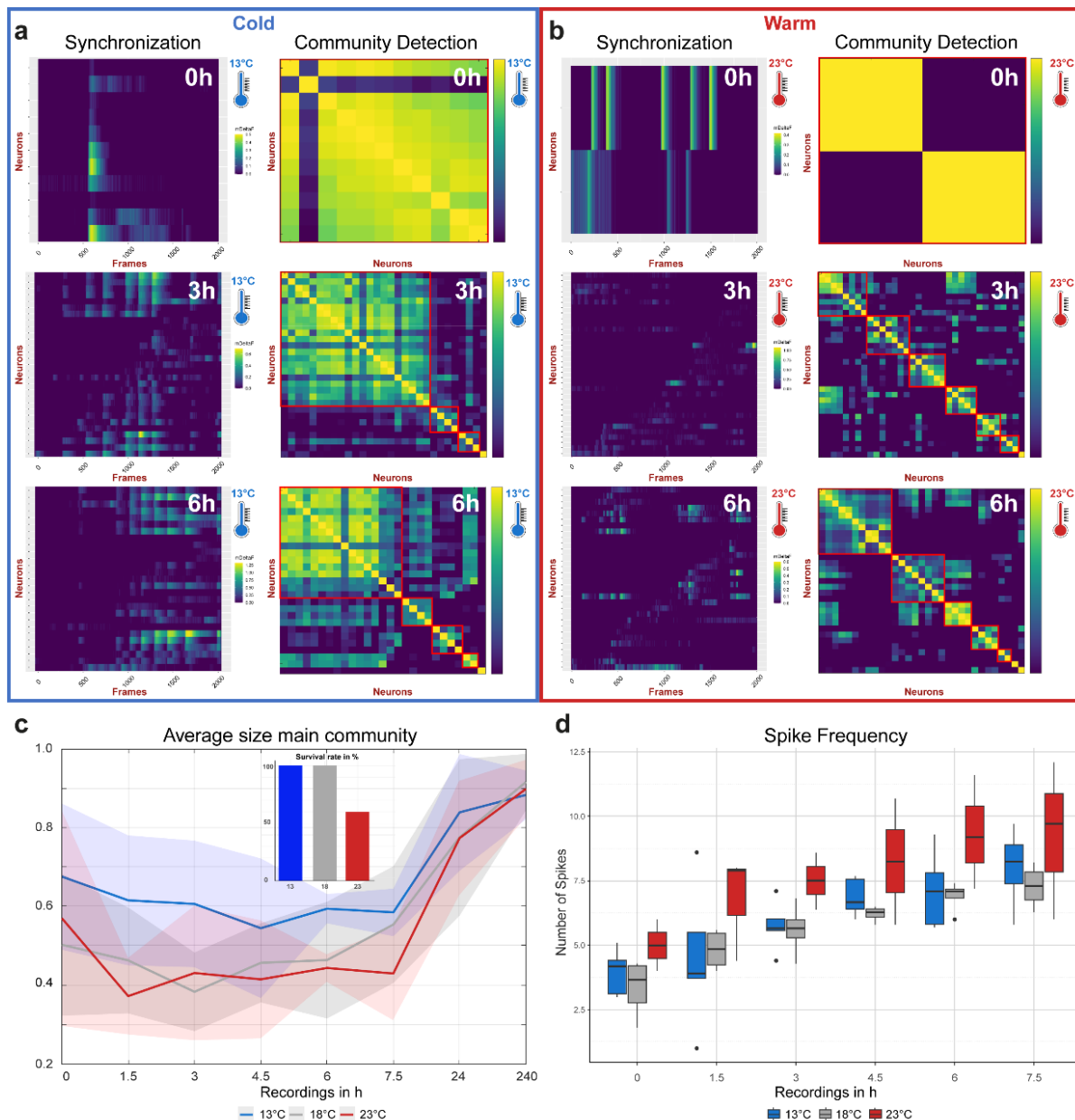
The previous experiments displayed the *de novo* nerve net assembly of the neuronal population N4 in *Hydra* embryos and early hatchlings. These nerve net constructions, however, occurred under standard lab conditions at 18°C and without the consideration of environmental factors, polyps are confronted within the natural habitat. To investigate whether external factors in the immediate environment of embryos affect the structure of the emerging nerve net, two external factors were examined: the effect of the temperature on the emerging neuronal N4 circuit and the impact of the colonizing microbiome during embryogenic development. Both, temperature and microbiome were used to reveal and provide information whether the nerve net construction follows a genetically determined inner gradient or an activity-guided nerve net assembly.

### Temperature changes exhibit an impact on the nerve net during the N4 assembly

To investigate the influence of temperature, the embryos were transferred from standard temperature (18°C) to either warm (23°C) or cold (13°C) conditions immediately after cuticle removal. To analyze the  $\text{Ca}^{2+}$  activity, the previously implemented pipeline (**Figure 17a**) was used and focused on the early hours after recording and cuticle removal (**Figure 19a,b**; Appendix Figure S9-S18). Interestingly, the observations exhibited significant deviations from previous experiments under standard conditions (18°C, see **Figure 18**) for both the average dominant community size and the neural spiking frequency (**Figure 19c,d**). Hatchlings exposed to the cold temperature (13°C) exhibited significantly increased synchronization levels. Furthermore, the analysis revealed a larger number of neurons and an enhanced neuronal connectivity considering the dominant community compared to the control group at 18°C (**Figure 19a,c**), indicating cold temperature to promote a rapid nerve net assembly. In stark contrast, hatchlings recorded at increased temperatures (23°C) displayed reduced synchronization levels. Additionally, the community detection for embryos at high temperatures revealed the dominant neuronal ensemble to be smaller in size if compared to the total number of detected neurons (**Figure 19b,c**). Remarkably, these structural and functional changes in the neuronal N4 circuit directly influenced the behavior of these hatchlings. Although the hatchlings maintained at 23°C were able to open their mouths, 40% of the samples were incapable to engulf prey and did not survive to the end of the 10-day



experiment (**Figure 19c**, BarChart). This is very strongly reminiscent of the behavioral defects shown in a previous study, where an ablated N4 population in adult polyps show the same behavioral defects (Giez et al., 2024). In addition,  $\text{Ca}^{2+}$  signals recorded in N4 neurons of embryos kept at warm temperatures during development indicated an increase in neural spiking frequencies (**Figure 19d**). Incorporating the temperature-driven alterations in the spiking frequency of the N4 neurons into an electrical circuit-based model, the model likewise predicts that the dominant community is smaller in size compared to the total number of detected neurons. Therefore, the developed model based on the presented data (**Figure 18**) can effectively emulate biological processes ((Noack et al., 2025, in final revision), Appendix Figure S19). To that extent, the compilation of the data clearly indicates that embryos during neuronal development face difficulties in assembling a functional N4 nerve net at high temperature, whereas cold temperatures suggests a beneficial environment for the assembly considering a rapid construction of the nerve net and the overall strength of connections between neurons within the dominant community.



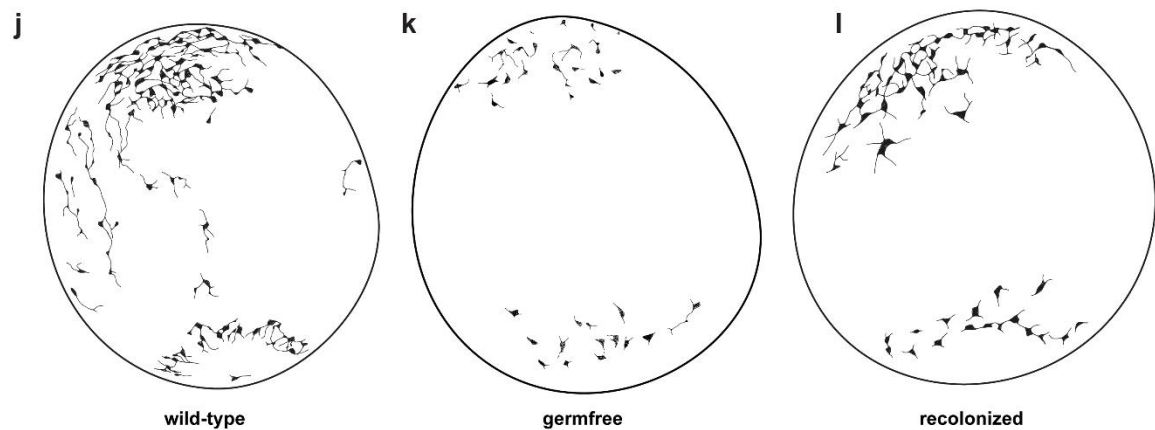
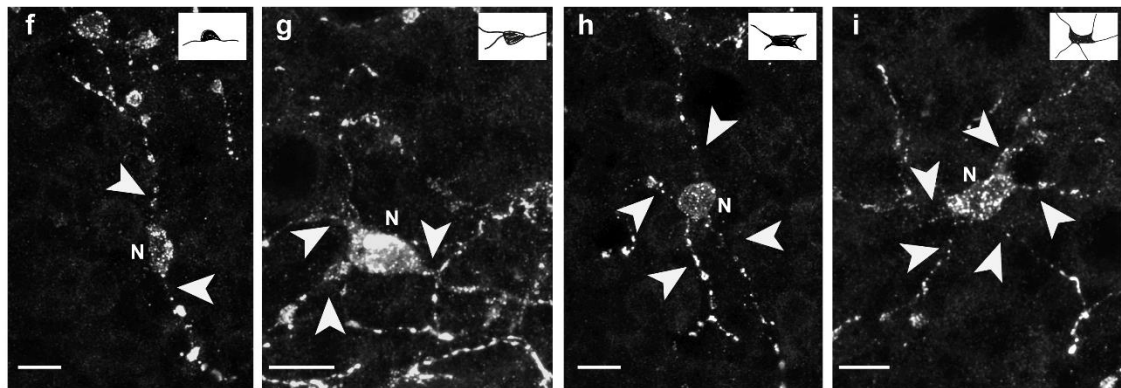
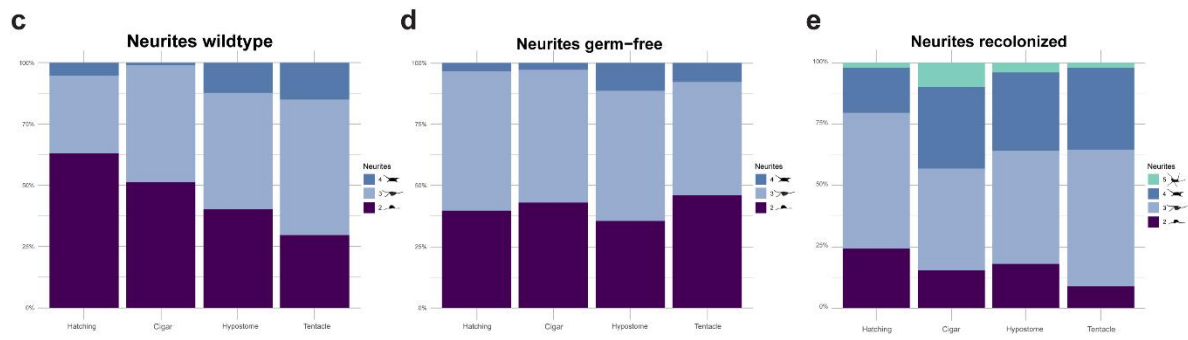
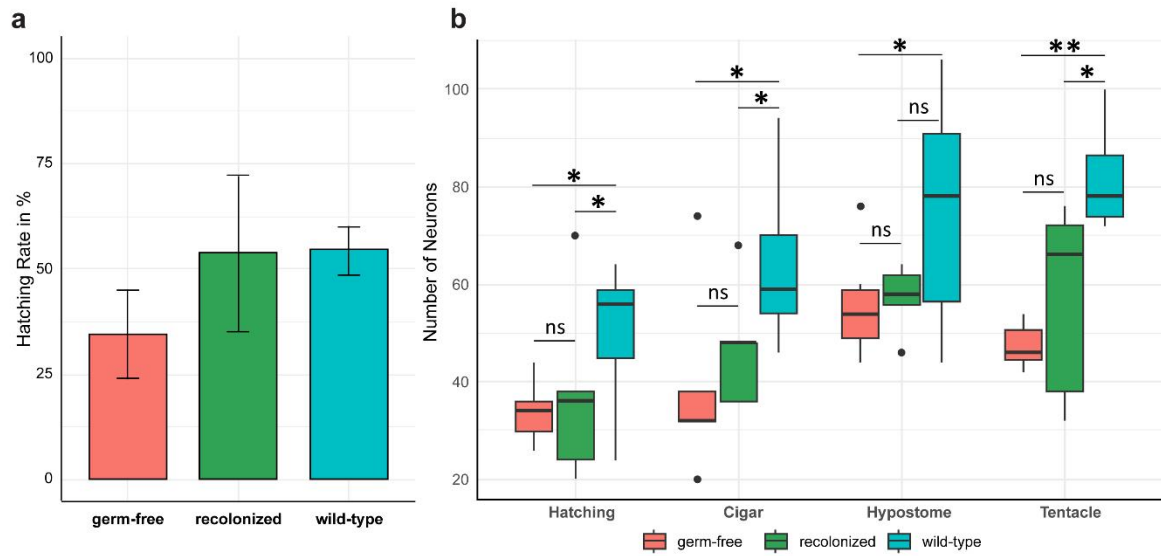
**Figure 19** Temperature-dependent nerve net assembly during early developmental stages. **a,b**, Single-cell analysis of neurons recorded under cold and warm conditions starting in the Bilayer stage. Total number of detected neurons in three representative early-staged recordings during development are shown. Significant differences were observed between both treatments in terms of synchronization and community detection. Reduced temperature conditions were found to be beneficial for synchronization levels during nerve net assembly, as well as convenient for the rapid development of a dominant community. In contrast, synchronization level and community size in **b** appear to be lower for those hatchlings recorded in a warm environment. **c**, Specific observations of the dominant community across all treatments (13°C – blue, 18°C – grey, 23°C – red) indicate a higher proportion of neurons connected within the main community: This suggests an increased assembly during early development under cold conditions, but did not display an advantage after 10d. In comparison, hatchlings exposed to normal and warm temperatures showed similar community sizes, except at 7,5h, where normal conditions exhibited a higher number of assembled neurons (shaded area represents the mean  $\pm$  SD). Additionally, data presented in the Barchart, show a decreased survival rate of polyps kept at warm temperatures to engulf prey ( $n = 15$ ). **d**, Spike frequency analysis revealed an increased number of signals in hatchling observed at 23°C, whereas the number of spikes measured for the cold and standard conditions displayed similar values. For the complete overview of all synchronization and community detection plots see Appendix Figure S9 – S18.

## Microbial influence during the development of *Hydra* embryos

To further investigate if the environment has an impact on the development of an emerging nerve net in *Hydra* embryos and early hatchlings, the absence of commensal bacteria, further referred to as microbiome, as the second environmental factor was examined. Based on previous findings in adult *Hydra* displaying the manifold influence of the microbiome on development, fitness, nervous system and behavior (Augustin et al., 2017; Domin et al., 2023; Giez et al., 2023; He and Bosch, 2022; Murillo-Rincon et al., 2017a), the absence of the microbiome may also affect the *de novo* nerve net assembly during embryogenesis.

Therefore, an antibody (RFamide) against a neuropeptide, specific for the neuronal populations N1 in the foot area and the populations N6 and N7 in the hypostome and tentacles was used (**Figure 15a-e**). Here, the analysis solely related to the aboral area of early hatchlings in each of the treatment groups. The role of the microbiome was uncovered by the absence of the natural microbiota after cuticle formation for wild-type embryos. Germ-free (gf) animals were compared with their wild-type (wt) counterparts and a third group, that was recolonized (rc) with a full complement of the microbiota. Intriguingly, embryos cultured under germ-free conditions displayed a decreased hatching rate, whereas a recolonization with the commensal bacteria completely rescued the hatching rate to the level observed in wild-type embryos (**Figure 20a**). In addition, by determining the number of RFamide-positive neurons in the future hypostome during early developmental stages, gf and rc embryos displayed a significantly reduced number of neurons compared to the wt control. Considering the differences between rc and gf embryos, the recolonized embryos exhibited a minor increase throughout the determined stages, however, no significant alteration was observed (**Figure 20b**). To further investigate the effects of the microbiome, the number of neuronal branches of neurons across the treatment groups was determined. Considering the branches in the wt samples, hatching-staged samples exhibited a high proportion of neurons with two neurites if compared to neurons with three and four neurites. Analyzing later staged hatchlings, the number of neurons with more than two branches increased, hence potentially indicating a process of neuronal maturation and an increase of connectivity in the newly formed network (**Figure 20c**). Remarkably, analysis for the germ-free samples furthermore and in addition to the decrease in the number of neurons, indicated fewer and constant numbers of branches throughout all examination time points for two, three and four neurites, suggesting a lack of

neuronal maturation during development (**Figure 20d**). Notably, the analysis for the recolonized treatment group exhibited a low number of neurons with two branches compared to the wt and gf conditions already in the Hatching stage. The highest number of neuronal branches was observed in neurons with three and four potential connections to neighboring cells, and this connectivity further increased during development. Within the recolonized treatment group, the hatchlings uniquely displayed neurons with more than four neural branches, suggesting a compensation of the lowered number of neurons by establishing an enhanced quantity of neural branches to adjacent cells (**Figure 20b,e,i**). Using Immunohistochemistry against RFamide in germ-free and recolonized hatchlings revealed the oral nerve net to be not properly formed in the absence of the commensal bacteria, compared to the equivalent wild-type samples of the same developmental stage (**Figure 20j-l**). In contrast to wt and rc samples, neurons in embryos cultured under germ-free conditions exhibited a decreased number of neurons and appeared to lack contact with other RFamide-positive cells probably also due to the lower number of neurites (**Figure 20k**). In the focused hypostome area, these connections to other stained cells were highly established in wild-type samples and to a smaller extent also in recolonized hatchlings (**Figure 20j-l**). These findings concerning the number of neurons and neuronal branches highlight the pivotal role of the microbiome during embryogenic development, neurogenesis and neuronal circuit formation. The observed decrease in network connectivity and stability suggests that external environmental factors, despite a thick protective layer throughout *Hydra's* embryogenic development, can influence the underlying developmental machinery.



**Figure 20.** Microbial impact on the nerve net construction. **a**, The absence of microbes exhibited a reduction in the hatching rate of germ-free embryos compared to the wild-type. However, exposing germ-free embryos to the natural microbiome could restore the hatching rate. Bars represent the mean  $\pm$  sd. (wild-type: n = 3242; germ-free: n = 209; recolonized: n = 107) **b**, Neuron counts in the subsequent head, as assessed by RFamide staining, revealed a significant reduction in neuronal density compared to the wild-type control. Recolonized embryos displayed no statistically significant difference in neuron counts, but consistently showed a slight increase across all developmental stages. (statistics: ANOVA, Tukey test, Kruskal-Wallis, Dunn test; p-values: ns:  $\geq 0,05$ ; \*:  $< 0,05$ ; \*\*:  $< 0,01$ ) **c-e**, Analysis of neuronal branches in wildtype, germ-free and recolonized hatchlings revealed distinct variations across all treatments. In wild-type samples, the number of neurites increased as development progressed. In contrast, germ-free hatchlings maintained a stable distribution, with neurons primarily showing 2, 3 and 4 branches. Recolonized hatchlings, however, displayed a higher proportion of neurons with 3 and 4 neurites even in the early developmental stages. Notably, neurons with 5 branches were absent in both wildtype and germ-free hatchlings, appearing exclusively in recolonized individuals (wild-type: n = 5-14; germ-free: n = 6-8; recolonized: n = 5). **f-i**, Immunohistochemistry for RFamide in germ-free and recolonized hatchlings. Arrowheads indicate the neurites. N, Neuron. Scale bars 10 $\mu$ m. **j-l**, Schematic representations of immunohistochemistry against RFamide for wild-type, germ-free and recolonized samples in the Hatching stage (Appendix Figure S20).

## Discussion

This work set out to investigate the emergence of a neuronal circuit and the nerve net formation at the early stage of animal evolution using *Hydra* embryos. The objective was to uncover the structural properties in one of the earliest nerve nets to emerge in phylogeny, to understand how emerging neurons assemble into functional neural circuits and to assess whether external environmental factors during early developmental stages affect the assembly and architecture of neural circuits. To achieve these aims a combination of immunohistochemistry, transgenic activity lines (GCaMP6s) and bioinformatic tools was used to reveal the mechanisms at single-cell resolution.

Based on current morphological definitions for nerve cells in *Hydra* (Charles N David, 1973), the findings in this work show the absence of morphologically differentiated neurons during late embryogenesis and early hatching stages. The results further indicate that both gap junctions and a vesicle-mediated communication between neurons and non-neuronal cells contribute to the rapid assembly of neural circuits during early developmental stages. Additionally, neurons are shown to emerge before they are functionally required, with the N4-specific neuronal circuit unfolding over time as a self-assembling system, driven and synchronized by  $\text{Ca}^{2+}$  signals and an increasing number of neurons. These insights, obtained at single-cell resolution, provide a detailed perspective on the *de novo* assembly of the specific N4 network in *Hydra* embryos and highlight the impact of environmental factors on the neuronal network assembly and architecture.

### Nerve net specific development in *Hydra* embryos and hatchlings

The human brain consists of 64 to 99 billion neurons (Goriely, 2024; Herculano-Houzel, 2009), each neuron can establish several thousands of connections and electron microscopic analysis of just 1mm<sup>3</sup> brain tissue produces approximately 1.4 petabyte of data (Shapson-Coe et al., 2024). While this complexity enables for sophisticated behaviors and processes, it proves to be an obstacle in understanding the fundamental mechanisms underlying nervous system formation, circuit assembly and functionality. Model organisms such as *Hydra* provide valuable opportunities for investigating principles of nerve net development, neurogenesis and potentially neurodegenerative disease mechanisms. In recent years, *Hydra* incited attention to address fundamental questions in neurobiology due to its behavioral repertoire, ongoing

neurogenesis in adult polyps and its regeneration capacity to fully restore the nervous system in decapitated body parts and even from dissociated cells (Badhiwala et al., 2021; Dupre and Yuste, 2017; Escobar et al., 2024; Giez et al., 2023; Lovas and Yuste, 2021; Primack et al., 2023; Shapson-Coe et al., 2024). Unlike the human brain with its vast number of neurons, *Hydra* possesses a relatively small and accessible nerve net, comprising approximately 3.000 – 5.000 neurons in adult polyps (Giez et al., 2021; Primack et al., 2023). An early study reported an even lower number of 70 neurons in early hatched polyps (0 – 5 hours post hatching), suggesting that this stage represents an ideal window for observing the earliest stages of nerve net formation and studying the emergence of behaviors. The defined time points investigated in this study (**Figure 7**) allowed for a detailed observation and revealed the absence of differentiated neurons during early time points (Bilayer and Hatching stage), based on the same method, in contrast to findings from a previous study (Martin et al., 1997). Additionally, a significant increase of neurons was observed within the first hours of development, indicating a rapid morphological transition and maturation of neuronal progenitor cells. This progression likely supports the formation of the nerve net and may contribute to early contractile behaviors in juvenile *Hydra* polyps. However, while the maceration technique can reveal the total number of precursor cells and neurons based on morphological traits during the previously described stages, it does not provide information about cellular connectivity or the topological organization of the developing nerve net. Immunostaining against neuropeptides, labeling all neuronal populations except for the population N5 (GLWamide, RFamide; (Klimovich et al., 2020)) revealed an already established nerve net-like structure during the Bilayer stage (**Figure 13**). In adult *Hydra*, precursor cells, derived from i-cells, migrate from the body column and complete their postmitotic differentiation at their final destination (David and Hager, 1994; Hager and David, 1997). This final cell cycle takes approximately six hours to complete, which may explain the post-hatching increase of differentiated neurons during the Cigar stage (**Figure 12**). However, it does not account for the presence of an organized nerve net-like structure during the Bilayer and Hatching stage, suggesting additional, earlier processes to be involved.

A possible explanation involves the hatching process itself, with three contributing factors: cuticle constitution, neuronal maturation and cell proliferation. During the Bilayer stage, the cuticle undergoes structural changes by developing cracks, making it slightly permeable,



thereby allowing the surrounding medium to seep into the cuticle (Martin et al., 1997). Given that the Bilayer stage lasts approximately 24 hours and the neural maturation from i-cells to mature neurons also requires 24 hours, these processes appear to be correlated and may suggest the Bilayer stage as a starting point for the nerve net formation. Moreover, the threefold increase in the total cell number between the Bilayer and Hatching stage (**Figure 9**) further suggests that the active proliferation may contribute to the mechanical and biological conditions necessary to induce the hatching process. All of these factors – cuticle weakening, neuronal maturation and cell proliferation – appear to correlate and may promote or even induce the hatching of an embryo. However, which of these factors serves as the primary inducer remains unknown. The increasing hydrostatic pressure, resulting from the swelling gastric cavity of the embryo and the cell proliferation, may exert additional pressure on the cuticle. The specific factors involved in the weakening of the cuticle in *Hydra* embryos remain unidentified but may involve enzymes similar to those reported in other model organisms such as the ZHE1 enzyme in zebrafish (*Danio rerio*) or the MHCE and MLCE enzymes in medaka (*Oryzias latipes*) (Sano et al., 2008; Yasumasu et al., 2010). The observations support the assumption that internal mechanisms preparing the hatchling for following development are initiated approximately 24h prior to the rupture of the cuticle. The presence of a nerve net-like structure during Bilayer and Hatching stage could be attributed to the 24h time window required for neuronal differentiation (David and Hager, 1994). As neuronal progenitor cells mature, the expression of specific precursor cell genes such as *Myb/Myc3* (Siebert et al., 2019) may overlap with the expression of the neuropeptides used for the immunostaining (GLWamide, RFamide) during this transitional phase. Additionally, neuronal cells may also begin to form branches, potentially facilitating and prepare for a rapid signal transduction after hatching. Targeting RFamide further revealed that the neuronal identity is already established during these early developmental stages, with clear spatial patterning in both oral and aboral regions (**Figure 15 a-e**). These findings extend a previous observation in terms of both the number of RFamide-positive neurons detected during Bilayer stage and the timing of patterning (Brumwell and Martin, 2002). In summary, this work provides novel insights into the structural characteristics of neurons and neuronal progenitor cells as well as the patterning and the neuronal identity during late embryogenesis and early *Hydra* hatchlings. However, the

functional state of the emerging nerve net remains unclear, as the fixed samples used for staining and macerations did not allow for neuronal activity measurements.

Previous studies have reported discrepancies regarding the number of neurons during hatching, which differ from the observations made in this study. In contrast to the number of 70 neurons observed by Martin and colleagues during hatching (Martin et al., 1997), this work was unable to validate these findings during the early determination timepoints (Bilayer, Hatching, Cigar stage). A possible explanation for this may be the differing time windows and staging criteria employed across both studies. In contrast to the study made by Martin *et al.*, this work considered time as a secondary factor and categorized the developmental stages based on morphological traits, from which the time was calculated (**Figure 7**), whereas Martin *et al.* selected two post-hatching stages: 0-5 hours and 2–20 hours. In their study, the post-hatching category (0-5h) appeared to encompass both the Hatching and Cigar stages, defined in the present work. When considering the mean neuronal count across these two morphologically defined stages, the present work's neuronal count even surpasses the ones reported in the previous study by Martin and colleagues (1997). Furthermore, differences in *Hydra* strains could contribute to the observed variations. Martin *et al.* used a cross of *Hydra vulgaris* PA1 (female) and CA7 (male), having in a nearly 100% hatching rate, whereas this study utilized the *Hydra vulgaris* AEP strain, which likely originated from multiple crossings between CA7 and PA1-derived offspring ((Martin et al., 1997), personal blog of Rob Steele (accessed on 21<sup>st</sup> April 2025: <https://tinyurl.com/nphysyh42>). Such genetic variation could have led to deviations regarding neurodevelopmental timing and neuronal counts.

Furthermore, the classification of individual hatchlings and embryos based on morphological stages facilitated the systematic collection of developmental stages. However, variations between individuals suggest differences in developmental timing, as evidenced by variations in the total number of cells (**Figure 9**) and the number of RFamide-positive neurons (**Figure 15 g**). These variations complicate the observation of a direct correlation between neuronal counts and morphological stages. An additional limitation arises from the maceration method itself. Distinguishing neuronal progenitor cells from mature neurons during specific developmental stages remains challenging - particularly during transitional phases. This is further compounded by a potentially harsh sample treatment, which may have damaged defining features of mature neurons such as neuronal branches. Taken together, these

observations suggest that morphology-based distinction alone may not provide a reliable method for identifying neuronal differentiation. Complementary approaches such as molecular markers, transgenic reporters or live imaging may be necessary to characterize the maturation state of neurons during early development more accurately.

The findings reported in this work provide an initial framework for understanding the structural properties of early nerve net formation in *Hydra vulgaris* AEP. However, open questions remain regarding the precise mechanisms driving the *de novo* nerve net formation. Future investigations are necessary to resolve these remaining gaps to further examine the involved cellular and molecular processes. As a first step, to unravel the underlying mechanisms, a molecular map based on transcriptional signatures would be valuable for identifying the molecular profiles of specific cell lineages or neuronal populations in *Hydra* embryos. Such an “atlas” would enable a deeper understanding of neuronal specification, trajectories and lineage relationships during embryogenesis, contributing to a more comprehensive view on the nerve net assembly in basal metazoans.

### **The *de novo* assembly of a functional neuronal circuit in *Hydra* and influential environmental impacts**

One aim of this work was to uncover how neurons in one of the phylogenetically oldest nervous systems can assemble into functional neuronal circuits and to question whether external environmental influences affect the assembly and architecture of the nerve net. This work focused on the ancestral metazoan *Hydra vulgaris* to decipher the *de novo* assembly of a population-specific neuronal circuit. Here, embryos expressing a neuronal population N4-specific transcript were used, this endodermal neuronal subpopulation is located throughout the body of the animal and participates in the eating behavior of adult polyps (Giez et al., 2024). The observations revealed that these population-specific neurons emerge before they are functionally required and that the neuronal circuit self-assembles over time, driven and synchronized by  $\text{Ca}^{2+}$  activity and an increasing number of neurons as development progresses. Furthermore,  $\text{Ca}^{2+}$  imaging demonstrated that the increase of active neurons paralleled the dynamic formation of a functional neuronal circuit.

## The overproduction of neurons and neuronal progenitors as a fundamental process

This work revealed a difference between active N4 neurons and cells expressing an N4-specific transcript, observed through  $\text{Ca}^{2+}$  imaging and immunohistochemistry (**Figure 16e**). This finding suggests that both during early development and in adult polyps, more neuronal cells are present than functionally required. Whether this reflects a unique *Hydra*-specific feature or follows an evolutionary fundamental process remains an open question.

Previous studies explored this topic, focusing on the interplay between the surplus generation of neurons or neuronal progenitor cells, apoptosis and pruning. The overproduction of neurons or neuronal progenitor cells during development and regeneration is a widely observed phenomenon across various species, indicating an evolutionarily conserved strategy to shape and optimize neural networks (Dori et al., 2021). This mechanism has been observed in model organisms such as planarians, *C. elegans* or mammals. Here, up to ~50% of the initially generated neurons are eliminated via apoptosis as synaptic connections are formed and refined (Dekkers et al., 2013; Karge et al., 2020; Yeo and Gautier, 2004). This mechanism ensures an optimization of the nervous system, allowing the mature nerve net to maintain an optimal number of neurons while refining synaptic connections by removing excess cells and incorrect wiring (Cowan et al., 1984; Deshmukh and Johnson, 1997). A possible mechanistic explanation for this phenomenon is provided by the “Neurotrophic Theory”, which states that developing neurons compete for limited trophic factors (survival signals) supplied by their target tissues. Neurons that fail to acquire a sufficient amount of these essential growth factors undergo apoptosis ensuring the assembly and formation of neural circuits while facilitating error corrections, target matching and optimization of connectivity (Deshmukh and Johnson, 1997; Oppenheim Ronald W, 1989).

This mechanism may contribute to the explanation of the rapid N4-specific neuronal circuit assembly observed in this work. An initial excess of neurons could be beneficial to reduce the physical distance between nerve cells, thereby increase the probability to form connections with neighboring cells. Additionally, the overproduction of neuronal precursor cells may enhance the probability of a simultaneous maturation and activation of neurons, potentially facilitating the nerve net assembly. Once a functional neuronal circuit is established, apoptosis

and pruning may fine-tune already existing connections. Moreover, an initial optimization of the connectivity in an emerging neuronal circuit could account for the temporary decrease in functional neurons observed in each of the individual samples as displayed in **Figure 17b**. In adult *Hydra*, the nerve net is maintained through a continuous neuronal turnover and apoptosis, ensuring that only required neurons can persist (Keramidioti et al., 2024). In contrast, embryos and early hatchlings may not initially require nerve net maintenance in the first place, but instead may prioritize the establishment of a functional nerve net to support emerging behaviors. However, following the basic establishment of a neuronal circuit, further optimization may serve as enhancement of signal efficiency and conserve energy for other growth-related processes. The overproduction of neurons and neuronal progenitor cells in *Hydra* embryos aligns with observations made in other model organisms with more complex nervous systems (Greenwald and Martinez-Arias, 1984; Lee and Park, 2021). This similarity underscores *Hydra*'s value as a model organism for investigating fundamental neurodevelopmental processes with relevance to higher metazoans.

While these findings support the hypothesis that neuronal overproduction contributes to neuronal network optimization, several limitations must be considered while interpreting the results. First, the evidence for apoptosis and pruning was not directly observed and demonstrated in the recordings of active N4 neurons, rendering this conclusion speculative rather than confirmed. Second, the reduced recording time may have limited the ability to capture long-term neuronal turnover for single individuals. Additionally, phototoxic effects during  $\text{Ca}^{2+}$  imaging cannot be fully excluded, raising the possibility that artefacts may have influenced the activity patterns of neurons. Third, these observations were limited to the observations for the endodermal N4 population. To strengthen the conclusions drawn from this work, future research should investigate whether similar patterns of neuronal overproduction and optimization occur in other neuronal populations during the early network assembly in *Hydra*. Finally, although the time between recording and fixation of the animals was minimized, deviations due to a rapid neurodevelopment cannot be completely excluded.

Given these limitations, further research is required to confirm and expand upon these findings, providing a more comprehensive understanding of the mechanisms underlying the neuronal overproduction. Investigating molecular markers of cell death and synaptic

remodeling could provide evidence for the mechanisms driving neural optimization. The sparse movement of late-staged embryos presents an opportunity to achieve a higher magnification and resolution in targeted areas, potentially enabling the visualization of neuronal connections and pruning during recordings. Molecular biological techniques such as TUNEL staining to detect apoptotic nuclei, along with morphological indicators of pruning (fragmenting neurites or phagocytic cells containing neural debris) could provide supporting evidence for the hypothesis that apoptosis and pruning are involved to refine the nerve net. Applying these tools to transgenic *Hydra* lines with neuron-specific labeling would allow for the direct observation of a neuronal elimination and apoptosis at specific developmental stages. Further, suitable transgenic lines for neuron-specific progenitor cells could provide additional information about neuronal progenitors. The use of double-constructs, combining late staged progenitor transcripts with neuronal population-specific transcripts, could reveal the transition from neuronal precursor cells to mature neurons. While commonly used markers such as *Myb/Myx3* have been employed to study progenitor populations, they are not ideal in this context due to their expression in non-neuronal precursor cells (Siebert et al., 2019). Identifying more selective markers will be crucial for accurately distinguishing neuronal progenitors. Additionally, functional experiments manipulating the neuronal turnover could provide insights into the role of apoptosis during early stages of nerve net assembly. Pharmacological inhibition of apoptosis by using caspase inhibitors or the genetical knock-down of progenitor cells using ablation systems could help to unravel the effects of an up- and down-regulation of neurons and neuronal progenitor cells. Such experiments would clarify the functional significance of apoptosis in shaping the neural network during early development and further determine whether neuronal overproduction is a regulatory mechanism conserved across species.

## Neuronal activity drives the assembly and development of a functional neural circuit

In this work, the formation and functionality of an emerging neuronal network in *Hydra* embryos and early hatchlings was investigated. By focusing exclusively on the N4 population,  $\text{Ca}^{2+}$  activity was analyzed to understand both the assembly of the network and the integration of neurons into the emerging network. Analysis of single-cell recordings and bioinformatic

applications revealed an increase of synchronization and number of neurons over time. Additionally, emerging neurons and neuronal ensembles of different sizes were observed across individuals, with an increasing dominant neuronal community at the oral region, acting as a main driver and suggesting an activity-driven nerve net assembly. Furthermore, environmental factors such as the microbiome and temperature appear to influence the neuronal nerve net development, highlighting the interplay between an intrinsic genetic programming and external factors in shaping the early assembly and connectivity of the N4-specific nerve net in *Hydra*.

Neural circuits in the central nervous system are well known for their roles in regulatory pathways for sensory function, motion control, learning, memory and their dysfunction is related to various neurodegenerative diseases (Ahrens et al., 2013; Bang et al., 2021; Kass-Simon and Pierobon, 2007; Tonegawa et al., 2015; Tully and Quinn, 1985). However, the *de novo* formation and assembly of an emerging network remains challenging due to the complexity of neural development. While studies in *C. elegans* or zebrafish embryos successfully used  $\text{Ca}^{2+}$  imaging to monitor neural activity, the differentiation and functional integration of individual neurons into networks is less documented (Kerr et al., 2000; Muto et al., 2013). The ability to use transgenic *Hydra* embryos provided the opportunity to examine the N4 population-specific assembly and the low complexity allowed for an observation of neurons at single-cell resolution. The findings reported in this work suggest strong evidence that the formation of the N4 nerve net is primarily driven by neuronal activity rather than a predefined molecular gradient.  $\text{Ca}^{2+}$  imaging and community detection analysis exhibited a steady expansion of neuronal synchronization, suggesting that neuronal activity plays a crucial role in the network assembly. The nerve net forms dynamically, with neurons integrated into an expanding and functional organized network rather than emerging from a spatially predetermined template.

## Gradients and the influence of temperature

Traditionally, neural circuit formation has been attributed to molecular gradients that guide neuronal differentiation and connectivity (Hartenstein and Stollewerk, 2015). Previous studies in cnidarians indicated that gradients of signaling molecules such as Wnt and BMP pathways contribute to nerve net development by guiding neuronal placement and connectivity (Galliot

et al., 2009; Hobmeyer et al., 2000; Knabl et al., 2025). However, the observations based on activity measurements in the work presented here contradict this model. First, the observed variability in  $\text{Ca}^{2+}$  activity across embryos and early hatchlings suggests the absence of a strict molecular gradient orchestrating the neuronal connectivity. Single neurons and small neuronal ensembles appear randomly across the observed samples without a spatially predetermined pattern. In a gradient-based model, neuronal differentiation would rely on molecular cues with a pre-programmed neuronal positioning which was not observed in this work. Second, rather than following a stepwise differentiation, neurons form functional ensembles as they become active and appear to be actively integrated into an emerging dominant community. This dominant community is an expanding network based on activity levels which incorporates smaller ensembles to increase the N4-specific network. This process more closely resembles self-organizing systems as reported in the neurodevelopment of vertebrates, where network assembly is driven by local interactions and an activity-dependent integration (Katz and Shatz, 1996; Sporns and Tononi, 2002). Third, although neural activity itself is influenced by environmental factors such as temperature, an activity-guided network assembly appears to offer a greater adaptability than gradient-based models. An activity-dependent assembly relies on local feedback and a functional integration, which can adjust more dynamically to external changes. In contrast, molecular gradients depend on a precise spatial distribution of signaling cues, which may react more sensitively to temperature-induced changes and potentially lead to a mispatterning or developmental delays. As a sessile organism, *Hydra* is highly dependent on ambient environmental factors such as temperature. Therefore, an activity-guided network assembly may have an evolutionary advantage by enabling a robust and flexible nerve net assembly under variable conditions.

These findings align more closely with self-organizing principles reported in more complex nervous systems where the neuronal activity influences synaptic integration and network maturation (Hiesinger and Hassan, 2018). Furthermore, the single-cell analysis of the neuronal activity revealed high spiking activity during early developmental stages (0h – 7.5h), which potentially facilitate the recruitment and rapid integration of new neurons into the emerging network. After one day, the network reaches a stabilized, synchronized state and the subsequent reduction of spiking neurons suggests a transition from an active recruitment of neurons into a fully functioning and synchronized N4 network. The observations in this work



suggest that the assembly of a functional network occurs through the gradual integration of smaller neuronal ensembles into larger communities. While the precise mechanism of integration remains unclear, the data indicate that incorporation into the larger, functionally synchronized community leads to a more coherent neuronal architecture over time. Since neuronal ensembles of varying sizes are located in different regions of the sample, long distances must be overcome between the dominant and smaller communities. Direct, long-distance connections were not observed in the recordings, but some other factors like endodermal epithelial cells or a different neuronal population may bridge the distances and facilitate connectivity within the population-specific network. The endodermal network in *Hydra* further contains the N5 population (**Figure 5b**) which is expressed throughout the body (Klimovich et al., 2020) and, together with epithelial cells, could serve as the missing link to connect long distances between small emerging neuronal ensembles and the dominant community, further supporting a rapid network assembly.

Including the observations for the different temperatures, both the activity-driven and gradient-driven network assembly hypotheses are challenged (**Figure 19**). While neuronal activity at 13°C and 18°C remains at similar levels, the number of spikes at 23°C increased across all recordings. Based on this, the network assembly at 23°C would be expected to benefit from an increased spiking activity. However, the observations indicate the opposite: cold temperatures appear to be advantageous, whereas warm temperatures (23°C) result in a delayed network assembly and even deficits at later developmental stages, resulting in a 40% mortality rate. Different factors may explain the disadvantage of a high spike frequency. First, a potential explanation could be the intensity and frequency of the  $\text{Ca}^{2+}$  spikes itself. Considering the correlation coefficient of the measurements, the results for the warm conditions indeed displayed a higher number of spikes although the intensity and spike duration remained lower compared to 13°C or 18°C. This suggests that not only is the neuronal activity important but also that the intensity and duration of  $\text{Ca}^{2+}$  signals are crucial for establishing connections with adjacent neurons to sustain an optimal network assembly. The implementation of an increased spiking frequency into a *Hydra*-based artificial circuit model also predicts a size-dependent reduction of the dominant community, indicating a disadvantage for an increased neuronal activity (Noack et al., 2025, in final revision). Second, the spike frequency adaptation (SFA) may be a contributing factor to neurodevelopmental

delays or disadvantages. An increased spiking frequency induced by external factors such as temperature can trigger SFA, a regulatory mechanism by which the neural system moderates high activity to prevent the system from an increased energy consumption, impaired information processing or overstimulation. SFA refers to the ability of neurons to reduce the spiking frequency during a sustained stimulation, thereby enhances the efficiency, ensuring a robust information processing and enables the nervous system to recognize relevant signals in the presence of background noises. During neurodevelopment SFA may lead to an ineffective signal transduction as the emerging network may be unable to adapt, reorganize and establish connections effectively. Furthermore, studies demonstrated that the integration of SFA into artificial neuronal networks enhances energy efficiency and improves learning capabilities, even in the presence of limited synaptic plasticity (Benda and Herz, 2003; Destexhe et al., 1999; Ganguly et al., 2024; Liu and Wang, 2001; Shaban et al., 2021; Subramoney et al., 2024). A third aspect that may be induced by a repeated neuronal activity is a decrease of the synaptic effectiveness due to a depletion of vesicles and neurotransmitters. The observations in this study revealed a vesicle-mediated communication between neuronal and non-neuronal cells, even during early neurodevelopmental stages (**Figure 11**). The high spiking activity observed under warm conditions may have triggered a rapid release of neurotransmitters, potentially depleting vesicles before they could be replenished. This phenomenon, known as synaptic depression or synaptic fatigue, occurs when neurotransmitters are released at a faster rate than vesicles can be restored, ultimately leading to a temporary incapability and reduction to transmit signals (Zhang and Huang, 2024). If the number of available vesicles at the presynaptic neuron is low, the signal propagation may become insufficient to reach neighboring neurons, resulting in a prolonged or dysfunctional nerve net assembly. Additionally, synaptic strengthening or weakening is particularly crucial during neurodevelopment to establish a functioning nerve net. N-methyl-D-aspartate (NMDA) receptors play a pivotal role in regulation of synaptic function, as they are highly permeable for  $\text{Ca}^{2+}$  and the influx of these signal molecules is essential for neuronal regulation, migration, synaptogenesis as well as synaptic remodeling and its efficiency. Weak signaling, known as long-term depression (LTD), caused by a low intensity or weak  $\text{Ca}^{2+}$  signals, as observed at 23°C, may lead to a reduced number of these NMDA receptors. Here, the synapses become less responsive and may prolongate or negatively influence the development of an emerging nerve net. In contrast a long-term

potentiation (LTP), caused by strong  $\text{Ca}^{2+}$  signals, as observed for 13°C, may promote synaptogenesis and enhance the neuronal responsiveness to stimuli. A dysregulation of NMDA receptors has been linked to brain disorders or dysfunctionalities in mammals, vertebrates and invertebrates (Choi et al., 2020; Lakhan et al., 2013; Napoli et al., 2024). This may also contribute to disorders during neuronal development as observed as an impaired feeding behavior (**Figure 19c**) (Carroll and Zukin, 2002; Lau et al., 2009; Lau and Zukin, 2007).

The correlation between an altered spiking frequency and changes in the duration in the network assembly suggests that neuronal activity directly influences both nerve net assembly and functionality. Notably, 40% of the warm treatment group exhibited a feeding incapacity, underscoring the pivotal role of neuronal activity during the network formation in *Hydra*. The increased spiking frequency in *Hydra* embryos at 23°C may trigger the mechanisms described above, influencing early neurodevelopmental processes. This further highlights *Hydra* as a valuable, yet sensitive, model organism to study mechanisms of central importance in nervous system development across higher metazoans. Further studies are required to determine how the spiking activity influences the network assembly and whether the proposed mechanisms are directly responsible for changes or deviations during the *de novo* formation of nerve nets.

The long-term recordings performed in this work allowed for tracking neuronal activity at single-cell resolution during early stages of neurodevelopment in *Hydra*. Unlike immunohistochemical approaches, which capture static snapshots in fixed animals, this method provided insights into a dynamic nerve net assembly. The use of a community detection based on the Leiden algorithm further strengthened the findings by objectively identifying functional connectivity changes over time. However, despite analyzing neuronal activity events in each recording (30min in total), only single events occurring within 2.000 frames were considered. Additionally, activity events within the one-hour break interval between recordings were not captured. Furthermore, while this study demonstrates an activity-driven approach, the influence of molecular gradients on the nerve net formation cannot be excluded completely. It might be possible that neuronal activity and molecular gradients work in conjunction rather than as opposing mechanisms. Previous studies suggest that molecular gradients could provide an initial framework for network formation, while the final steps of network construction may require neuronal activity. This dual role was observed in systems such as the development of the mammalian cortex, where early activity refined

axonal targeting established by molecular gradients (Ackman et al., 2012; Katz and Shatz, 1996; López-Bendito and Molnár, 2003). This interplay could also explain the deviations between the findings of a nerve net-like structure during antibody staining of early stages (**Figure 13**) and the neuronal activity observed for the N4-specific  $\text{Ca}^{2+}$  measurements (**Figure 18, 19**). A further limitation concerns the potential phototoxic effects of the 488nm wavelength used for the live-cell imaging, particularly in relation to the observed inability to engulf prey in 40% of the samples at 23°C. Transgenic neurons expressing blue-light-sensitive proteins such as GFP are more susceptible to phototoxicity, which can lead to a reduced percentage of cells performing mitosis, a decreased cell cycle and an increased percentage of cell death (Douthwright and Sluder, 2017; Icha et al., 2017). Phototoxicity may also contribute to the observed inability to perform the full set of the eating behavior including the engulfment of the prey. However, since all samples were exposed to the same laser intensity and recording conditions, the phototoxic effect is more likely to contribute to, rather than directly cause, the 40% mortality rate observed at 23°C. This may also explain why the neuronal N4 population was particularly affected. Additionally, all treatment groups have a limited sample size of five. Increasing the number of biological replicates would support the findings and improve the reliability of the conclusions drawn in this work. Furthermore, the removal of the cuticle during the Bilayer stage may have caused developmental artefacts as an artificial and premature hatching process. However, as the Bilayer stage appears to represent an early phase of the N4-assembly, the presence of the opaque cuticle and the inaccessibility of earlier embryogenic stages impeded the observation of how initially disconnected neurons integrate into a functional N4.

In addition, whole transcription RNA sequencing of early and late embryogenic stages would provide deeper insights into the involvement of molecular- and activity-driven gradients. State-of-the-art sequencing, such as spatial transcriptomics, could reveal the expression levels of key neurodevelopmental molecules like Wnt or BMP or identify neurogenesis- and neuron-specific transcripts, indicating the appearance of first neurons and which neuronal population emerges first (Piwecka et al., 2023). Furthermore, extended research is required to investigate different populations during the nerve net formation as well as the contribution of endo- and ectodermal epithelial cells. Emerging tools such as optogenetic activation and inactivation of

neurons could further enhance the understanding of the *de novo* nerve net formation and assembly in *Hydra* embryos and early hatchlings.

## The microbiome and its role during nerve net development

In the following section, the findings and influence of the microbiome as the second environmental factor, will be discussed. The findings revealed that the absence of a microbiome in *Hydra* embryos leads to a reduced hatching rate and a significantly lower number of RFamide-positive neurons in the hypostome. Additionally, germ-free embryos produced a lower number of neuronal branches and indicated a lack of maturation, suggesting an impaired network formation. Although recolonized embryos exhibited a lower number of neurons compared to the wild-type, the number of neuronal branches increased to more than four, likely enhancing the connectivity to compensate the reduced neuronal count. These findings highlight the pivotal contribution of the microbiome during embryogenic neurogenesis and network formation, demonstrating its influence on network stability and maturation despite the coverage by a protective cuticle throughout embryogenesis.

The interest in microbiome research increased in the 2000s with the rise of new sequencing technologies. The Human Microbiome Project (HMP), launched in 2007, was crucial in accelerating microbiome studies by mapping microbial communities using 16S rRNA sequencing and metagenomics (Gevers et al., 2012; Proctor et al., 2019; Turnbaugh et al., 2007). Over the last two decades, *Hydra* was developed as an excellent exploratory model organism for studying host-microbe interactions (Bathia et al., 2024; Franzenburg et al., 2013a; Fraune et al., 2010; Fraune and Bosch, 2007; Giez et al., 2023) but to date, the microbial influence during embryogenesis remains unclear. Throughout embryogenesis, the developing embryos are covered by a thick and impermeable cuticle which prevents direct contact to the surrounding microbiome. However, the microbial environment may have primary or secondary influences during embryogenesis, and therefore may further influence neurogenesis and the formation of neuronal circuits. On the one hand, it may be possible that antimicrobial peptides (AMPs), bacterial-produced molecules of small size such as bacteriocins (Bahar and Ren, 2013; Simons et al., 2020), are able to cross this thick barrier and directly influence various developmental processes. Previous studies demonstrated that AMPs, produced by *Hydra* polyps, indeed play a role as they are part of the innate immune system and can shape the

bacterial composition of the microbiome (Augustin et al., 2017; Bosch, 2012). Furthermore, maternally produced AMPs of the periculin family are used as protective molecules during early embryogenesis (Fraune et al., 2011, 2010). A potential role of AMPs, either produced by the embryo itself to influence the composition of its surrounding or the ambient bacteria, cannot be fully excluded. Defense mechanisms against pathogens during embryonic development is not unusual in the animal kingdom, for example organisms such as *Drosophila* can release AMPs (drosocin, dipterocin) during larval stages to protect the organisms from bacterial pathogens (Imler and Bulet, 2005) or *Philanthus triangulum*, which is able to emit nitric oxide against fungal infection (Strohm et al., 2019). However, if embryos are able to release AMPs to actively influence the composition of the ambient bacteria or even hold an active microbiome remains unknown. Initial findings of 16S rRNA demonstrated a divergent bacterial community on *Hydra* embryos compared to adult polyps from the same culture. As adult animals are able to actively shape the composition of the microbiome, the findings for the embryos might display the overall composition of the surrounding media, suggesting the inability to influence the colonizing bacteria on the surface of the cuticle (Appendix Figure 21).

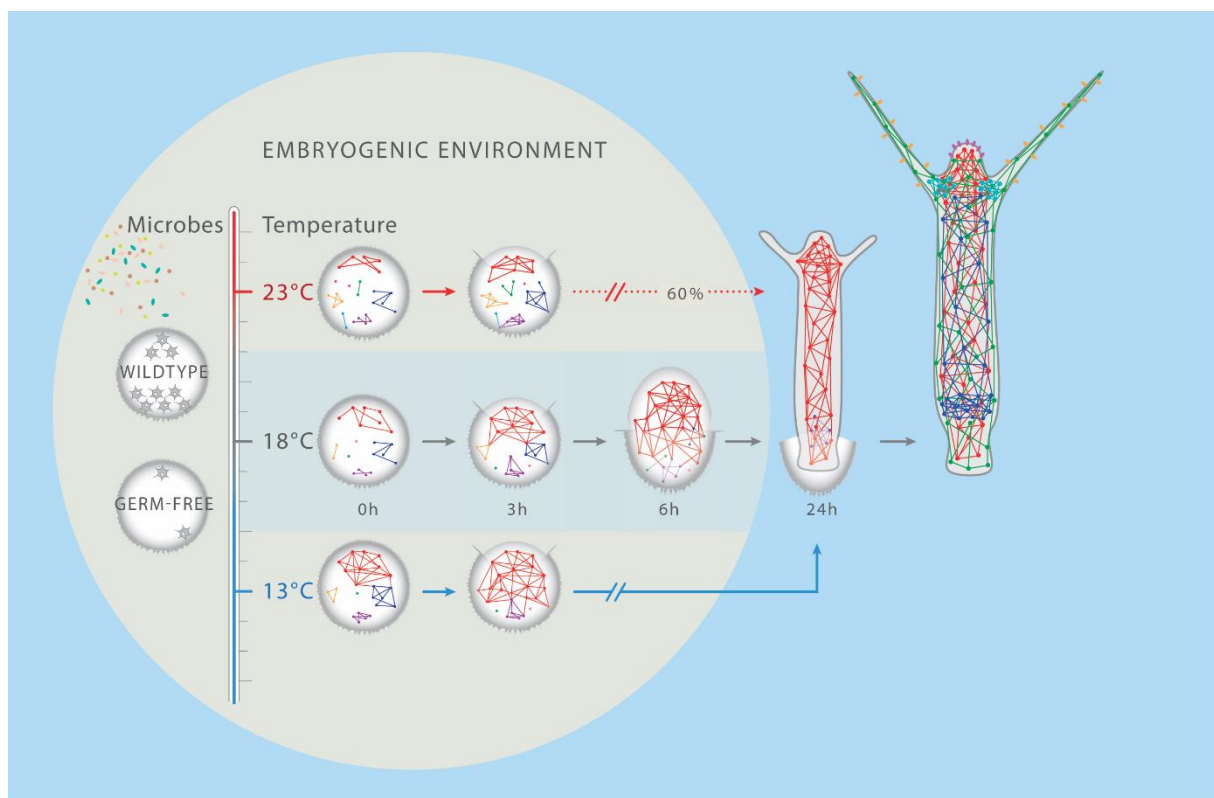
On the other hand, the microbiome can function as a secondary factor by suppressing the growth of potential pathogens or their toxic metabolites which may influence the development of the embryo. It remains unknown if a direct interaction occurs between the microbiome and the embryo but the absence of bacteria, caused by antibiotic treatment, may open niches for the growth of other organisms such as fungi. Previous research indicated that the absence of bacteria can lead to an increased susceptibility to fungal infections in germ-free organisms. Additionally, the absence of commensal bacteria can disrupt the balance of microbial communities, allowing for the overgrowth of fungi such as *Candida albicans* (Drummond et al., 2022; Fraune et al., 2015; Hou et al., 2023; Seelbinder et al., 2020). In principle, the microbiome may have a direct and/or indirect effect on the embryos by potentially facilitating an undisturbed development during embryogenesis. One critical factor which needs to be considered here is the antibiotic treatment, implemented to generate germ-free embryos. Embryos during the spike stage (24hpf) were treated with an antibiotic cocktail (Franzenburg et al., 2013b) which may have directly caused negative effects on the embryo and especially neurodevelopment. It cannot be excluded that the reduced number of neurons observed in early hatchlings of the germ-free and recolonized groups is to be attributed to the

antibiotic treatment during early embryogenic stages. Nevertheless, the recolonized embryos exhibited a restored hatching rate similar to the wild-type, which indicates rather a beneficial involvement of the microbiome than a negative effect of the antibiotic treatment.

Furthermore, the absence of a microbiome demonstrated an impaired neuronal circuit formation, indicating that successful development and assembly depends on the continuous presence of a co-evolved microbiome. The results displayed for germ-free and recolonized individuals, in comparison to the wild-type control, support and extend previous observations in mice, which demonstrated a defective development of the nervous system in embryos especially regarding neuronal connections in the absence of maternal microbial signals (Vuong et al., 2020). The ability to compensate for neural deficiency in early *Hydra* hatchlings by establishing an increased number of neural branches in present neurons by restoring the microbiome provides evidence for the crucial role in neurodevelopmental processes. While antibiotic treatment may directly affect nervous system formation, the microbiome likely plays a crucial role in mitigating additional impacts from opportunistic microorganisms such as fungi or archaea by maintaining the microbial balance.

As a next step, increasing the sample size will be essential to validate the initial observations for germ-free and recolonized embryos. To eliminate the potential influence of the antibiotic treatment, embryos developed under germ-free conditions would provide deeper insights and could additionally reveal the role of maternal signals during oogenesis. Furthermore, the mono- and multiple-association with *Hydra*-specific bacteria can reveal the influence of the main colonizers during embryogenesis. Previous studies on adult *Hydra* have already demonstrated the pivotal involvement of symbiotic bacteria in the eating and contractile behavior (Giez et al., 2023; Murillo-Rincon et al., 2017). In addition, the antibody staining exclusively revealed developmental snapshots of different samples during circuit formation. Therefore, neuronal activity measurements could disclose the microbial contribution to the nerve net assembly of population-specific neuronal circuits during long-term recordings in single animals. Moreover, 16S rRNA analysis of embryos could reveal an advanced understanding of differences or similarities between the bacterial composition of adult polyps and embryos. Here, the surrounding media needs to be included to verify whether ambient bacteria simply colonize on the cuticle or if the embryo is somehow able to directly shape the bacterial composition as demonstrated in adult *Hydra* (Augustin et al., 2017).

In summary, the determination of environmental factors that influence neural circuit assembly and stability is essential for understanding the maintenance of nerve nets and their influence in the face of global environmental changes, such as increasing temperatures and loss of microbial diversity. Similar to the observations in this work, previous studies have shown that external factors, such as hypoxia or maternal infection, influence neural circuit formation during critical phases of development in organisms like *Drosophila* or vertebrates such as *Danio rerio*, contributing to neurological diseases (Coulson et al., 2022; Lee et al., 2017; Marín, 2016). The new findings of the microbial influence on developmental processes could further extend the current knowledge about host-microbe interactions. Model organisms such as *Hydra* enabled valuable insights at the base of metazoan evolution. *Hydra* embryos could further provide the ability to understand the influence of the microbiome on *de novo* mechanisms such as neurogenesis, pattern formation or cell-cell interactions and information processing. These findings demonstrate that *Hydra* represents a valuable system for bridging molecular, environmental and evolutionary perspectives on nervous system development.



**Figure 21.** Graphical summary highlighting the findings for the emergence of the N4-specific nerve net in *Hydra* embryos and early hatchlings. Assembly and network construction is affected by external influences such as microbes and different temperatures during (Graphics by Katja Duwe-Schrinner).



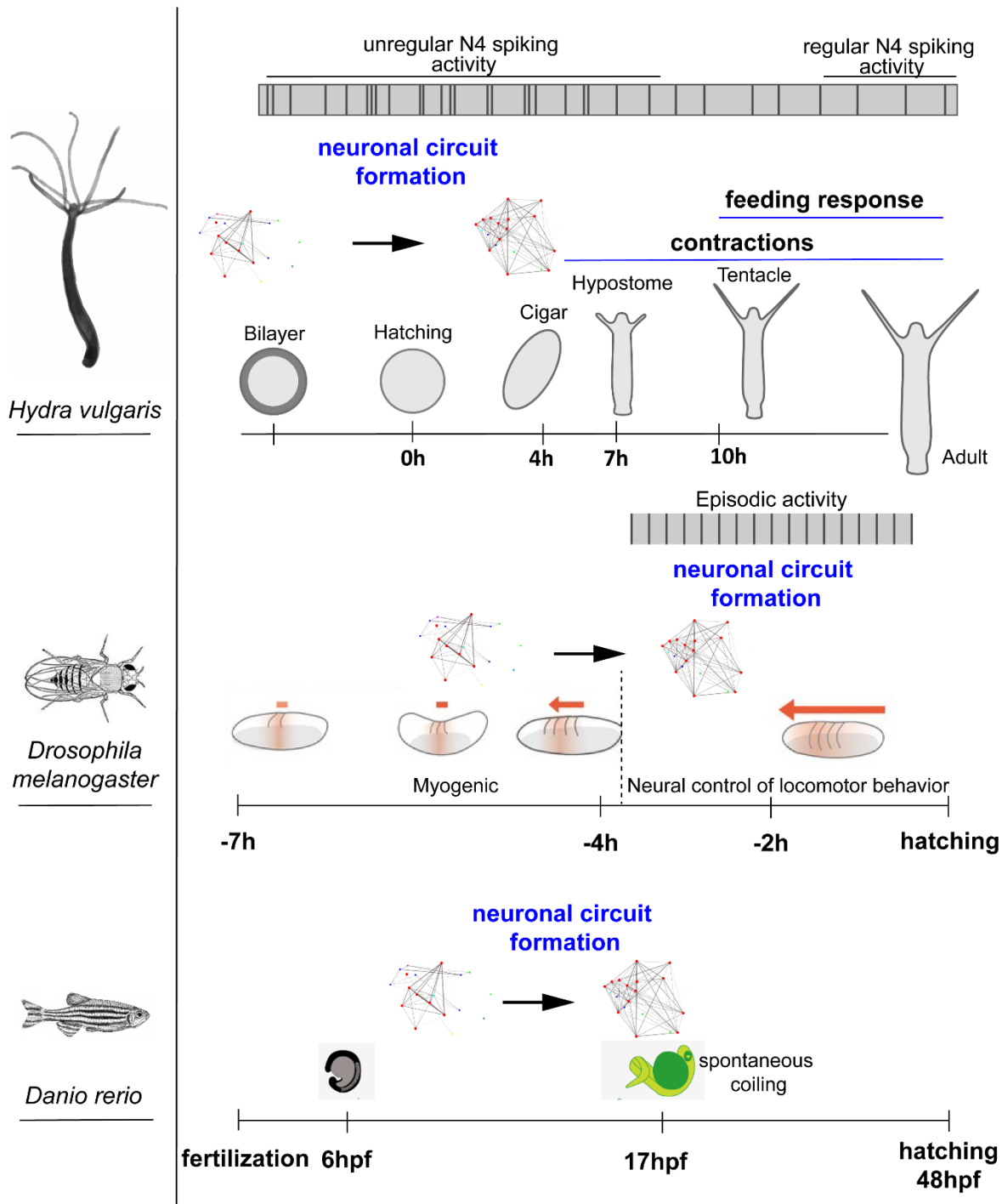
## Early behavior and the correlation to the emergence and formation of the nerve net in *Hydra* hatchlings

One of the major questions in the field of neurobiology is the relation between neuronal activity and behavior. How can animals perform complex behaviors without any prior experience? What are the minimal neuronal requirements and what cellular structures are involved in performing complex behaviors? The complexity of the nervous system and the vast number of neurons and neuronal connections enhance the difficulty to understand fundamental concepts. The model organism *Hydra* is able to perform complex behaviors with a comparatively low number of neurons and without a centralized information processing. The discussion in the previous sections, intended to contribute to the understanding of these basic mechanisms by examining the neuronal landscape in *Hydra* embryos, the functional assembly of the population-specific N4 network, the emergence of behaviors during early stages of development and the influence of environmental factors on the nerve net formation. Together, these insights may help to elucidate how functional behaviors can emerge from simple, self-organizing neural systems in the absence of prior experience or a centralized control.

In this work, the first contractile behavior emerging during the transition from the Cigar to the Hypostome stage was identified, which correlates with a rapid increase in matured neurons (**Figure 12**). This correlation suggests a potential role of the nervous system in initiating this behavior. Additionally, ultrastructural analysis of late-staged embryos and early hatchlings revealed a vesicle-mediated communication between neuronal and non-neuronal cells as well as the involvement of gap-junctions (**Figure 11**), supporting a rapid signal transmission between neuronal and non-neuronal cells. In addition, early inducible behaviors such as nodding and mouth opening, were first observed during the Hypostome stage and were accompanied by the emergence of first sensory neurons (**Figure 10 and 14**). These findings indicate that rapid developmental processes may enable early behavioral functions necessary to perform an independent energy intake. Moreover, immunostaining against the neuropeptide RFamide revealed an increasing neuronal density and connectivity within the nerve net across examined developmental stages (**Figure 15**). An increase in neuronal activity during early circuit formation, compared to neuronal activity after one day, further supports the assumption that the maturation of neuronal circuits may contribute to the emergence of early behaviors.

The results of this work demonstrate that the first hours after hatching are marked by rapid changes in an emerging nerve net, including an increase in the neuronal numbers and functionality, particularly in terms of synchronicity and the assembly of an N4-specific network. After the break of the cuticle, *Hydra* hatchlings undergo immediate neurodevelopmental changes to acquire the ability to perform essential behaviors important for eating, movement or contractions. Several mechanisms, including rapid cell proliferation (**Figure 9**), vesicle-mediated communication (**Figure 11**), the maturation of neurons (**Figure 12**), and the optimization and strengthening of neuronal connectivity (**Figure 18d**), likely contributing to the implementation and ability for early behaviors.

The rapid formation of neural connections is not unique to *Hydra* but has also been reported in more complex organisms such as *Danio rerio* or *Drosophila* (**Figure 22**). Here, the initiation of neural connections in the spinal cord of zebrafish embryos implement basic reflexes, while in *Drosophila larvae* the locomotive behavior is initiated by episodic neural activity (Kohsaka et al., 2012; Nelson and Granato, 2022). In *Hydra*, spontaneous contractions as the first behavior emerge around eight hours after hatching, during which hatchlings undergo both morphological and cellular changes. Since this rapid growth requires energy, the emergence of behaviors that can provide for a continuous energy supply is crucial. However, energy is also required for processes such as cell proliferation and differentiation, which are essential for behavioral development. Previous studies in *Hydra* demonstrated that nurse cells contribute to early embryogenesis by undergoing apoptosis to support oocyte growth but remain morphologically unchanged until hatching. Following hatching, apoptosis resumes, leading to the degradation of nurse cells within 72h (Alexandrova et al., 2005; Technau et al., 2003). Degraded cellular components may be recycled through efferocytosis or autophagy, indirectly providing energy for further and rapid developmental processes (Lin et al., 2021; Moon et al., 2023). The post-hatching activation of apoptosis in nurse cells likely serves both as a clearing mechanism to remove unnecessary cells and as a metabolic recycling process, facilitating an efficient development.



**Figure 22. Neuronal circuit formation is universal and emerges in the absence of a centralized information processing.** Across the animal kingdom, neuronal circuit formation seems to follow a universal principle long before any centralization of information processing. To ensure rapid circuit formation *Hydra* embryos and hatchlings exhibit an increased and irregular spiking activity, supporting the implementation of early behaviors. In contrast, adult polyps with full behavioral repertoire display regular spiking patterns (Giez et al., 2024). Similarly, in *Drosophila*, locomotor behavior begins 7h before hatching, with episodic activity as the first behavior emerging 3.5h later, driven by neural activity (Sadeqzadeh et al., 2014) (mod. from Kohsaka et al. 2012). In zebrafish, spontaneous coiling, resulting from the development of early spinal circuits, appears as early as 17h after fertilization (Nelson and Granato, 2022) (graphic by Zoe Zorn).

According to previous studies different neuronal subpopulations are involved in behaviors such as contractions, feeding or phototaxis (Dupre and Yuste, 2017; Giez et al., 2023; Kim et al., 2024; Klimovich et al., 2020; Noro et al., 2021). Initial longitudinal contractions are accompanied by morphological changes in early hatchlings (Appendix Video S1) and involve populations located in both the hypostome and the foot area. In particular, the populations N1 and N2, also termed contraction burst network (CB) (Ec1A, Ec1B, Ec5 (Siebert et al., 2019)), are associated with longitudinal contractions (Dupre and Yuste, 2017). Among these, only the neurons of the N2 populations may function as pacemaker cells controlling the spontaneous contractions (Klimovich et al., 2020). Although this work was not able to identify the first emerging neuronal population in *Hydra* embryos, antibody staining against RFamide revealed an early patterning involving the oral (N6/N7) and aboral (N1) neuronal populations (**Figure 15**). However, activity measurements of the neuronal population N4 demonstrated deviations between neuronal activity and the population-specific detection, making direct correlation between neuronal emergence and functionality speculative (**Figure 16e**). The involvement of the N1 population in early contractile behavior remains a possibility, but further experiments, especially activity measurements, are necessary to confirm its role in contractions. Further involvement is attributed to the pacemaker population N2, which is located beneath the hypostome at the oral end of *Hydra* (**Figure 5a,b**), or, more specifically lying within the predicted region beneath the hypostome for the subtentacle network (STN) (Dupre and Yuste, 2017). This network, which governs the nodding behavior in adult polyps, exhibits a rapid signal propagation ( $251 \pm 54.6 \mu\text{m/s}$ ), suggesting efficient bidirectional signal transmission (Dupre and Yuste, 2017). Experiments in this work found that nodding, similar to the first contractions, is among the earliest behaviors *Hydra* hatchlings can perform, although induced by external stimuli. While the involvement of N2 in the STN was not directly confirmed in this work, its hypothesized role in both contraction and nodding indicates a multifunctional contribution to *Hydra* behaviors. The findings in this work highlight a temporal correlation between the first contractions and the inducible nodding behavior, suggesting the Hypostome stage as an important milestone in behavioral development (**Figure 10**). This stage may provide the minimal neuronal requirements for early emerging behaviors, potentially supported by the emerging N2 network. A previous study in *Hydra* polyps, using a combination of live imaging, calcium activity and biomechanical simulations, demonstrated the pivotal role of neurons in

behavioral contractile sequences. Specifically, neurons in the CB network activate epitheliomuscular cells which further induce the contractile behavior (Wang et al., 2023). However, since nerve-free *Hydra* polyps can still perform a reduced number of contractions (Campbell et al., 1976; Kass-Simon, 1972; Terada et al., 1988), neurons are not the sole drivers of behaviors. Instead, they likely function alongside endo- and ectodermal epithelial cells to coordinate early motor behaviors. The early emergence of behaviors in *Hydra* hatchlings raises questions about the minimal requirements necessary to perform a functional behavior. The findings of an increasing RFamide-positive network in the oral and aboral area, as well as the inducible nodding behavior during early development to potentially facilitate behaviors is also observed in vertebrates. These findings demonstrated that basic motor behaviors such as spontaneous muscle twitches in developing embryos occur before the full maturation of sensory circuits (Blankenship and Feller, 2010). The ability of *Hydra* hatchlings to perform early behaviors such as contractions and nodding, accompanied by rapidly assembling networks may suggest that essential motor functions arise from minimal, currently connecting networks rather than complete mature neural circuits. This supports the assumption that performing behaviors does not require a fully established nerve net but can emerge in minimal yet functioning circuits.

This work highlights that the emerging nerve net in *Hydra* undergoes a rapid functional and activity-driven assembly after hatching. Further, the ability of the nervous system to adapt in response to environmental influences indicates a certain level of neural plasticity, even for *Hydra* as an early metazoan. The changes observed during the emergence of the nervous system, including the differences of neuronal densities and branches and the beneficial influence of temperature, supports the assumption of neural plasticity and highlights the similarities to higher model organisms such as *Drosophila* (Züfle et al., 2025). Similar mechanisms are observed in vertebrates where synaptic refinements and activity-dependent plasticity are crucial for a functional circuit formation, especially during sensitive periods (Hensch, 2005; Knudsen, 2004). For example, early spontaneous activity plays a crucial role in shaping the connectivity in mammalian cortical networks, similar to the episodic neural activity initiating locomotion in *Drosophila* larvae (Kohsaka et al., 2012; **Figure 21**). This may indicate that neural architecture and plasticity appear to be universal and valid not only for *Hydra* polyps, but also for vertebrates including metazoans with an increased nervous system

complexity. However, the nerve net in *Hydra* represents an alternative for neural organization compared to centralized nervous systems found in higher metazoan clades. The rapid assembly of neuronal circuits such as the endodermal N4 nerve net and the emergence of early behaviors in *Hydra* without a centralized brain challenges models of hierarchical neural control. However, this shares similarities to studies in *Caenorhabditis elegans* which used tools such as  $\text{Ca}^{2+}$  imaging, cell ablation and electrophysiology to demonstrate that small, distributed networks can effectively generate behaviors (Gao et al., 2018). The presence of a pacemaker population in *Hydra* further points to the fact that organisms without a centralized brain may rely on local circuits to induce or coordinate motor behaviors. Similar mechanisms have been demonstrated in pacemaker-driven networks in invertebrates, such as the stomatogastric ganglion of crustaceans, where rhythmic motor patterns are generated by small neural circuits without a centralized control (Marder and Bucher, 2001).

The assumptions made in the previous section are limited because indirect observations cannot provide conclusive evidence for direct causal relationships. To overcome these limitations further studies and transgenic lines are needed to prove the direct involvement of the neuronal population N2 in behaviors such as nodding or contractions. Moreover, *Hydra* hatchlings display a high time-dependent variance within morphological stages and cellular characteristics (**Figure 9, 15, 16**). This renders it difficult to predict emerging behaviors during specific stages and the number of population-specific neurons may vary between determination timepoints as morphology does not allow any conclusion about the number of neurons. A staining of neurons in polyps with the emerging behavior could reveal the minimal requirements to perform complex behaviors. However, the pacemaker population N2 may be a highly interesting target to unravel the participation and involvement of early emerging neuronal circuits in behaviors of *Hydra* hatchlings. In addition, reducing a behavior to a single neuronal population may not reflect the complexity and interconnection between neurons in *Hydra*. Previous studies already demonstrated the activity and participation of different populations in behaviors such as eating (N3, N4, N6) or somersaults (N3, N4). Thereby, especially the globally distributed populations N3 and N4 in ecto- and endoderm participate in multiple functions like contractions, detachment, phototaxis, somersaults or engulfment (Dupre and Yuste, 2017; Giez et al., 2024, 2023; Yamamoto and Yuste, 2023). In addition, also

the role of endodermal and ectodermal epithelial cells is still not understood and their involvement in the emerging behaviors remains unknown also in early hatchlings.

In prospective studies, transgenic lines to visualize the  $\text{Ca}^{2+}$  activity of N2 neurons could reveal the involvement in emerging behaviors such as contractions and nodding. Furthermore, future studies could benefit from the implementation of optogenetic lines which allows for an activation or inactivation of specific neuronal populations to observe their involvement in specific behaviors. In addition, these optogenetic lines could reveal information about the minimal requirements to induce behaviors by the activation of single neurons. Combining these approaches with behavioral assays that facilitate the observation of free-moving hatchlings could shed light on the relation between behavior and the nervous system. These integrated strategies could clarify how specific neurons or neuronal circuits contribute to the emergence of behaviors and offer a functional framework for understanding how behavior is encoded in decentralized nerve nets. Taken together, *Hydra* hatchlings are a powerful model system to gain a deeper understanding of the correlation between neuronal activity and behavioral output.

## Outlook

*Hydra* is a fascinating and versatile model organism, offering various advantages due to its phylogenetic position, its rich behavioral repertoire despite a comparatively low complexity and its accessibility for the generation of transgenic lines. *Hydra* became a powerful system across various fields including developmental biology and regeneration, cell biology, genomics and molecular biology, ageing and longevity, evolution, research on symbiosis and host-microbe interactions as well as in the field of neurobiology. However, most of the current knowledge and techniques in all of these research fields are based on adult polyps, neglecting the possibility to explore *de novo* mechanisms close to the base of the metazoan evolution.

In the field of neurobiology, *Hydra* embryos could improve and support the understanding of fundamental concepts regarding the assembly, connectivity and plasticity of emerging nervous systems. Furthermore, the impact of external stimuli on embryogenesis may indicate whether different developmental processes and morphological phenotypes are affected by environmental influences. Building on the tools and pipelines generated in this work, new transgenic lines for neuronal populations could add further insights and prove the fundamental concept of an activity-driven nerve net assembly. Implementing new tools such as optogenetics would allow for examining neuron-neuron and neuron-non-neuron interactions, revealing the specific role and hierarchy in behavioral outputs. Furthermore, comparative analyses between *Hydra* and further developed metazoans can contribute to the understanding of universal concepts in nerve net architecture across the animal kingdom to overcome complexity and neuronal diversity.

Taken together, *Hydra* embryos could contribute to and extend *Hydra's* already diverse research repertoire. However, adult polyps are widely used and successfully add to the conceptual understanding of early metazoans, but the investigation of *de novo* mechanisms and particularly examinations of the emergence and assembly of neuronal circuits is exclusive to *Hydra* embryos and early hatchlings. Expanding *Hydra's* research applications by including embryos further underscores its versatility and importance as a model organism and extends the possibility to understand mechanisms hidden from higher metazoans due to the high complexity.



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## List of publications

1. Giez C, **Noack C**, Sakib E, Hofacker L-M, Repnik U, Bramkamp M, Bosch TCG. Satiety controls behavior in *Hydra* through an interplay of pre-enteric and central nervous system-like neuron populations. *Cell Reports* (2024), 43(6), DOI: 10.1016/j.celrep.2024.114210
2. **Noack C**, Jenderny S, Giez C, Merza O, Hofacker L-M, Wittlieb J, Repnik U, Bramkamp M, Ochs K, Bosch TCG. Assembly of a functional neuronal circuit in embryos of an ancestral metazoan is influenced by environmental signals including the microbiome. *BioRxiv*(2024), DOI: 10.1101/2024.12.10.627692, under final revision in PNAS

## Appendix

### Figures

Figure S1: Emergence of first contractions and somersaults – extending and related to Figure 10



<https://tinyurl.com/2v7cbzy2>

Contractions are the first emerging behavior in *Hydra* hatchlings and occur three to 14h after cuticle disruption. First somersaults are observed between 12 – 59h and displayed a high variance between single animals. (contractions n = 17; somersaults n = 12)



Figure S2: Potential communication via DVs and gap junctions – related to Figure 11



<https://tinyurl.com/mtrrf58r>

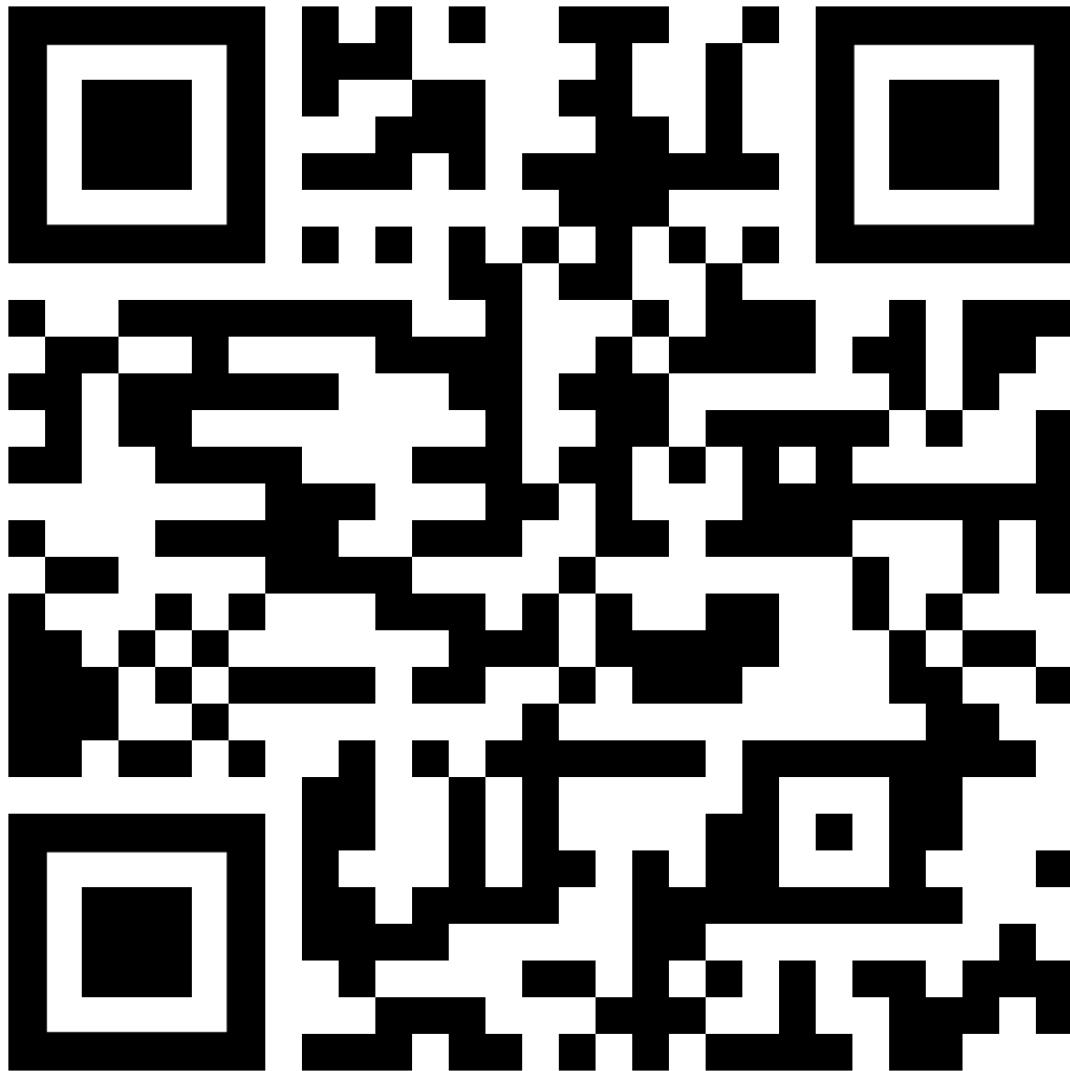
Figure S3: Cell-cell connections – related to Figure 11



<https://tinyurl.com/ypcefcf8>

Enlarged structures focusing on plasma membranes and different junctions found in early *Hydra* hatchlings. Red arrowheads: gap-junctions; yellow arrowheads: septal-junctions.

Figure S4: Drawing\_TEM – related to Figure 11



<https://tinyurl.com/495ee7a7>

Schematic representation of the core findings in the TEM analysis starting in the Bilayer stage and ending in the Tentacle stage, covering a developmental timeframe of approximately 24h. Over the course of time we observed a maturation of neurons as well as an increase of connections between endodermal and ectodermal epithelial cells throughout the mesoglea. Gap-junctions as well as electron-dense vesicles appear to drive and support a rapid development of the nervous system. TEM analysis revealed a widespread communication potential between neurons and mesoglea, neurons and epithelial cells, epithelial cells and mesoglea as well as among endodermal and ectodermal epithelial cells.

**Figures S5-S8      18°C**

Figure S5: Sample1 (H63) – activity analysis N4 neurons – related to Figure 17b &  
18



<https://tinyurl.com/ysfyuzj8>

Figure S6: Sample3 (H65) – activity analysis N4 neurons – related to Figure 17b &

18



<https://tinyurl.com/5wur2hn4>

Figure S7: Sample4 (H66) – activity analysis N4 neurons – related to Figure 17b &

18



<https://tinyurl.com/yskkhf88>

Figure S8: Sample5 (H67) – activity analysis N4 neurons – related to Figure 17b &

18



<https://tinyurl.com/yk2udv27>

**Figures S9-S13    13°C**

Figure S9: Sample6 (H72) – activity analysis N4 neurons – related to Figure 19



<https://tinyurl.com/53tt97v6>



Figure S10: Sample7 (H75) – activity analysis N4 neurons – related to Figure 19



<https://tinyurl.com/22e2mds4>

Figure S11: Sample8 (H77) – activity analysis N4 neurons – related to Figure 19



<https://tinyurl.com/4bne5ray>

Figure S12: Sample9 (H80) – activity analysis N4 neurons – related to Figure 19



<https://tinyurl.com/fpefbazc>

Figure S13: Sample10 (H81) – activity analysis N4 neurons – related to Figure 19



<https://tinyurl.com/e2fs3m9u>

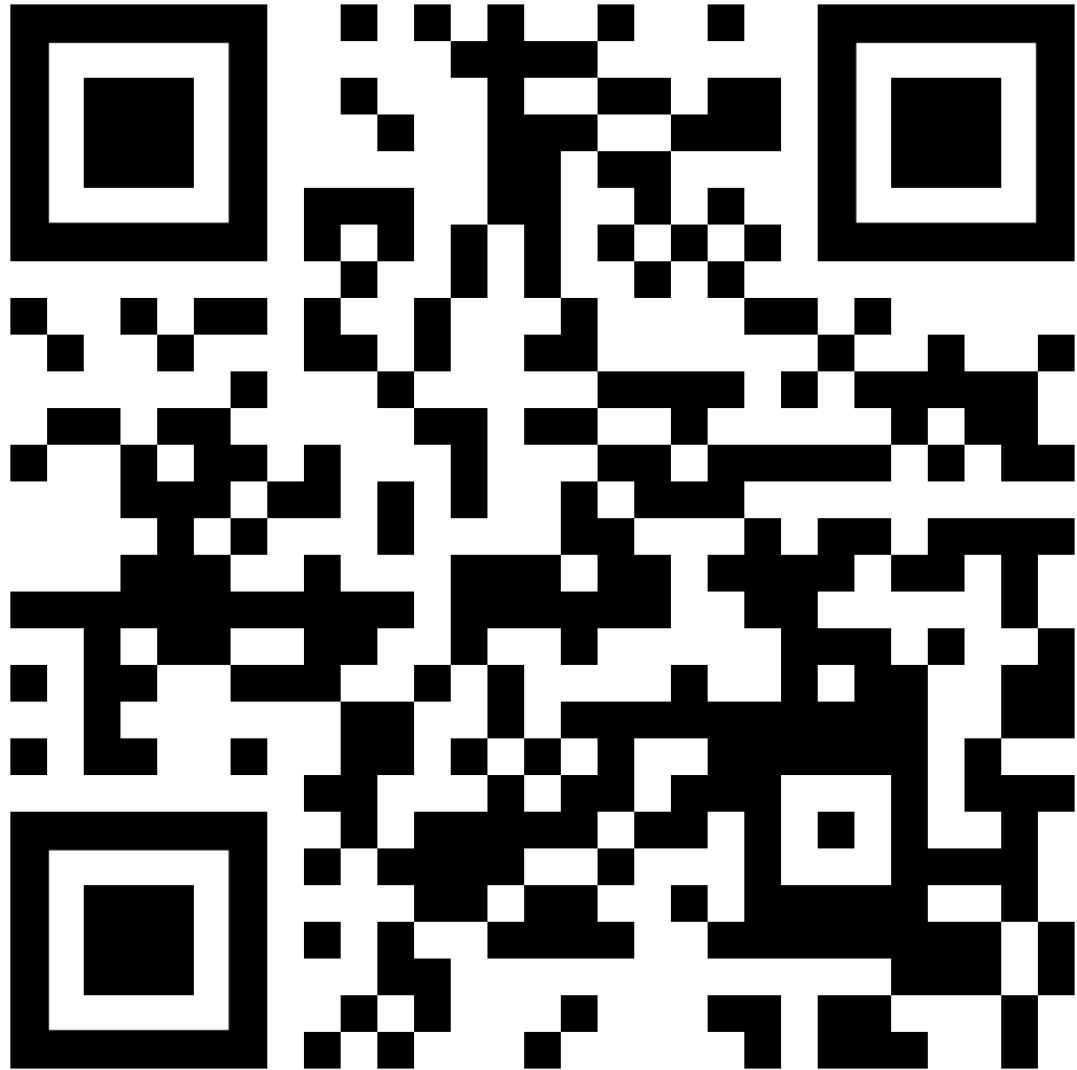
**Figures S14-S18 23°C**

Figure S14: Sample11 (H82) – activity analysis N4 neurons – related to Figure 19



<https://tinyurl.com/yv889vt7>

Figure S15: Sample12 (H83) – activity analysis N4 neurons – related to Figure 19



<https://tinyurl.com/3vszkvaz>

Figure S16: Sample13 (H84) – activity analysis N4 neurons – related to Figure 19



<https://tinyurl.com/y7nrxn6n>

Figure S17: Sample14 (H85) – activity analysis N4 neurons – related to Figure 19



<https://tinyurl.com/bdz87fev>



Figure S18: Sample15 (H86) – activity analysis N4 neurons – related to Figure 19



<https://tinyurl.com/dy5erce9>

Figure S19: Circuit model and additional simulations – related to Figure 18 & 19



<https://tinyurl.com/pwdw9rzs>

**a**, Electrical circuit diagram for the vector-valued circuit model described in the methods section. **b**, Wave digital flow diagram corresponding to the vector-valued circuit model. **c,d**, Emulation results for higher frequency variations exhibited by the deployed neuron model based on observation in *Hydra* hatchlings recorded under warm (23°C) conditions. **c**, Simulated calcium activity. **d**, Detected communities for a simulated nerve net with identical neuron parameters are highlighted by red squares. **Conducted, analyzed and implemented by Sebastian Jenderny.**

Figure S20: Immunohistochemistry of microbial impact – related to Figure 20



<https://tinyurl.com/5acxtddd>

Immunohistochemistry of hatchlings during Hatching stage against RFamide, related to the schematic overview at Figure 20j-l

Figure S21: 16S rRNA Sequencing

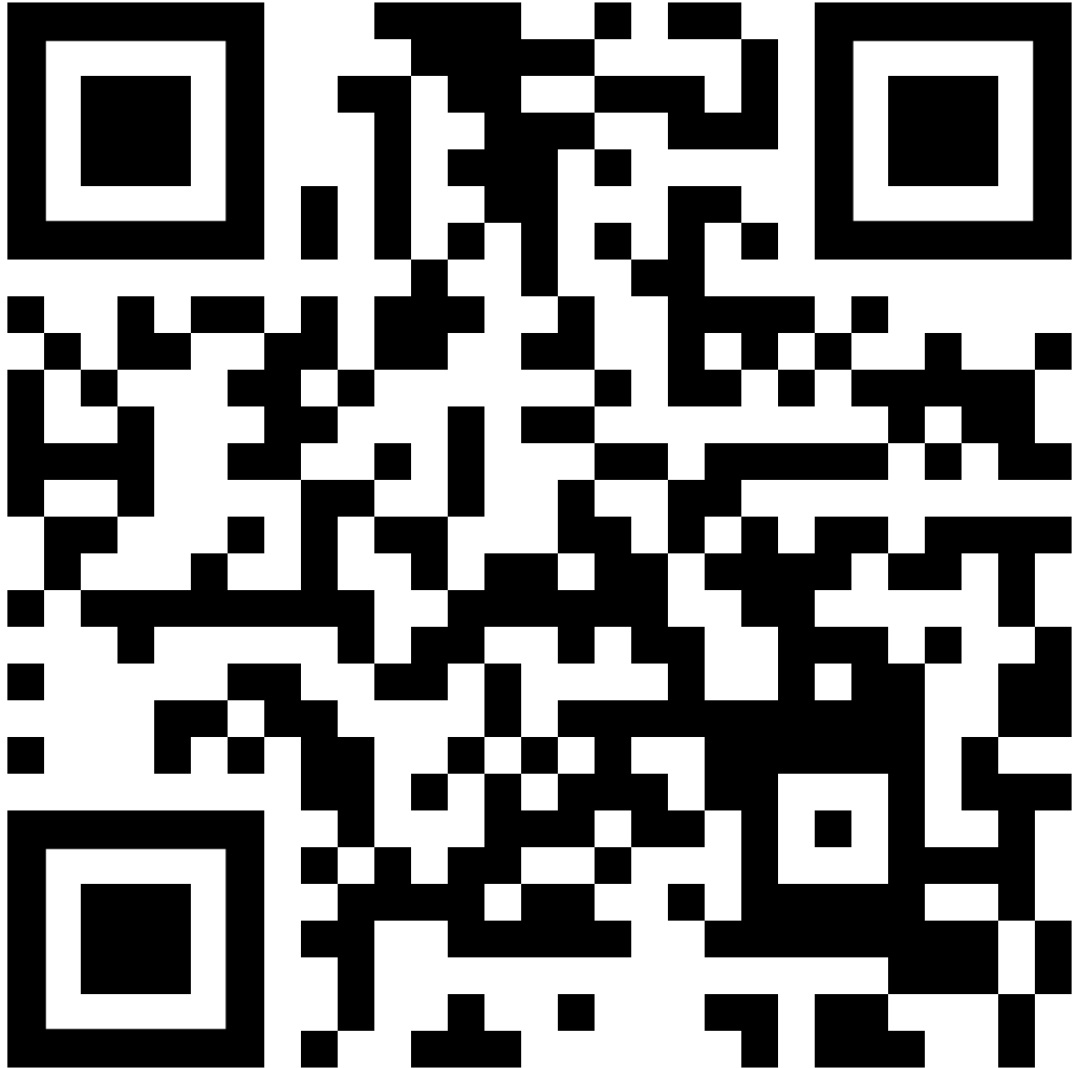


<https://tinyurl.com/4s4ds8a5>

16S rRNA analysis revealed differences in the composition of colonizing bacteria between adult polyps and embryos, despite both being collected from the same transgenic N4 culture. However, since the surrounding media was not analyzed, it remains unclear whether the microbial composition on embryos reflects that of the environment. Further experiments are needed to understand the interaction between *Hydra* embryos and their colonizing bacteria (Adult polyps: n = 4; embryos: n = 6). **(conducted and analyzed by Ornina Merza)**

## Videos

Video S1: Hatching of *Hydra* – related to Figure 7



<https://tinyurl.com/yubu8zyb>

Video S2: Calcium activity of the N4 network – Hatching stage – related to Figure

18



<https://tinyurl.com/p4b2at4x>

Video S3: Calcium activity of the N4 network – Cigar stage – related to Figure 18



<https://tinyurl.com/35m397nu>

Video S4: Calcium activity of the N4 network – Tentacle stage – related to Figure

18



<https://tinyurl.com/yw4cnc3w>



Video S5: Calcium activity of the N4 network – Adult polyp – related to Figure 18



<https://tinyurl.com/37e3w8zp>

## Abbreviations

BSA	Bovine serum albumin
cm	Centimeter
CNS	Central nervous system
d	Day(s)
dH <sub>2</sub> O	Distilled water
DMSO	Dimethyl sulfoxide
dpf	Days post fertilization
DVs	Dense-cored vesicles
e.g.	exempli gratia
ECM	Extracellular matrix
fps	Frames per second
fpm	Frames per minute
GFP	Green fluorescent protein
GSH	Glutathione
h	Hours
HM	Hydra media
hpf	Hours post fertilization
IgG	Immunoglobuline G
IgY	Immunoglobuline Y
LTD	Long-term depression
LTP	Long-term potentiation
mg	Milligramm
min	Minute
ml	Milliliters

mm	Milimeter
MO	Mouth opening
n	Number
nm	Nanometer
NMDA	N-methyl-D-aspartate
Nod	Nodding
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline + Tween
PFA	Paraformaldehyde
rpm	Revolutions per minute
RT	Room temperature
SFA	Spike frequency adaptation
TEM	Transmission electron microscopy
TM	Tentacle movement
μl	Microliter
μm	Micrometer
μM	MicroMol
°C	Celsius
%	Percent

## **Definition of terms used in this work**

### **Neuron**

A neuron is a specialized cell that has the ability to receive, process and transmit information through electrical and chemical signals. It is the primary building block and basic functional unit of the nervous system. Following criteria has to be fulfilled to call a cell a neuron: excitability, polarized structure, synaptic communication, expression of neuron-specific genes and proteins.

Neurons communicate via synapses and release neurotransmitters which influence the activity of connected neurons. Various types of neurons (e.g. sensory-, motor-, interneurons) are specialized for different functions within the nervous system. They are essential to all brain functions, from sensory perception to movement and higher cognition. Neurons are organized into circuits and networks that collectively drive complex behaviors and/or cognitive processes (Dale Purves et al., 2011; Hodgkin and Huxley, 1952; Kandel et al., 2000; Santiago Ramon y Cajal, 1894; Spitzer, 2006).

### **Ensemble**

An Ensemble is defined by a dynamic physical synaptic connectivity and refers to a group or population of neurons that collectively participates in a specific function or response. These neurons tend to be synchronized in their activity and work together to represent a specific piece of information, process a particular stimulus, or carry out a defined behavior.

Ensembles are often dynamic and emerge from the coordinated activity of neurons, meaning they can be composed of different neurons at different times depending on the task or environmental conditions. The concept of neural ensembles is critical for understanding how the brain encodes and represents complex information, as well as for memory, perception, and decision-making (Donald O. Hebb, 1949; Georgopoulos et al., 1986; Yuste, 2015b).

### **Circuit**

A neuronal circuit is defined by physical synaptic connectivity. It is a stable, anatomically connected group of neurons that communicate via synapses to process information and generate specific outputs. A neuronal circuit is fundamental to sensory perception, motor

coordination, emotional responses, and cognitive functions (Luo, 2020; Shepherd, 2004; Sporns, 2011).

### **Network**

A network refers to a group of neurons that are functionally and/or structurally interconnected, allowing information to flow between them to perform complex processing tasks. This interconnected system communicates to transmit information throughout the nervous system (Bassett and Sporns, 2017; Bullmore and Sporns, 2009; Sporns, 2011).

### **Nerve net**

A nerve net is a comparatively simple, decentralized network of neurons without a centralized brain. Neurons are diffusely connected and communicate directly to coordinate basic responses to environmental stimuli. They are primarily found in radially symmetrical animals such as Cnidarians (e.g., jellyfish, *Hydra*, sea anemones) and Ctenophores (comb jellies). Unlike the nervous system of bilaterian animals, nerve nets lack a clear hierarchical organization and typically allow a bidirectional signal transmission across neurons to enable simple reflexes and body movements (Mackie, 2004; Moroz, 2009; Satterlie, 2011; Technau and Steele, 2011).

### **Nervous system**

The nervous system is a specialized network of neurons and glia cells that enables the organism to perceive, process, and respond to internal and external stimuli through rapid electrical and chemical communication. It is divided into central nervous system (CNS), responsible for integrating information and generating behavior, and the peripheral nervous system, which is linking the CNS to limbs, organs, and tissues. Unlike a nerve net, the nervous system exhibits centralization and specialization, allowing for more complex behaviors, faster responses, and sophisticated processing (Bear et al., 2020; Dale Purves et al., 2011; Kandel et al., 2000).

### **Connection and connection strength**

A “connection” is defined as functional link between two neurons, followed by the correlation of their calcium activity over time. Here, connections represent rather functional associations based on temporal co-activity, than direct anatomical synapses or gap junctions. “Connection strength” is defined as the Pearson correlation coefficient between the calcium activity traces of two neurons. A higher correlation coefficient implies a stronger functional connection.

## **Declaration**

I, Christopher Noack, declare that:

Apart from my supervisor's guidance, the content and design of this thesis is all my own work.

The thesis has not already been submitted neither partially nor wholly as part of a doctoral degree to another examining body. Apart from the included published papers no other part of this thesis has been published nor submitted for publishing. Furthermore, I declare that I have not yet attempted a doctoral degree.

The thesis has been prepared subject to the Rules of Good Scientific Practice of the German Research Foundation (DFG) and apart from the listed reference no further resources have been used.

Signature:

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Christopher Noack