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Synaptic patterns for reliable circuit function require postembryonic maintenance by Jeb-Alk and normal network activity during embryogenesis

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Abstract

A functioning nervous systems results from complex developmental processes. One requirement is that Individual neurons need to form sufficient synaptic connections with adequate partners. Here, molecular signaling and neural activity control morphological development of axons and dendrites and synaptogenesis in order to establish and maintain stable networks. However, mechanisms maintaining stable postembryonic circuits are not well understood and the long-term effects of embryonic neural activity on neuronal morphology and connectivity are unkown. This thesis investigates trans-synaptic, anterograde Jelly-Belly-Anaplastic lymphoma kinase signaling in postembryonic circuit development and elucidates the establishment of synaptic patterns by embryonic neural activity in the motor circuit of *Drosophila* larva.

I demonstrate that Alk activity inhibits the formation of postsynaptic specializations on motoneurons during postembryonic circuit growth by analyzing single cell connectivity. I employ a new Bxb1 integrase-based technique for targeted mutations to show that presynaptic release site number of an upstream interneuron is unchanged but Jeb-Alk seems to elicit a negative feedback that limits the formation of presynaptic filopodia. These Jeb-Alk devoid circuits with altered synaptic patterns produce epilepsy-like seizure behavior. Additionally, *In vivo* time lapse imaging of dendrites reveals that dendritic growth and postsynaptic synaptogenesis are regulated independently and presynaptic filopodia likely promote dendritic elaboration. During embryogenesis, neural activity adjusts the establishment of synaptic patterns in motoneurons. In a picrotoxin-induced epilepsy-like model, dendritic growth is unaffected, but synaptic input is increased. The number of release sites of an upstream interneuron is again unaffected.

In summary, I identified cellular and molecular mechanisms required for the establishment and maintenance of synaptic patterns for reliable circuit function. With novel genetic and imaging techniques, I show embryonic neural activity is pivotal for the formation of functionally stable synaptic patterns, and establish Jeb-Alk signaling as a negative regulator of circuit expansion maintaining embryonically established connectivity. These developmental mechanisms highlight that balancing pre- and postsynaptic growth and synaptogenesis is central to stable network function.

Zusammenfassung

Ein funktionierendes Nervensystem ist das Ergebnis komplexer Entwicklungsprozesse. Eine Anforderung ist, dass einzelne Nervenzellen ausreichend synaptische Kontakte mit adäquaten Partnern bilden. Dabei steuern molekulare Signalwege und neuronale Aktivität die morphologische Entwicklung von Axonen und Dendriten sowie die Synaptogenese, um stabile Netzwerke aufzubauen und aufrechtzuerhalten. Die Mechanismen, die stabile postembryonale Schaltkreise aufrechterhalten, sind nur wenig verstanden, und die langfristigen Auswirkungen der embryonalen neuronalen Aktivität auf die Morphologie und Konnektivität der Neurone sind unbekannt. Diese Dissertation untersucht den trans-synaptischen, anterograden Signalweg Jelly-Belly-Anaplastische Lymphoma Kinase während der postembryonalen Entwicklung sowie die Etablierung synaptischer Muster durch embryonale Netzwerk- Aktivität im motorischen Schaltkreis der *Drosophila*-Larve.

Ich zeige, dass Alk-Aktivität die Bildung von postsynaptischen Spezialisierungen auf Motoneuronen während des Wachstums der postembryonischen Schaltkreise hemmt, indem ich die Einzelzellkonnektivität analysiere. Ich verwende eine neue, auf der Bxb1-Integrase basierende Technik für gezielte Mutationen, um zu zeigen, dass die Zahl der präsynaptischen Spezialisierungen eines vorgeschalteten Interneurons unverändert ist, aber Jeb-Alk scheint ein negatives Feedback hervorzurufen, das die Bildung präsynaptischer Filopodien limitiert. Schaltkreise ohne Jeb-Alk besitzen veränderte synaptische Mustern und erzeugen epilepsie-ähnliches Krampfanfälle. Zusätzlich zeigt *In vivo* Imaging von Dendriten, dass dendritisches Wachstum und postsynaptische Synaptogenese unabhängig voneinander reguliert werden und präsynaptische Filopodien wahrscheinlich die dendritische Elaboration fördern. Während der Embryogenese justiert neuronale Aktivität die Entstehung der synaptischer Muster in den Motoneuronen an. In einem durch Picrotoxin induziertem, epilepsie-ähnlichem Modell ist dendritisches Wachstum unverändert, aber der synaptische Input ist erhöht. Die Anzahl der präsynaptischen Spezialisierungen eines vorgeschalteten Interneurons ist wiederum unbeeinflusst.

Zusammenfassend habe ich zelluläre und molekulare Mechanismen identifiziert, die für die Entstehung und Erhaltung synaptischer Muster zur zuverlässigen Funktion der Schaltkreise erforderlich sind. Mit neuen genetischen und bildgebenden Verfahren konnte ich zeigen, dass neuronale Aktivität während der Embryogenese entscheidend

für die Bildung funktionell stabiler synaptischer Muster ist, und den Jeb-Alk Signalweg als negativen Regulator der Schaltkreiserweiterung einführen, der die embryonal entstandene Konnektivität erhält. Diese Entwicklungsmechanismen verdeutlichen, dass die Balance zwischen prä- und postsynaptischem Wachstum und Synaptogenese von zentraler Bedeutung für eine stabile Netzwerkfunktion ist.

Contents

Contributions

Over the course of this project several colleagues have contributed to the data presented within this thesis

Franz Bauer and Eunchan Lee have contributed data on the influence of picrotoxin during their bachelor theses.

Tatjana Kovacevic has contributed data on the influence of Jeb-Alk on postsynaptic synapse number.

Linda-Joel Manhart has performed the cloning of the dFLEx constructs for Alk, Jeb, Drep2 and Brp.

Aaron Ostrovsky has contributed to the R script used throughout this Thesis and designed the Bxb1-dependent dFLEx vectors.

Jan Felix Evers has helped with the collection of the data on axonal filopodia and larval body size under elav>AlkDN expression.

CHAPTER 1

Introduction

The brain is a highly complex organ containing millions of neurons in humans and over one hundred thousand neurons in adult fruit flies. Via specialized cell-cell connections, the so-called synapses, neurons are assembled into sophisticated circuits capable of sensing information, processing information, and making decisions to elicit behavior. In order to form functional circuits, neurons have to establish the adequate number of synapses with the right partners. For the formation of proper connections neurons are compartmentalized into dendrites and axons. Dendritic morphology determines what information a neuron receives (London and Michael, 2005) and how this information is processed, while axonal structure determines how the information is passed on (Ofer et al., 2017). In humans, misregulation neurodevelopmental processes leads to mental disorders or intellectual disabilities like autism (Mullins et al., 2016) or epilepsy (Bonansco and Fuenzalida, 2016). Thus, by increasing our understanding of the development and maintenance of neuronal connectivity, we can also increase our understanding of these pathologies and possibly open new possibilities for treatments. It is clear that the functionality of the central nervous system (CNS) highly correlates with animal's fitness. One defining characteristic of the nervous system is its ability to adapt and stay plastic. Established neural circuits are modified throughout an animal's life in order to adapt to a changing environment and organismal growth, or during the process of learning and memory formation. This plasticity is accompanied by a continuous maintenance of proper function. How plasticity and functional stability are balanced on a molecular level is important for our understanding of neuronal development and function. While the initial establishment of neuronal circuits has been studied in detail, we lack understanding of the pathways and mechanisms coordinating circuit stability and expansion during organismal growth. Furthermore, it is largely unclear to what degree neuronal activity during circuit formation affects the development of

individual cells long-term. Lastly, how do molecular mechanisms of maintenance and activity-dependent circuit development work together to create a functional and resilient neuronal network?
The aim of this thesis is to analyze the factors regulating neuronal connectivity during postembryonic circuit expansion and organismal growth. Using the central motor system of *Drosophila melanogaster* larvae, I analyzed the specific connectivity of identified synaptic partners. With the anterograde, trans-synaptic Jelly belly Anaplastic lymphoma kinase signaling I identified a molecular mechanism that limits the postembryonic expansion of neuronal circuits and as such maintains circuit stability. Furthermore, I provide the first quantitative analysis on how embryonic neural activity levels regulates connectivity and influences the architecture of in the motor circuit of the CNS.

1.1 Development of connectivity of the central nervous system

The nervous system is a highly complex organ characterized by its high degree of cellular connectivity and the sensitive processes it controls. Hence, the formation of the CNS is tightly regulated throughout development and occurs in a defined order of developmental steps including cell specification, axon guidance, dendritic growth and synaptogenesis. Neuroscientists have extensively described the development of the CNS across animal phyla (Sanes et al., 2019). The fruit fly *Drosophila melanogaster* has served as a model for neuroscientists due to its limited size but sufficient complexity (Bellen et al., 2010), especially for studying neurodevelopment in detail (more in section 1.8). In what follows I briefly recapitulate, the embryonic development of the *Drosophila* CNS, which has been characterized thoroughly over the last 50 years (Hartenstein and Campos-Ortega, 1984; Hartenstein et al., 1987; Truman and Bate, 1988).

1.1.1 Formation of the CNS during embryogenesis

During the 21 hours of embryogenesis, from fertilization of the egg to hatching of the larva, a functional nervous system forms from the ectoderm germ layer. At stage nine of embryonic development, around six hours after egg laying (AEL), neuroblasts, insect specific stem-cell-like progenitors, delaminate from the ectoderm to form the neuroectoderm. These cells and their daughter cells will later differentiate into the larval CNS. During differentiation, a neuroblast undergoes asymmetric mitotic divisions to regenerate a neuroblast and a ganglion mother cell. In most cases the ganglion mother cell then undergoes relatively symmetric division, yielding two neurons or one neuron

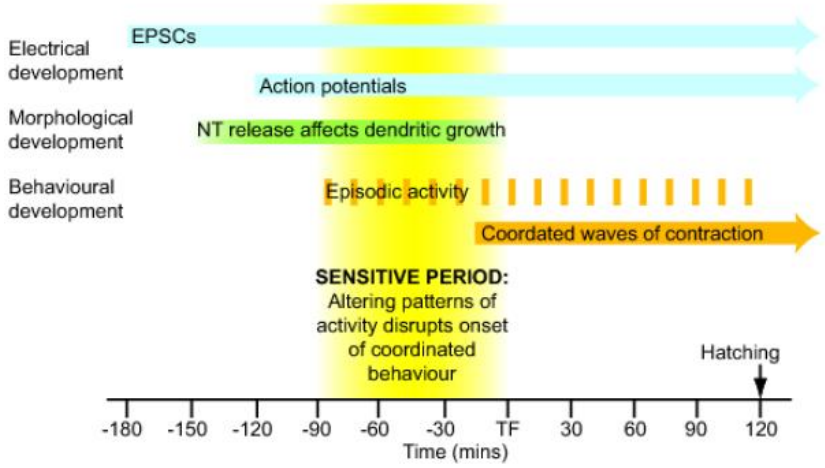

FIGURE 1.1. **Embryonic motor system development.** The graph indicates electrical, behavioral and morphological development during embryogenesis. Currents in the motor system can be recorded as early as 5 hours before hatching. Coordinated activity occurs two hours before hatching but can be delayed by altered activity during a sensitive period in development. Dendritic development is also influenced by neurotransmitter release during this period of neurodevelopment. NT - neurotransmitter, EPSCs - excitatory postsynaptic currents. (Crisp et al., 2011)

and one glial cell. Neuroblasts have been uniquely identified and are conserved across animals, and each neuroblast produces an identifiable lineage as it undergoes multiple rounds of division during embryogenesis (Doe, 1992; Bossing et al., 1996; Schmid et al., 1999). These neuronal cells stay in spatial proximity and will fasciculate together, a paradigm that holds true also for adult neurogenesis (Hartenstein and Campos-Ortega, 1984; Pang and Clandinin, 2018). Neuronal differentiation starts at embryonic stage 13, approximately ten hours AEL. At this time the CNS also condenses revealing its distinct morphology consisting of the two brain lobes and the ventral nerve chord (VNC). After neuronal axons start to grow from the cell body towards their intended target area, the formation of dendrites is first observed during early stage 17 of embryonic development at 15 hours AEL (Landgraf et al., 2003; Brody, 1999). Subsequently, axons and dendrites locate within the inner volume of the tissue, called the neuropil, where intercellular contacts form. Cell bodies remain on the outside of the neuropil to form the cortex. Once cellular protrusions ramify within the neuropil, synapses are established and first action potentials in the motor system can be detected about four hours before larval hatching at 17 hours AEL, (figure 1.1 light blue bars) (Crisp et al., 2008).

1.2 Elaboration of cellular morphology and establishment of synaptic connections

Within the CNS distinct domains form during development. First, the brain lobes are structurally and functionally different from VNC. The VNC, consisting of three thoracic segments and nine abdominal segments, is characterized by a segmentally repeated structure, with mostly similar neuronal subpopulations in equitable arrangement. Each abdominal hemisegment contains the same set of approximately 300 neurons (36 motor neurons plus around 270 uniquely identifiable interneurons) (Landgraf et al., 2003; Rickert et al., 2011). The neuropil of the VNC contains mostly sensory circuits in the ventral regions, while neurons responsible for the regulation of movements reside to a large degree in the dorsal motorneuropil. The latter is the target of this study, as I focused on identified pairs of synaptic partners within the locomotor circuit (details in section 1.8).

For the motor system, it has been shown that motoneurons grow their dendrites quite stereotypically into the neuropil of the VNC and these dendritic arbors form a myotopic map representing the relative arrangement of the target muscles (Landgraf et al., 2003). The proper topographical localization of dendrites and axons is regulated through pathfinding cues: Targeting of motoneuron dendrites is regulated by the midline signaling systems Slit-Robo and Netrin-Frazzled. Both pathways also regulate axonal pathfinding together with other signals together with other signals (e.g. Semaphorin, Plexin, Dscam) (Mauss et al., 2009; Howard et al., 2019).

Proper targeting is a prerequisite for circuit formation. After cells reach their target territory outgrowing dendrites and axons allow for contact with and selection of synaptic partners. During this period, dendritic and axonal arbors are characterized by a high density of filopodia (Gallo, 2013). Filopodia have been described across different invertebrate and vertebrate phyla (Niell et al., 2004; Meyer and Smith, 2006; Sheng et al., 2018; Kanjhan et al., 2016). These cellular protrusions are characterized by very dynamic outgrowth and retraction, which increases the chance of contact between axons and dendrites. Consequently, they can be seen as tools of exploration for the neuron that serve to sample the environment for synaptic partners. Accordingly, increased dynamics in filopodial growth have been correlated with synaptogenesis (Gallo, 2013; Sheng et al., 2018). Once contact between pre- and postsynaptic membranes is made the formation of synapses can ensue (Menna et al., 2011). In vertebrates, postsynaptic filopodia can induce the accumulation of presynaptic proteins and thus induce functional synaptogenesis (Ziv and Smith, 1996). Filopodia can further serve as precursors of dendritic or axonal branches and synaptogenesis is capable of stabilizing these cellular protrusions (Niell et al., 2004; Heimann and Shaham, 2011). A study in *Xenopus* has suggested that dynamic dendrites and axonal filopodia have different

roles in synaptogenesis: immature dendrites are denser in synaptic contacts, while stable axonal branches carry synapses rather than dynamic, axonal filopodia (Li et al., 2011). Hence, it remains unclear, how dynamic axonal filopodia contribute to synaptic connectivity, when they do not correlate with axonal growth.

Regarding the molecular assembly and development of synapses, the clearly defined structure of the *Drosophila* larval neuromuscular junction (NMJ) has served as a model for decades (Jan and Jan, 1976; Salkoff and Kelly, 1978). Especially presynaptic structures in the axonal boutons have been analyzed in detail (Ghelani et al., 2018). Several proteins and their localization within this well-studied presynaptic complex have been described of which some can also be found in synapses within the CNS (Ghelani et al., 2018). Among them, the scaffolding protein Bruchpilot (Brp), a CAST/ERC family member, is a central protein at presynaptic release sites. It makes up and can be found at all synapses in the periphery and the CNS (Wagh et al., 2006; Kittel et al., 2006). Therefore, various tagged versions have been developed to be used as synaptic markers, and recently endogenous, conditionally tagged brp alleles were established by the Evers lab (Manhart, 2019; Gärtig et al., 2019).

We know much less of the composition of postsynaptic specializations. Neurotransmitter receptors and ion channels have been identified, but reliable, global markers of postsynaptic specializations in *Drosophila* are less established. Consequently, tools for studying central neuron-neuron synapses in *Drosophila* have been missing largely. Studies have very recently used endogenously labeled alleles of Dopamine receptors (Kondo et al., 2020) and the two potassium ion channel Shaker and ShaI (Gür et al., 2020). DNA fragmentation factor related protein 2 (Drep2) has previously been identified to be postsynaptically localized in Kenyon cells (Andlauer et al., 2014; Hussain et al., 2018). The molecular function of Drep2 has not yet been identified, but olfactory learning is reduced in mutant animals (Andlauer et al., 2014). These studies employed a Drep2 antibody or overexpressed a GFP-tagged allele. In this thesis, I demonstrate the reliability of an endogenously, conditionally tagged Drep2 construct as a marker for cholinergic postsynaptic specializations in the larval CNS.

1.3 Maintenance of neural circuits after initial assembly

After initial circuit assembly, there is a period where connections are being revised. One important process is the selective pruning or removal of contacts and neurite structures (Stoneham et al., 2010; Tessier and Broadie, 2009). Unneeded or obsolete connections can be removed in an activity-dependent process, which possibly optimizes the circuitry by improving the signal-to-noise ratio. Subsequently, established circuits need to function reliably, but also retain flexibility. Adjustments of circuitry to environmental

requirements are crucial for animal fitness, one well known example being learning and memory formation. Additionally, the nervous system has to adjust with growing body size. The molecular mechanisms regulating the adjustments of neuronal circuits during the massive growth in larval development are not well understood. During this process it is necessary that neuronal growth is coordinated with organismal growth to ensure continuing function. For example, larger muscles need more excitation to induce muscle contraction. In this context, size of larval NMJ scales with larval growth (Schuster et al., 1996). The dendrites of motoneurons located in the CNS scale accordingly and also increase their synaptic drive (Zwart et al., 2013) as well as their synaptic contacts with an identified presynaptic interneuron scale (Couton et al., 2015).
In the sensory system, manipulation of Tao kinase activity regulates both the dendritic expansion of class IV dendritic arborization neurons in the body wall (Hu et al., 2020) as well as their connectivity to interneurons in the CNS (Tenedini et al., 2019). In both cases, manipulations of the molecular pathways change functional output of the circuits likely through changes in connectivity. In the motor circuit, one identified regulator of neuronal growth with organismal growth is a receptor of the steroid hormone ecdysone, EcR-B2 (Zwart et al., 2013). EcR-B2 is cell-autonomously responsible for any postembryonic dendrite expansion, as expression of a dominant negative allele halts growth after hatching. However, it is unclear what molecular signaling mechanisms orchestrate adequate dendritic scaling on a finer scale. Furthermore, mechanisms that coordinate growth of pre- and postsynaptic neurons as reported by Couton et al. (2015) and scaling of their synaptic connections have not been identified yet. This thesis focuses on these topics with the aim to elucidate the coordinated growth of synaptic partners and its role in the maintenance of adequate connectivity during circuit expansion.

1.4 Interneuronal signaling pathways regulating neuronal development

Above I described the intricate development of neuronal circuits. This development is to a large extent regulated by intercellular signaling. Molecular, intercellular signaling is needed to establish synaptic connectivity. Synaptic partners present extra-cellular molecules or release ligands that can be recognized by receptors on the other side of the synapse. Studies focusing on the early phases of circuit formation or on the synapses at the NMJ described several mechanisms of trans-synaptic pathways regulating the development of synaptic partners (Wu et al., 2010; Harrison and Perrimon, 1993).
A major component of the intercellular communication between synaptic partners are cell adhesion molecules (CAM) (Schwabe et al., 2014). CAMs play a unique role in the coordination of cellular development. Some are directly linked to the cytoskeleton,

which is essential in filopodial growth and stabilization as well as structural assembly of synaptic contacts (Togashi et al., 2009; Tallafuss et al., 2010). The synaptogenic function of N-cadherins (Arikkath and Reichardt, 2008), leucine-rich repeat transmembrane protein (LRRTM) (Linhoff et al., 2009; Wit et al., 2009; Schroeder and Wit, 2018), Nectins (Mizoguchi et al., 2002) and neurexin-neuroligin (Chih et al., 2005; Graf et al., 2004) has been demonstrated in vertebrate models but also in *Drosophila* most prominently at the larval NMJ (Sun et al., 2011; Carrero-Martínez and Chiba, 2009). In adult *Drosophila*, neurexin-neuroligin adhesion complexes promote the arborization of motoneuron axons independent of neuronal activity (Constance et al., 2018). Another CAM regulating neuronal development in *Drosophila* is Dscam1, a homologue of human Down syndrome cell adhesion molecule (DSCAM), which regulates dendritic branching via self-avoidance and tiling in sensory dendritic arborization neurons in the larval body wall (Soba et al., 2007; Matthews et al., 2007) and is essential for dendritic growth of adult flight motoneurons (Hutchinson et al., 2014). DSCAMs also regulate pre- and postsynaptic connectivity in the visual system of frogs (Santos et al., 2018). Finally, CAMs orchestrate the specification of connectivity in certain circuits as demonstrated by the array of genes involved in the synaptic specification in the visual system of adult *Drosophila* (Pecot et al., 2013; Berger-Müller et al., 2013; Millard and Pecot, 2018). Taken together several CAMs work together to promote adequate neuronal development based on direct contact of cell surfaces.

In addition to CAMs, trans-synaptic signaling via secreted ligands orchestrates pre- and postsynaptic development. A major role has been attributed to retrograde signaling pathways. In retrograde signaling, ligands released from the postsynaptic cell, for example the muscle at the NMJ, activate receptors in the presynaptic compartment to regulate presynaptic development.

In vertebrates, neurotrophins are major regulators of neuronal development (Harris and Littleton, 2015). This family consists of the secreted ligands nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurottrophin-3 and -4/5, which bind to their receptors, tropomyosin kinase receptors (Trk). Neurotrophins regulate various aspects of neuronal development including neuronal survival, synaptic targeting or synaptic plasticity and are classically understood as growth promoting factors (Harris and Littleton, 2015; McAllister et al., 1999; Gómez-Palacio-Schjetnan and Escobar, 2013). Interneuronal BDNF signaling affects axonal as well as dendritic arborization and synaptogenesis on either side of the synapse (Inoue and Sanes, 1997; Sanchez et al., 2006; Hu et al., 2005; McAllister et al., 1997; Causing et al., 1997). In *Drosophila*, neurotrophins were described a few years later and are less prominent. However, Neurotrophin signaling through Toll receptors supports neuronal survival and axon targeting in the CNS (Zhu et al., 2008; Mcilroy et al., 2013) and promotes synaptic growth at the NMJ (Ballard et al., 2014). More common and complex than *Drosophila* neurotrophin signaling is the evolutionary conserved transforming growth factor beta (TGF-β) signaling (Meyers

and Kessler, 2017); both subfamilies of TGF-β signaling, bone morphogenetic protein (BMP) and Activin signaling, are involved in neurodevelopmental processes (Upadhyay et al., 2017). Mutation of the ligand and BMP 4/5/6 homologue glass bottom boat (Gbb) or one of its receptors disrupts presynaptic structure leading to larval NMJ undergrowth and impairs neurotransmitter release. Furthermore, Gbb intersects with activity-dependent mechanisms as knockdown suppresses activity-dependent growth plasticity (Berke et al., 2013; Piccioli and Littleton, 2014; Berke et al., 2020). Gbb is also required to strengthen synapses in the central motor circuit (Baines, 2004). Gbb acts in a retrograde fashion and regulates gene expression of motoneurons (McCabe et al., 2003; Vuilleumier et al., 2019).

On the other hand, anterograde Activin signaling regulates GluRIIA un GluRIIB receptor accumulation in the muscle at the larval NMJ (Kim and Connor, 2014). Activin further regulates neuronal proliferation in the larval brain (Zhu et al., 2008) and axon targeting of larval motoneurons (Serpe and Connor, 2006) as well as photoreceptor axons in the adult visual system (Ting et al., 2007). In the visual system it further restricts dendritic growth of first order interneurons preventing formation of aberrant synapses (Ting et al., 2014). This process is antagonized by growth promotion from anterograde insulin like peptide signaling (DILP2) via insulin receptor (Luo et al., 2020). Activin signaling has further a neuroendocrine function in the regulation of the transition between developmental stages (Gibbens et al., 2011) and induces expression of ecdysone receptor B1 to promote pruning during metamorphosis (Zheng et al., 2003).

Another anterograde signaling pathway is Wnt signaling. Motoneurons at the NMJ secrete Wnt ligands that regulate both pre- and postsynaptic differentiation (Koles and Budnik, 2012). Wnt signaling likely also acts autrocrine, and lack of the Wnt wingless (Wg) leads to disruption of NMJ development measurable as a reduced number of synaptic boutons.

Neuronal growth in *Drosophila* is further regulated by the steroid hormone ecdysone. Ecdysone is essential developmental progression of larval stages and is involved in the remodeling of various tissues, including the pruning neurons during metamorphosis (Schubiger et al., 1998; Truman, 1996). The ecdysone receptor isoform B1 regulates dendritic growth in sensory neurons (McParland et al., 2015), dendrite pruning during metamorphosis (Zheng et al., 2003) and arbor morphology across several neuroblast lineages in the adult CNS (Brown and Truman, 2009). Isoform B2 is essential for the postembryonic elaboration of the dendritic arbor of RP2 motoneuron (RP2) in the VNC) (Zwart et al., 2013). As such it is the only known regulator crucial for the scaling of dendritic arbors in the CNS with organismal growth.

In conclusion, a wide array of trans-synaptic signaling mechanisms orchestrate the development of neurons in the CNS reflecting the complexity of the organ and its function. The majority of these pathways act synaptogenic and growth promoting. Additionally, synaptic signaling is more often than not retrograde and the above mentioned studies

lack in different regards. (1) Most vertebrate studies are conducted *in vitro* omitting the developmental and tissue dependent mechanisms. (2) Studies in *Drosophila* focus - mostly due to methodological simplification - on the neuron-muscle connection at the NMJ, where the simple two-cell situation does not reflect the complexity found in the densely packed neuropil of the CNS. Or, (3) studies in the CNS focused on early phases of circuit formation, for example embryos or the formation of the visual system in pupae. The latter is a relatively well spatially organized and structured tissue. Therefore, we lack an understanding of neuronal development during the stages of development, where neuronal growth and connectivity scale with organismal growth (Zwart et al., 2013; Couton et al., 2015; Gerhard et al., 2017) especially in a less strictly organized volume of the CNS.
On the other hand, only two studies demonstrate mechanisms of postembryonic synaptic development using electrophysiology. First, the CAM Fascillin II (Fas II) regulates specifically postembryonic development of synaptic connectivity in the larval CNS (Baines et al., 2002). Here, synaptic connectivity at 48h ALH but not before is dependent on Fas II levels. Furthermore, retrograde BMP signaling via the ligand Gbb strengthens synaptic contacts as measured by increased invoked synaptic currents (Baines, 2004). These studies limited their analysis to electrophysiological measurements. However, detailed anatomical analysis regarding cell morphology or synaptic structure were not provided. This thesis aims to close this gap by focusing on postembryonic neuronal development after the initial establishment of circuits, when synapses have already formed and circuits expand, and by analyzing cellular connectivity on at nanometer resolution and dendritic growth dynamics with live imaging.

1.4.1 ALK signaling during neurodevelopment is evolutionary conserved

During the last fifteen years the relevance of the oncogene Anaplastic lymphoma kinase (ALK) for neuronal development was discovered (Palmer et al., 2009; Hallberg and Palmer, 2013). ALK is a receptor tyrosine kinase (RTK) that was first identified as part of a gene fusion in anaplastic large-cell non-Hodgkins lymphoma (Morris et al., 1997). Since then, the ALK-related malfunctions have been described to occur within various types of cancers including neuroblastoma and non-small cell lung cancer (Miyake et al., 2002; Hallberg and Palmer, 2013; Toyokawa and Seto, 2014; Umapathy et al., 2019). Research over the last twenty years stressed the relevance of ALK signaling in cancer biology leading to therapeutic applications targeting ALK (Hallberg and Palmer, 2013). Additionally, research has focused on defining the developmental role of ALK signaling (figure 1.2). This thesis focuses on *Drosophila* anaplastic lymphoma kinase (Alk) and Jelly belly (Jeb) signaling in the CNS as a regulator of circuit maintenance in regards

to connectivity and thus its role in stabilizing postembryonic, expanding circuits.
ALK is a highly conserved RTK and orthologues have been found in the invertebrates *C. elegans* (Ishihara et al., 2002; Liao et al., 2004), *D. melanogaster* (Loren et al., 2001; Weiss et al., 2001) and across different vertebrate model systems, such as chicks (Hurley et al., 2006), zebrafish (Yao et al., 2013) and mice (Iwahara et al., 1997; Vernersson et al., 2006). Closely related to ALK is the Leukocyte receptor tyrosine kinase (LTK) group of receptors and they have thus been grouped together in the ALK/LTK RTK family (Morris et al., 1997; Palmer et al., 2009). LTKs can be found in vertebrates, but ALK is the only member of the ALK/LTK family found in C. elegans (SCD-2, suppressor of constitutive dauer formation 2) and *Drosophila* (Alk) (Hallberg and Palmer, 2013). The structure of ALK is very similar across these species consisting of an intracellular domain with the insulin receptor like kinase domain and the extra-cellular domain containing two MAM domains and one type-A LDL receptor repeat (Weiss et al., 2012). Upon ligand binding ALK dimerizes and is activated through autophosphorylation within the intra-cellular domains (Hallberg and Palmer, 2013). The evolutionary conservation of ALK/LTK family receptors imply the relevance and applicability of research focusing on invertebrate models for these RTKs.
Regarding the activation of ALK various potential ligands have been mentioned since the discovery of ALK. Most commonly, midkines (MK) (Stoica et al., 2002; Reiff et al., 2011; Nagashima et al., 2019) and pleitrophins (PTN) (Stoica et al., 2001) have been assumed to activate LTKs and ALK in vertebrates (Bilsland et al., 2008). However, these results could not be reproduced in other studies (Mathivet et al., 2007; Moog-Lutz et al., 2005). More recently, studies described FAM150A and FAM150B as activating ligands for LTK and ALKin vertebrates (Zhang et al., 2014; Reshetnyak et al., 2015; Guan et al., 2015). These have since been renamed to ALK and LTK ligands (ALKALs) and their evolutionary conservation was demonstrated as human and zebrafish ALKALs are capable of activating zebrafish Ltk as well as human ALK (Fadeev et al., 2018).
The identified activating ligands of the ALK in *C. elegans*, called HEN-1 (hesitation behavior 1) (Ishihara et al., 2002), and *Drosophila*, called Jelly Belly (Jeb) (Lorén et al., 2003; Englund et al., 2003) (see figure 1.2), which are structurally related as both contain low-density lipoprotein (LDL domain), differ significantly in their sequence from MK, PTN or FAM150A and B. Interestingly, the *Drosophila* MK/PTN homologues Miple 1 and 2 are dispensable for Alk signaling in the visceral mesoderm despite miple2 mRNA being expressed there (Englund et al., 2006; Hugosson et al., 2014). While miple1 mRNA is also expressed in the larval CNS, no studies have addressed its function there.
Across vertebrate model systems, ALK and the suggested ligands are expressed in the CNS and regulate neurodevelopmental processes such as neuronal differentiation (Fadeev et al., 2018), glial differentiation (Nagashima et al., 2019), neurite outgrowth, and cell growth (Motegi et al., 2004). In invertebrates, a role of ALK signaling in the

development of neurons and synapses has been suggested (Ishihara et al., 2002; Liao et al., 2004; Kitazono et al., 2017; Bazigou et al., 2007; Gouzi et al., 2011; Rohrbough et al., 2013b; Gouzi et al., 2018). More specifically, HEN-1 is a component important for sensory integration and behavioral plasticity in *C. elegans* (Ishihara et al., 2002). The ligand likely acts synaptically and the ALK homologue receptor, SCD-2, can be found at presynaptic active zones (Liao et al., 2004). Moreover, mutation of SCD-2 in *C. elegans* reduces associative learning (Wolfe et al., 2019) and its activation by HEN-1 facilitates olfactory forgetting (Kitazono et al., 2017).
In *Drosophila*, the importance of Jeb-Alk signaling has first been described in mesodermal development (Weiss et al., 2001; Loren et al., 2001). Activation of Alk by Jeb is essential for the development of the visceral mesoderm during midgut formation (Lorén et al., 2003; Englund et al., 2003). Jeb-Alk is crucial for visceral musculature differentiation and as this tissue lines the intestine of the animal, mutation of Alk or Jeb results in failure of proper gut formation. Consequently, animals die shortly after hatching. In this developmental process activation of Alk leads to stimulation of the MAPK/ERK pathway, for which the scaffolding Cnk (connector enhancer of kinase suppressor of Ras) is essential (Wolfstetter et al., 2017). Subsequently, downstream transcription of target genes like Duf (dumb-founded)/Kirre (kin of irregular chiasm) (Lee et al., 2003; Varshney and Palmer, 2006), Org-1 (Lee et al., 2003), Hand (Varshney and Palmer, 2006), Dpp (decapentaplegic, Shirinian et al. (2007)) and the transcription factor Lmd (lame duck, Popichenko et al. (2013) is induced.
Dpp is a ligand of the TGF-β subfamily BMP (section 1.4). Therefore, the regulation of Dpp signaling by Alk demonstrates linkage of TGF-β and Jeb-Alk signaling. Similarly, it was also demonstrated in *C. elegans*, that SCD-2 activation influences TGF-β signaling to regulate dauer phase initiation (Reiner et al., 2008). Taken together this supports another similarity between Alk signaling in these two invertebrates.

1.4.1.1 Alk function in the central nervous system

A large part of this thesis focuses on the relevance of Alk activation by Jeb for the development of neuronal morphology and connectivity in the CNS. Therefore, this section is dedicated to summarize the current status of research on the role of ALK signaling in the nervous system across animal phyla but with a focus on *Drosophila* studies.
In the CNS of larval *Drosophila*, Jeb-Alk signaling has been implied in sparing neuroblast growth during nutrient restriction suggesting action of Alk in neuroblasts in late (after 60 h ALH) larval life (Cheng et al., 2011a). In a similar context, secretion of Jeb by cholinergic neurons and Alk activation in the neuroendocrine insulin-producing cells seems to regulate the expression of Insulin-like peptide 5 (dilp5), further linking

nutrient sensing and body growth with Jeb-Alk signaling (Okamoto and Nishimura, 2015). Together these finding suggest (1) Alk expression in non-neuronal cells of the CNS and (2) Jeb secretion by cholinergic neurons in *Drosophila*.

Furthermore, studies have demonstrated the significance of anterograde Jeb-Alk signaling at glutamatergic synapses of the larval NMJ where it negatively regulates synaptic coupling strength Rohrbough and Broadie (2010)). As such Jeb-Alk signaling is the only described negative regulator of synaptic coupling at the NMJ (Rohrbough and Broadie, 2010). Rohrbough and colleagues further showed that while Jeb-Alk affects neurotransmission early, effects of missing Jeb-Alk signaling in NMJ morphology are solely postembryonic and only measureable in late larval life (Rohrbough et al., 2013b). Lastly, they suggest that Jeb-Alk acts through Ras/MAPK/Erk signaling as it does during gut development (Englund et al., 2003; Lee et al., 2003). In Jeb or Alk mutants, the level of phosphorylated Erk in the CNS is reduced and Jeb function at the NMJ depends on the secreted protein Mind the gap (Mtg) (Rohrbough et al., 2013b; Rohrbough and Broadie, 2010). Interestingly, in *C.elegans*, HEN-1 acts through SCD-2 at the NMJ to regulate presynaptic differentiation and NMJ morphology demonstrating a similar synaptic function of the ALK family from worms to flies (Liao et al., 2004) (figure 1.2).

In *Drosophila*, neuronal Jeb-Alk signaling is also regulated by heparin sulfate glycans (HSPG, Friedman et al. (2013)). In a *Drosophila* model for the cognitive disorder Fragile X syndrome HSPG expression is elevated, which reduces Jeb-Alk signaling. This effect can be rescued by restoring physiological levels of HSPG (Friedman et al., 2013). Conclusively, this study provides a link of Jeb-Alk to altered neurotransmission levels in a disease model with clinical relevance.

In the adult *Drosophila* CNS, Jeb is secreted at axon terminals and Alk locates postsynaptically (Bazigou et al., 2007). In visual system anterograde Jeb-Alk signaling is crucial for circuit assembly: Jeb is secreted by photoreceptor axons and controls their morphology and thus target selection (Bazigou et al., 2007). Consequently, R8 axons overgrow past their intended target or into neighboring columns when Jeb is absent from presynaptic R-cells or Alk is missing in target neurons in the medulla. Later, it was shown that Jeb induced Alk activation in budding dendrites is crucial for survival of L3 neurons specifically and targeting defect of R-call axons are more likely due to the missing of L3 neurons axon terminals in the medulla (Pecot et al., 2014). Thus, Jeb-Alk acts anti-apoptopic in the adult visual system and regulates circuit connectivity through cell survival.

Additionally, Alk / ALK was shown to be relevant for the regulation of the response to ethanol in flies, mice, and also humans (Lasek et al., 2011; He et al., 2015; Schweitzer et al., 2016). Human polymorphism at the ALK locus has even been suggested to correlate with sensitivity to ethanol (Mangieri et al., 2017). Furthermore, Alk acts in *Drosophila* to negatively regulate sleep (Bai and Sehgal, 2015). The ethanol- and

sleep-related phenotypes of Alk correlate with its localization within the mushroom body of adults flies, that is (among other functions) central to these behaviors. These studies demonstrate the importance of Jeb-Alk in the regulation of complex behaviors, which are also plasticity-related processes within the CNS.
The most relevant function of ALK signaling in the context of this thesis is related to the plasticity of the CNS taking place during learning and memory. Across various model organisms ALK orthologues are involved memory and learning processes (Gouzi et al., 2011, 2018; Weiss et al., 2012, 2017; Kitazono et al., 2017; Wolfe et al., 2019). In *Drosophila*, olfactory associative learning is negatively regulated by Alk activity as over-activation decreases memory performance, and inhibition of Alk in adults enhances learning (Gouzi et al., 2011)). More specifically, Alk activity in a subset of neurons in the mushroom body (α-, β- lobes) is necessary to specifically regulate protein synthesis dependent long-term memory (Gouzi et al., 2018). Similarly, ALK knock-out mice show improved memory performance in water maze tests (Weiss et al., 2012) and associative learning in *C. elegans* is regulated by SCD-2 (Wolfe et al., 2019).
Studies in flies further demonstrate that acute function of Alk in the adult influences learning performance rather than simply developmental disturbances (Gouzi et al., 2018). In the adult, Alk localizes within the dendrites of Kenyon cells in the mushroom body calyx directly at the synaptic microglumeruli. Upon olfactory conditioning Alk expression increases, further underlining the relevance of acute Alk activity and suggesting a local synthesis of Alk (Gouzi et al., 2018).

In mice, knock-out of ALK is sufficient to rescue decreased memory performance in Neurofibromatosis type 1 (NF1) models (Neurofibromin 1 (Nf1) mutants) (Weiss et al., 2017). Interestingly, a similar counteractive mechanism of Alk to Nf1 has been demonstrated in *Drosophila*, measured as animal body size. Here, chemical inhibition of Alk rescues the smaller body size phenotype of Nf1 mutants (Gouzi et al., 2011). A likely link of Nf1 and Alk is the Ras/MAPK/Erk signaling pathway: Inhibition of Alk restores the elevated levels of phosphorylated Erk under reduced Nf1 levels to a near physiological level (Gouzi et al., 2011). Thus, Nf1 and Alk counteract each other regarding the activation of Ras/MAPK/Erk signaling, meaning Alk is a negative regulator of Nf1-dependent Ras/MAPK/Erk activation. Lastly, Nf1 and Alk have further been linked by their direct colocalization within the mushroom body (Gouzi et al., 2011) and their effects on circadian activity in mice (Weiss et al., 2017).
The link of Nf1 and ALK is relevant regarding the medical implications of ALK signaling beyond cancer research. Neurofibromatisis 1 is a genetic disorder that affects the nervous system and is characterized by a high occurrence of tumors within the nervous system (Brown et al., 2013). Additionally, patients show cognitive impairments and exhibit a higher prevalence of seizures and autism spectrum disorders (Santoro et al., 2018; Eijk et al., 2018). Hence research suggests that mutation of Nf1 greatly

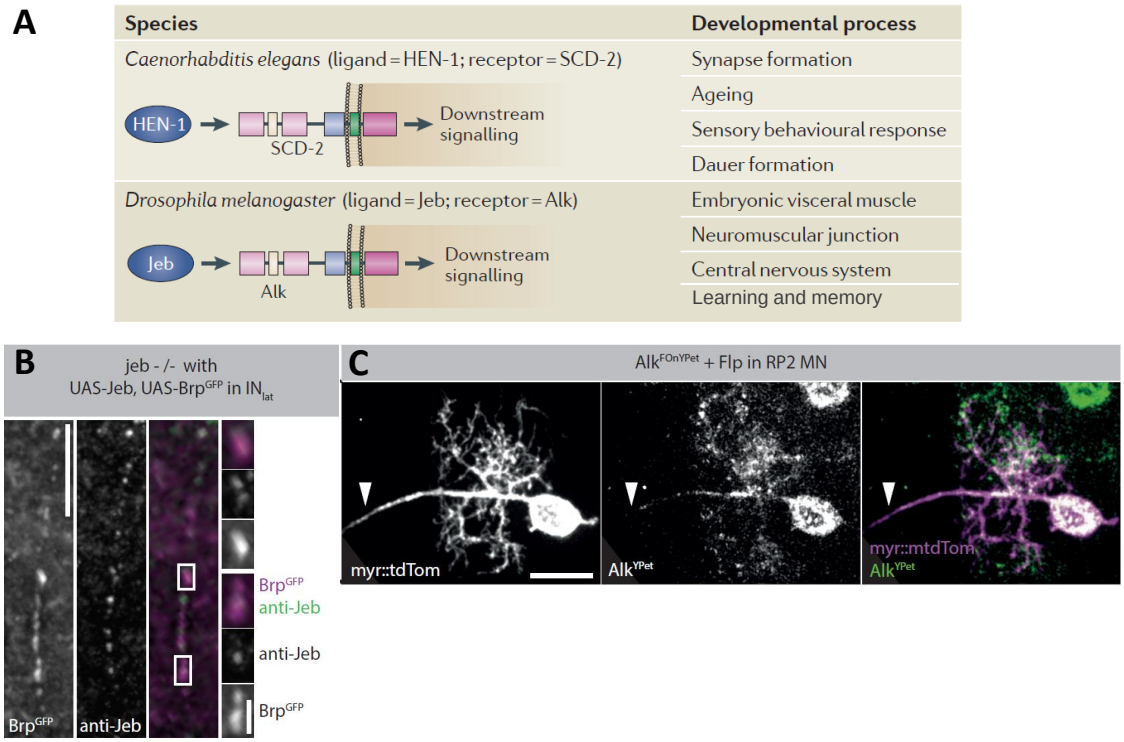

FIGURE 1.2. **Alk-Jeb signaling in the central nervous system. A** - Overview over the processes that are regulated by HEN-1-SCD-2 and Jeb-Alk signaling in invertebrates. The pathways are crucial for several neurodevelopmental processes in both organisms. Table modified form Hallberg and Palmer (2013). **B** - Immunohistochemistry against Jeb (anti-Jeb) reveals its localization to presynaptic release sites (BrpShort^{GFP}) along IN$_{lat}$ axons of the lateral interneuron (IN$_{lat}$). Jeb expression was rescued only in IN$_{lat}$ within jeb mutant animals. (Gärtig et al., 2019) **C** - "Alk^{YPet} localizes to RP2 soma, primary neurite and dendrites, but does not enter the axon leaving the VNC (arrowhead)". (Gärtig et al., 2019)

affects neuronal development and complex nervous system function. Moreover, Jeb-Alk signaling is reduced in a *Drosophila* model for the cognitive disorder Fragile X syndrome (Friedman et al., 2013). The correlation of defects in ALK signaling and models of cognitive diseases has medical implications. These observations might support further research in ALK as a therapeutic target for reducing the cognitive impairments in NF1 or Fragile X patients.

Lastly, there has been previous work on larval Jeb-Alk in *Drosophila* by the Evers lab. This work established the synaptic localization of Jeb and Alk within the larval CNS. Immunohistochemistry against Jeb revealed its localization at the release sites of a cholinergic interneuron (Gärtig et al., 2019) (figure 1.2 B). Activation of a conditional fluorophore tag of Alk in larva motoneurons demonstrated the localization of Alk protein within the dendritic arbor (Gärtig, 2016; Gärtig et al., 2019) (figure 1.2 C). These data suggest a synaptic function of Jeb-Alk also in the central motor circuit of larvae as an anterograde, trans-synaptic signaling pathway. Taken together with the suggested regulation of dendritic growth by Alk (Gärtig, 2016), Jeb-Alk is a promising candidate for studying postembryonic connectivity.

In summary, work in recent years has demonstrated the conserved importance of ALK orthologues for the development of a properly functioning CNS. ALK signaling not only regulates development of CNS as cell number, cell size, and apoptosis but acts acutely during the experience-dependent plasticity of memory and learning. Based on the role of Alk in neuronal plasticity, I aimed to understand its function within the highly plastic, growing CNS of larvae. I addressed the influence of Jeb-Alk signaling on cellular growth and synaptogenesis during postembryonic development. Thus, elucidating the function of Alk aims to improve our understanding of a pivotal player in (1) plasticity-dependent functions of the nervous system and (2) malfunctioning of such processes in pathologies, e.g. specifically intellectual disabilities as seen in NF1 patients (Weiss et al., 2017). In *Drosophila*, Jeb-Alk acts within the CNS to regulate wiring of the adult visual system (Bazigou et al., 2007; Pecot et al., 2014). Jeb-Alk signaling further orchestrates the plastic process of olfactory memory formation and regulates synaptic strength at the NMJ. These findings demonstrate Jeb-Alk as a promising, yet only very little studied player in the regulation of connectivity and disease models further support to the neurodevelopmental role of Jeb-Alk signaling. So far, no studies have addressed the effects of Jeb-Alk on the connectivity of expanding circuits in the growing larval CNS. This study aims to fill this gap to further elucidate mechanisms of postembryonic neural development.

1.5 Neural activity is essential for circuit formation

As demonstrated above, an array of intercellular signaling pathways coordinates neuronal development to a large extent. Additionally, neural activity is needed to establish and orchestrate the proper connectivity of neuronal circuits (Spitzer, 2006). In several cases, molecular signaling and neural activity act to regulate each other (Zhang and Poo, 2001).

A well-known example of activity-dependent development are classical experiments in the visual system of cats by Wiesel and Hubel (1963a). They demonstrated that closure of one eye during certain periods in development leads to changes in neuronal wiring that cannot be reversed resulting in life long loss of visual acuity (Hensch, 2005). Specifically, closing of one eye leads to atrophy of neurons in the lateral geniculate nucleus that receive input from that eye (Wiesel and Hubel, 1963b). On the next level, after opening of the closed eye, visual cortical neurons are unresponsive to stimuli from the formerly closed eye, instead most cells are responsive to stimuli from the open eye (Wiesel and Hubel, 1963a); an observation that is also true in primates (Hubel et al., 1977).

However, neural activity in the visual system occurs already before sensory input as

spontaneous activity (Katz and Shatz, 1996). Therefore, neural activity during circuit development is not limited to experience-dependent activity. Across the vast variety of emerging circuits that have been studied, all exhibit spontaneous activity soon after first synapses have formed or even before (Blankenship and Feller, 2010). This first uncoordinated spontaneous activity quickly transitions to a patterned spontaneous network activity within the developing CNS (Kirkby et al., 2013). For example, during *Drosophila* embryogenesis first spontaneous burst at 17 h ALH result in coordinated waves of activity after another hour of development (figure 1.1) (Crisp et al., 2008).
Indeed, spontaneous activity is crucial to the assembly of neuronal circuits (Blankenship and Feller, 2010; Kirkby et al., 2013). Activity interacts with genetic determinants to wire neural circuits. In the well-studied mammalian visual system spontaneous activity that occurs as retinal waves is necessary to organize the lateral geniculate nucleus (Penn et al., 1998). In ferrets, the eye specific layer formation is inhibited by blocking activity in one eye. However, blockage in both eyes rescues layer specificity demonstrating a competitive mechanism based on spontaneous activity that is essential for adequate wiring (Penn et al., 1998). Spontaneous activity further occurs in the mammalian auditory system (Gummer and Mark, 1994; Jones et al., 2001; Tritsch et al., 2007; Sonntag et al., 2009) and disruption affects synaptic connectivity (Mckay and Oleskevich, 2007; Kandler et al., 2009; Tritsch and Bergles, 2010). Glomeruli structure in the olfactory circuits of mice is also affected by blockage of spontaneous activity (Yu et al., 2004).
Furthermore, spontaneous activity is not limited to sensory circuits. First uncoordinated and subsequent episodic spontaneous activity also occurs in the locomotion circuits of both vertebrates (Nishimaru et al., 1996; O'Donovan et al., 1998; Warp et al., 2012) and invertebrates (Crisp et al., 2008, 2011). In the spinal cord of mice, cholinergic activity is necessary to form proper rhythmically active circuits and flexor-extensor coordination (Myers et al., 2005) demonstrating the importance of proper neural activity for circuit maturation. Similarly, optogenetic manipulation of spontaneous episodic activity in *Drosophila* embryos disturbs the maturation of the network as the onset of crawling-like movement in embryos is delayed (Figure 1.1) (Crisp et al., 2011). The timely onset of these peristaltic movement is further dependent on input from sensory neurons (Crisp et al., 2011; Fushiki et al., 2013). Thus, neural activity as spontaneous network activity and sensory feedback is essential for adequate development of the larval motor circuit.
Mechanistic links of spontaneous activity to synaptic development have been demonstrated as well: Patterned activity in the spinal cord of *Xenopus laevis* orchestrates neurotransmitter specifications (Borodinsky et al., 2004) and spontaneous network activity in the chick embryo regulates the strength of inhibitory and excitatory synaptic contacts (Gonzalez-Islas and Wenner, 2006). These findings provide examples that directly link spontaneous network activity and changes in neuronal connectivity. However, the regulation of developmental processes on a cellular level by spontaneous

neural activity and thereby the mechanistic role of this activity is not well understood. Spontaneous activity seems to be a crucial component for the establishment of proper connectivity and the maturation of circuits. Investigation of the role of intrinsic activity may contribute to our understanding of network maturation and how inappropriate activity may misdirect developmental processes resulting in life-long inadequacies.

1.6 Plasticity of circuits to spontaneous neuronal activity

Above I described activity-dependent (spontaneous or sensory-evoked) effects on neuronal circuits. The capability for changes in the function or anatomy of parts of the nervous system in response to stimuli is called plasticity. Plasticity describes the ability of neural networks to stay flexible while maintaining proper function. A vast amount of research has identified different mechanisms of plasticity throughout development. In general, plastic responses can be categorized as Hebbian and homeostatic plasticity. In Hebbian plasticity, coordinated neuronal excitation between pre- and postsynaptic cells increases synaptic efficacy of individual connections (Hebb, 1949); a lack of coordinated activity decreases synaptic efficacy, respectively. Therefore, Hebbian plasticity creates a positive feedback, where excitation increases synaptic strength making further excitation more likely. This Hebbian-based positive feedback is understood as the basis for experience-dependent learning in vertebrates as well as invertebrates (Cassenaer and Laurent, 2012; Sachse et al., 2007). Homeostatic plasticity, on the other hand, is seen as a mechanism that maintains existing function. Here, neurons or neural networks compensate missing excitation by increasing excitability or vice versa. Conclusievly homeostatic plasticity constitutes a mechanism that counteracts Hebbian plasticity and might as such also limit memory formation (Raman et al., 2019; Tetzlaff et al., 2012). Various examples for both mechanisms of plasticity have been demonstrated across model organisms and even between different circuits or cell types within a nervous system.

Principally, both Hebbian and homeostatic plasticity of neural circuits can be achieved through various cellular adaptions. Neuronal properties of individual cells can change, e.g. intrinsic excitability, or circuit connectivity may be adjusted. The plasticity of connectivity can itself be separated into two distinct categories synaptic plasticity and structural plasticity - which may occur individually or simultaneously to various degrees (Fauth and Tetzlaff, 2016).

Synaptic plasticity is the strengthening or weakening of synaptic connections via the adaption of synapse composition (Vactor and Sigrist, 2017). Here long-term and short-term depression or potentiation have been described: Specific patterns of induced

neuronal activity alter the efficacy of synapse transmission long-term or short term, which can be measured with electrophysiology or calcium imaging. Hebbian long-term potentiation is a classic example thought to underlie learning (Bliss and Collingridge, 1993). An example for homeostatic synaptic plasticity is the presynaptic homeostatic response at the *Drosophila* NMJ, where neurotransmitter release is adjusted in response to impaired receptor function (Davis and Martin, 2015).
Both Hebbian and homeostatic plasticity can also be seen in a second mechanism, called structural plasticity (Fauth and Tetzlaff, 2016). Structural plasticity describes the formation or degradation of synapses themselves upon changes in neuronal activity or of, as a more drastic but slower response, synapse-carrying cellular protrusions (axons or dendrites). Compared to what we know about synaptic plasticity our knowledge of structural plasticity is far more limited. In the adult fly brain, homeostatic responses were measured in the mushroom body (Kremer et al., 2010), where less activity increases synapse density pre- and postsynaptically. In the growing larval nervous system structural plasticity according to the Hebbian theory occurs at the NMJ as axonal structures grow upon increased activity (Ataman et al., 2008). On the other hand, reduced light exposure induces the homeostatic response of dendritic outgrowth of ventral lateral neurons in the larval visual system (Yuan et al., 2011). Similarly, overexcitation of motoneurons with the warmth-gated cation channel Trp1A decreases their dendritic arbor size (Oswald et al., 2018).
In conclusion, our understanding of activity-dependent structural plasticity is limited. Especially the effects of variations of spontaneous activity on developmental processes is unclear. We know little about what perturbations can induce structural plasticity and to what degree. However, several findings point out, that plasticity varies along nervous system development. The next section, aims to illustrate the temporal importance of varying plasticity of neurons and neural circuits along the developmental timeline.

1.7 Critical periods during neural development

Plasticity of neuronal circuits changes across development (Oberman and Pascual-leone, 2013). For example, there is a decline in plasticity with age, which correlates with the onset of cognitive disorders in humans (Oberman and Pascual-leone, 2013). Furthermore, there are time frames of heightened plasticity during brain development that have significant impact on later development (Hensch, 2005). These periods that occur early in development have been termed critical periods. During critical periods neurons are more susceptible to alterations in molecular signaling or neuronal activity which allows changes in circuitry that are more drastic than at other times in development and are irreversible (Hensch, 2005, 2008).
The classical experiments by Wiesel and Hubel mentioned above (section 1.5) also

demonstrated that the plastic effects of eye closure on the wiring of the visual system are time sensitive and limited to a period shortly after birth (Wiesel and Hubel, 1963a,b). Hence, experience-dependent plasticity of the visual system is said to be confined to a critical period. A critical period also exists in the auditory system in mice, where intensified exposure to a certain tone during the critical period changes the cortical, tonotopic map of frequencies within the auditory system (Barkat et al., 2011).
In invertebrates, structural changes due to experience-dependent mechanism in a critical period are also present. In honeybees, early olfactory learning in a sensitive period can change the wiring within the antennal lobe (Arenas et al., 2012). Depending on the learned odor, the size of glomeruli within the antennal lobe changes and thus hardwires this experience into the structure of the CNS, resulting in a lifelong change in the animals behavior. Similarly, a two-day, posteclosion critical period for experience-dependent structural changes in the adult *Drosophila* olfactory system exists (Golovin et al., 2019). Exposure to an odorant during this time, but not later (7-9d post eclosion), reduces the innervation of the activated olfactory sensory neuron within the respective microglumerulus in the antennal lobe.
These examples demonstrate the structural changes that can be induced by experience-dependent neuronal activity during a critical period. These periods of heightened plasticity seem to set up the network for future demands. This setup occurs based on the currently available information, meaning activity, and thus depends on the current requirements to the network (e.g. visual stimuli or auditory range). However, even before sensory input is available spontaneous network activity plays a role in the coordination of proper wiring of circuits (section 1.6). Here the role of neural activity is less intuitive than experience-dependent wiring of sensory circuits. Spontaneous activity interacts with genetic determinants of circuit formation as an additional parameter in the establishment of neural circuits (Blankenship and Feller, 2010). A regulatory role of spontaneous network activity is not limited to sensory circuits but is also present in motor circuit formation (Nishimaru et al., 1996; O'Donovan et al., 1998; Warp et al., 2012; Crisp et al., 2008, 2011). What and how spontaneous activity orchestrates neural wiring is less clear. Furthermore, the role of critical periods during the wiring of motor circuits is also not well understood.
For larval *Drosophila* development, previous studies demonstrated that manipulations of the spontaneous network activity during embryogenesis can change a neurons properties long-term, for example changing excitability of larval motoneurons (Baines et al., 2001; Giachello and Baines, 2015). The time frame during which activity manipulations are sufficient to alter network properties has been refined into a critical period of *Drosophila* embryogenesis, which ranges from 17 to 19 hours AEL (Giachello and Baines, 2015). This time span correlates with first spontaneous action potentials of motoneurons and the transition to patterned spontaneous network activity and coordinated movements of the muscles in the body wall (Baines and Bate, 1998; Crisp et al., 2008) (figure 1.1).

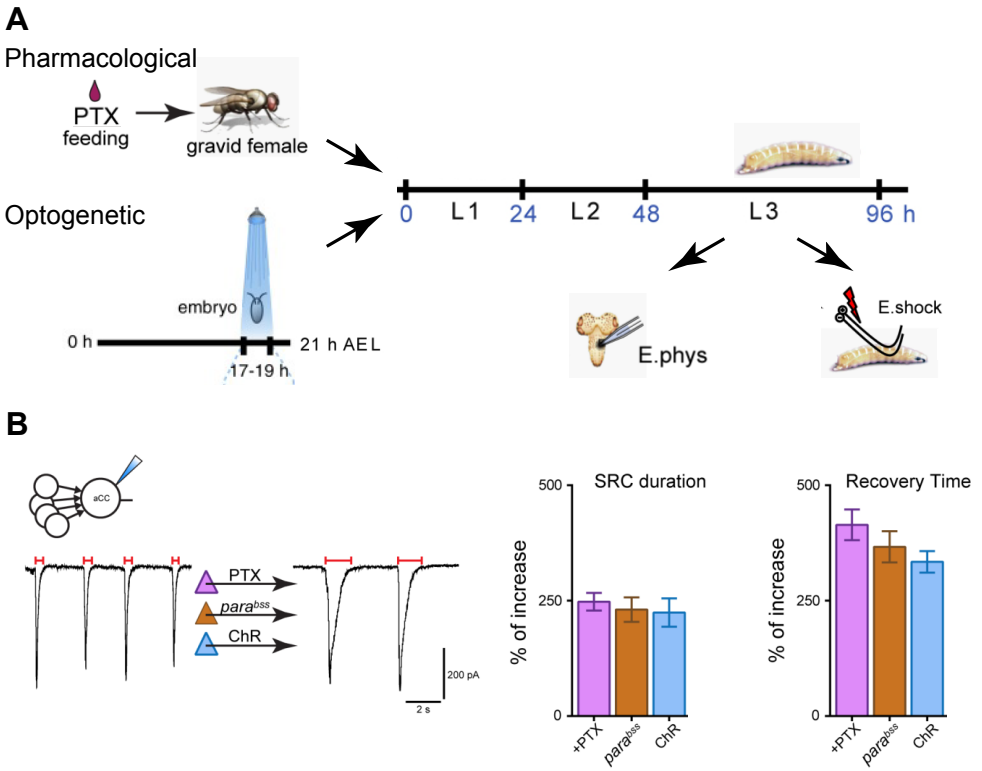

FIGURE 1.3. **Pharmacological and optogenetic manipulations create less alter neuronal properties and reduce network resilience. A** - Effects of the feeding of picrotoxin (PTX) to gravid females (pharmacological) or the activation of Channelrhodopsin (ChR) with blue light pulses from 17 to 19 hours after egg laying (AEL) can be measured in thrid instar animals with electrophysiology or electroshock tests. **B** - Whole-cell patch recordings (traces shown) reveal that PTX feeding and ChR activation (see A) recapitulate the longer duration of spontaneous rhythmic currents (SRC) measured in paralytic (bang-sensitive) mutants ($para^{bss}$) with a similar 200% increase in duration (left bar graph). Increase of SRC duration correlates with a longer recovery time after electroshock (right bar graph). Panels were modified from Giachello and Baines (2015, 2017); Giachello et al. (2019)

During this time, spontaneous episodic activity is necessary for the maturation of the network as disturbances delay the onset of crawling-like movement in embryos (Crisp et al., 2011) (figure 1.1). Therefore, it is suggested that maturation of the motor circuit is dependent on neural activity. Furthermore, neurotransmitter release influences the onset of coordinated network activity as well as morphological development of neurons (figure 1.1) (Tripodi et al., 2008). Together these findings demonstrate the relevance of this critical period of development for the establishment of connectivity within the larval motor circuit. The exact changes in motor circuit connectivity upon manipulations are however not fully understood. It is unclear how morphology and connectivity of motoneurons is adjusted by spontaneous activity during a critical period to achieve the intended tuning of the network, however electrophysiological measurements described changes of neuronal properties (Giachello and Baines, 2015).

Neuronal activity during the critical period (17 to 19 h AEL) above physiological levels leads to development of a less resilient network and changes electrophysiological

properties of motoneurons (Giachello and Baines, 2015, 2017) (figure 1.3). Previous studies used optogenetic as well as pharmacological methods of over-activation to induce hyperexcitation. Optogenetically, blue-light-dependent activation of the light-gated ion-channel Channelrhodopsin expressed in all neurons from 17 to 19 hours is sufficient to destabilize circuits long-term (Giachello and Baines, 2017) (figure 1.3 A). Pharmacologically, feeding of a proconvulsant to gravid females is enough to induce network over-activation during embryogenesis and consequently life-long network hyperexcitability: Wild-type animals treated with the known proconvulsant picrotoxin (PTX) explicitly during embryogenesis show a less resilient network (Giachello and Baines, 2015) (figure 1.3 B). PTX itself is a non-competitive gamma-aminobutric acid (GABA)-receptor inhibitor that blocks inhibitory transmission causing a hyperexcitability of the nervous system (Usherwood and Grundfest, 1964). Methodically, less resilient, destabilized circuits are identified by a longer recovery time from electroshock-induced seizure. This larval seizure phenotype is seen as an epilepsy-like condition and is reminiscent of the phenotype that was first described using the set of genetic mutants termed bang-sensitive (e.g. para^{bss}, figure 1.3), which are characterized by neuronal hyperexcitability (Fergestad et al., 2006).

In conclusion, it is captivating that genetic predispositions elicit the same epilepsy-like behavior of neural circuits as external activity manipulations during embryogenesis. Both, genetic precondition as well as acute, drug-induced activity manipulations, produce less resilient, hyperexcitable networks. While the electrical properties of these altered networks have been studied quite extensively, it is unclear how hyperexcitability is encoded physiologically. In human epilepsy patients, a reduced spine density is seen in hippocampal neurons and sometimes changes in dendritic length and shape can be measured (Wong and Guo, 2013). What the cell biological effects of altered activity during a critical period in *Drosophila* embryos are is unclear. Do changes in circuit excitability manifest in altered structures of dendrites and axons or changes in synapse number? Are specific connections of identified partners affected? Studying critical periods including their molecular regulation and influence on neuronal plasticity in the relatively simple and technically very accessible fly model, is a promising path to increasing our understanding of these periods and their role in complex circuit function. This study provides an analysis of the anatomical effects of embryonic activity on postembryonic circuit development in an effort to understand neural activity as parameters defining circuit connectivity.

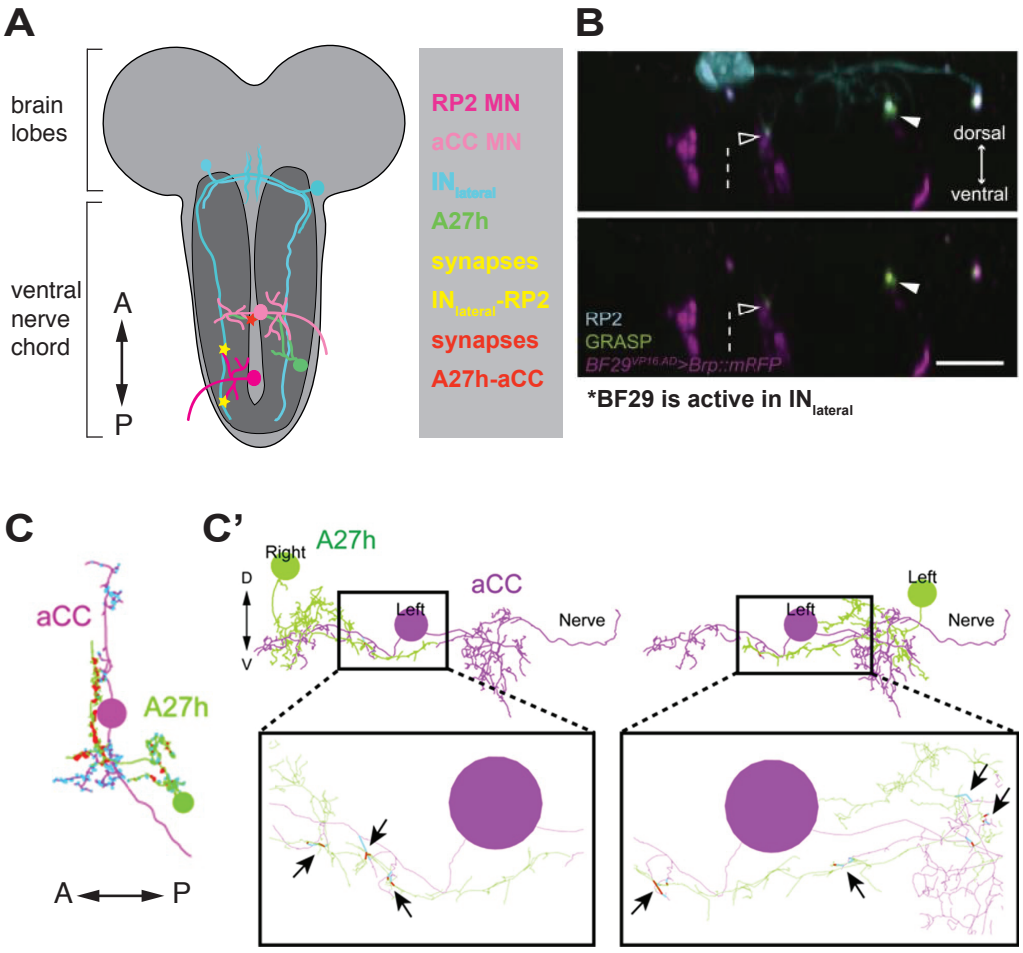

FIGURE 1.4. **Identified synaptic partners of the larval motor circuit. A** - The simplified scheme indicates the position of four neuronal types of the motor circuit. This thesis uses the well studied motoeurons RP2 (magenta) and aCC (light rose), as well as the interneurons IN$_{lat}$ (blue) and A27h (green) as models. Not indicated is the segmental repetition of RP2, aCC and A27h; for each one neuron exists per hemisegment. Modified from Gärtig et al. (2019) **B** - GRASP experiments showed that IN$_{lat}$ synapses onto both RP2 (also aCC, not shown). Modified from Couton et al. (2015). **C-C'** - Reconstructions of aCC and A27h from electron microscopy (C) identified that synapses between A27h and aCC mostly locate in the commissure, where axons and dendrites cross the midline. Modified from Fushiki et al. (2016).

1.8 *Drosophila* larval motor circuit as a model organism to study development of neuronal connectivity

To summarize the above, precise developmental regulation coupled with sufficient plasticity are crucial for emergence of functional neural circuits. The disruption of either molecular signaling pathways or the adequate neuronal activity can lead to neurodevelopmental diseases. However, the complexity of the CNS and its circuits makes studying of these correlations immensely difficult.

Drosophila melanogaster has been used as a model system for neural development for decades. Over the last years, the usefulness of *Drosophila* larvae in comparison to adult

flies has gained attention (Thum and Gerber, 2019): The small size of the CNS with about 10.000 neurons (vs. over 100.000 in adult flies), the availability of connectomes and the identification of behavioral tests including learning paradigms widened the application of this model and thus raised its relevance for biological research. Foremost the morphological and electrical development of motoneurons during embryogenesis has been studied in detail (Baines and Bate, 1998; Landgraf et al., 1997). Importantly, the larval motor circuit has been suggested as a model to show similarities to higher processing centers in the vertebrate brain (Sanchez-Soriano et al., 2005).
The larval motoneurons on the VNC have been characterized morphologically, in regards to the position of their cell body, the shape and size of their dendritic arbors and their axonal projections (Landgraf et al., 1997; Kim et al., 2009). Recently, selectron microscopy (EM) provided the field with detailed analysis of the circuitry of the segmentally structured locomotor system in the VNC (Kohsaka et al., 2014; Schneider-Mizell et al., 2016; Fushiki et al., 2016; Gerhard et al., 2017; Zarin et al., 2019). Here, synapses onto motoneurons and even synaptic connections of upstream interneurons were quantified. Combined with the wide toolset of driver lines available for the via EM described neuronal subpopulations in *Drosophila* larvae, the analysis of identified neuronal circuits *in vivo* is possible in a so far unseen detail.
This thesis focuses on two well studied motoneuron subclasses: The anterior corner cell (aCC) motoneuron and the RP2 motoneurons that innervate dorsal muscle of the body wall. For both motoneuron subclasses one cell per hemi segment can be found (figure 1.4). They fasciculate together within the intersegmental nerve (ISN) to their target muscles. RP2 innervates multiple dorsal muscles, while aCC motoneuron (aCC) has a neuromuscular junction only on DA1 muscles (Kim et al., 2009). Within the VNC neuropil the dendrites of both cell types are found in the dorsal motor neuropil. The RP2 dendritic arbor branches within the ipsilateral side, where the cell body is located and axon leaves the neuropil. The dendritic arbor of aCC is bipolar with two distinct arbors: A larger ipsilateral part and smaller second arbor that projects through the commissure into the contralateral hemisegment. Besides broader driver lines, like OK6, which is expressed in all motoneurons, more specific promoters have been described (Sanyal, 2009). This study used the RN2 promoter construct, that is a partial sequence of the even skipped promotor to specifically drive transgene expression in aCC and RP2 motoneurons (Ou et al., 2008). Together, our detailed knowledge of the development of these motoneurons combined with the available genetic tools, makes these neurons of the locomotor system a powerful and promising model to study the development of circuits in the CNS. The well described anatomy allows us to see how changes during development affect morphological and synaptic development of these neurons.
In order to analyze the nature of neuronal connectivity and the influence of genetic and activity manipulations in detail, previously identified presynaptic partners were selected (figure 1.4). Here again, two neurons will be pivotal to this study: the cholinergic lateral

interneuron (IN_{lat}) (Couton et al., 2015), and another cholinergic interneuron termed A27h interneuron (A27h) (Fushiki et al., 2016; Carreira-Rosario et al., 2018a; Zarin et al., 2019).

IN_{lat} is presynaptic to both aCC and RP2 motor neuron. The cell body of IN_{lat} is located close to the suboesophageal ganglion in the brain lobes and the neurons receives its synaptic input in this area. The axon of IN_{lat} projects laterally along the entire length of the VNC and makes en-passant synaptic connections across all segments. At the end of embryogenesis and throughout larval development IN_{lat} synapses onto aCC as well as RP2 (Couton et al., 2015) (figure 1.4 B). Interestingly, during postembryonic development, the number of aCC/RP2-IN_{lat} synapses scales with organismal growth from an average of approximately one synapse at 0 h ALH to over ten at 48 h ALH for RP2 and roughly twice as many in across the dendritic arbor of aCC (Couton et al., 2015). The synaptic pair of RP2 and IN_{lat} will be the model for the analysis of Alk-Jeb signaling during larval development.

The second neuron upstream of motoneurons analyzed in this thesis is A27h (Fushiki et al., 2016; Carreira-Rosario et al., 2018b; Zarin et al., 2019). This premotor interneuron repeats segmentally and arborizes into the motor domain of its own segments as well as its more proximal segment. Together with its synaptic partners A27h forms a segmentally modular circuit. A27h receives input from the Mooncrawler Descending Neuron (MDN) (Carreira-Rosario et al., 2018b) and is essential for forward locomotion where it is suggested to coordinate inter-segmental regulation (Fushiki et al., 2016). In this context, it drives feed-forward inhibition by exciting inhibitory interneurons thus facilitating the propagation of coordinated contractions. Furthermore, each A27h neuron forms synapses onto motoneurons of the ISN, primarily aCC and RP5 (Fushiki et al., 2016). Here, one A27h neuron projects axonal branches into both hemisegments, thus synapses onto motoneurons on either side (figure 1.4 C). EM data revealed 12 / 11 (left A27h / right A27h) A27h-aCC synapses in abdominal segment 1 in a first instar larva and 18 / 16 A27h-aCC synapses in abdominal segment 3 of a second animal (Fushiki et al., 2016). Most of these connections, but not all, are located close to the soma and thus lie in the commissure (figure 1.4 C). Hence, A27h is a pivotal neuron in locomotion and its connectivity has been described through EM.

Taken together, the described neurons and their identified synaptic connections allow for a detailed analysis of the effects of developmental perturbations onto identified, previously quantified connections. Using this model, specific manipulation and analysis of either pre- (IN_{lat}, A27h) or postsynaptic neurons (RP2, aCC) are possible. In combination with the expression of specific markers both cell morphology and connectivity as synaptic proteins can be analyzed reliably. Thus, the larval motor system of *Drosophila* provides a great model for the study of developmental aspects of neuronal connectivity. Lastly, the comparability to vertebrate central circuits (Sanchez-Soriano et al., 2005) and the usage of disease models (epilepsy-like models) underline the broader relevance

of detailed studies in this simple, yet powerful model circuit.

1.9 Objective of this Thesis

The previous chapters introduced the development of neural connectivity within the CNS focusing on the fruit fly *Drosophila melanogaster*. Research over the last decades has stated the relevance of signaling pathways and neural activity for the establishment of circuits. It is largely unclear how network properties that are set by intrinsic neural activity during a critical period are manifested on a cellular basis, e.g. via changes in circuit connectivity. Furthermore, we lack an understanding of mechanisms that balance continuing function and the plasticity in response to organismal growth during postembryonic development. How the connectivity of synaptic partner is maintained and correlated with tissue and cellular growth, has not been described.
Using the relatively simple, yet sufficiently complex central motor system of *Drosophila melanogaster* larvae, I analyzed the specific connectivity of identified synaptic partners. I aimed to tackle postembryonic circuit expansion from the signaling side, using the anterograde, trans-synaptic Jelly belly Anaplastic lymphoma kinase signaling. I used a novel genetic technique to inhibit Jeb-Alk signaling and analyzed morphological development of synaptic partners as well as synaptic sites in single neurons using expansion microscopy and recently developed conditional, endogenous synaptic markers. I further aimed to demonstrate the importance of embryonic neuronal activity for circuit connectivity, using a PTX-induced, epilepsy-like model. Here, I also utilized an identified pair of synaptic partners and analyzed the growth of dendrites and the development synaptic input onto motoneurons. With the application of novel techniques, I aimed to provide quantitative data on neuronal structures and connectivity of identified neurons in the CNS, allowing for new conclusions on postembryonic neural development.
Taken together, this thesis uses recent technical advances and demonstrates them as reliable tools for producing quantitative data on connectivity of the CNS. The identification of common features of genetic and activity manipulations allows new insights into mechanisms regulating network properties and the role of pre- and postsynaptic partners during plastic development of central circuits.

CHAPTER 2

Results

2.1 Alk^{YPet} verifies Alk expression is limited to neurons

In order to understand the mechanism of a specific signaling pathway within an organism a detailed analysis of gene expression and protein localization is valuable. Besides the expression and activity of Jeb-Alk signaling during mesodermal development in *Drosophila* (Loren et al., 2001; Weiss et al., 2001; Englund et al., 2003; Lorén et al., 2003), various studies have demonstrated a role of Jeb-Alk signaling in the fly nervous system (Bazigou et al., 2007; Gouzi et al., 2011; Cheng et al., 2011a; Rohrbough and Broadie, 2010; Rohrbough et al., 2013b; Pecot et al., 2013). Additionally, work in the Evers lab has supported the notion that Jeb-Alk acts in the central nervous system (CNS) (Gärtig, 2016; Gärtig et al., 2019). While several studies demonstrate a neuronal action or localization of the receptor *Drosophila* anaplastic lymphoma kinase (Alk) (Bazigou et al., 2007; Rohrbough et al., 2013b), it has also been suggested that Alk is active in neuroblasts and possibly glia cells (Cheng et al., 2011a). To verify whether activity of Alk is limited to neurons or also acts in other cell types of the nervous system, I employed a new genetic tool, called dFLEx, with an endogenous fluorophore tag (Manhart, 2019; Gärtig et al., 2019).

2.1.1 An endogenous Alk^{YPet} tag at MiMIC10448 resembles published Alk expression

Work by Aaron Ostrovsky in the Evers lab has produced a conditional YPet fluorophore-tag within the dFLEx cassette at the endogenous Alk locus (Alk^{FOnYPet}) (Gärtig et al.,

2019) using a landing site introduced by the Bellen lab, called MiMIC10448 (Venken et al., 2011). From this landing site the Ypet flourophore was introduced to the genomic 3' end via same strand directed homologous recombination (for details see Gärtig et al. (2019)). The dFLEx cassette contains flippase recognition target (FRT) sites that allow cassette inversion by the flippase recombinase (FLP) (figure 2.1 A). Thus, targeted activation of the tag can be achieved by selective expression of Flp. Using this genetic tool, I previously showed the specific localization of ($Alk^{FOnYPet}$ in the dendrites of larval motoneurons (Gärtig, 2016). However, data about possible artifacts of the YPet tag affecting the functionality of Alk were missing.

First, I created a constitutively labeled Alk^{YPet} protein via the inversion of the dFLEx cassette (figure 2.1 A) in the germ line. This was achieved by activating flippase expression controlled by a heat shock promoter in male larvae. Animals carrying the constitutive Alk^{YPet} allele are viable and show no behavioral deficits. Furthermore, organismal development occurs with no obvious delay or alterations suggesting that the YPet tag does not majorly interfere with Alk functionality.

Previous immunohistochemistry experiments have demonstrated localization of Alk in the mushroom body of adult flies (Gouzi et al., 2011). Using the constitutively labeled Alk^{YPet} I could similarly demonstrate localization of Alk^{YPet} within the alpha, beta and gamma lobes of the mushroom body (figure 2.1 B). Additionally, strong expression was found within the mushroom body calyx in microglumeruli structures (figure 2.1 C). Alk^{YPet} localizes synaptically as previous Alk immunohistochemistry has shown (Gouzi et al., 2011). These results suggest that localization of Alk as well as functionality are not affected by the introduction of a C-terminal YPet flourophore. Additionally, localization within microglumeruli supports the notion that Alk is postsynaptically localized as suggested by the dendritic localization of Alk^{YPet} in the RP2 motoneuron (RP2) (Gärtig et al., 2019) (figure 1.2 C).

2.1.2 Alk is expressed in neurons specifically

Several roles of Alk in developmental neuronal processes have been suggested in previous publications: Pathfinding in the visual system (Bazigou et al., 2007) as well as learning and memory performances in adults are regulated through Jeb-Alk signaling (Gouzi et al., 2011; **?**). However, Jeb-Alk signaling has also been implicated in the development of neuroblasts (Cheng et al., 2011b). Here, Jeb-Alk seems to protect neuroblast growth under nutrient restriction via glial Jelly belly (Jeb) release and Alk activity in neuroblasts. To further elucidate the possible role of non-neuronal Alk activity for neuronal development, I aimed to pinpoint the expression pattern of Alk within the CNS.

Therefore, I activated the conditional YPet fluorophore at the alk locus ($Alk^{FOnYPet}$, figure 2.1 A) with flippase expression under the pan-neuronal driver nSyb-Gal4. Induced

Alk^{YPet} locates throughout the entire neuropil of the larval CNS under neuronal flippase expression (figure 2.1 D). Significantly weaker signal was detected in the cortex. Hence, Alk is endogenously expressed in neurons.
Next, I limited activation of the YPet tag to glial cells using flippase expression under the promotor repo-Gal4. Interestingly, ventral nerve chord (VNC)s at 24 h after larval hatching (ALH) showed no expression of Alk^{YPet} under repo-Gal4 (figure 2.1 E). Thus, I demonstrated that Alk activity in glial cells is in not required for the regulation of neuronal development in early larval life as no detectable endogenous Alk expression exists. However, I could not exclude that Alk expression might initiate later than 48 h ALH of larval life as I restricted my analysis to this time periods, that is also suitable for *in vivo* imaging of axons (figure 2.6) and dendrites (figure 2.13). Conclusively, Alk expression and therefore Jeb-Alk signaling seems to be exclusively neuronal within the first 48 h ALH.

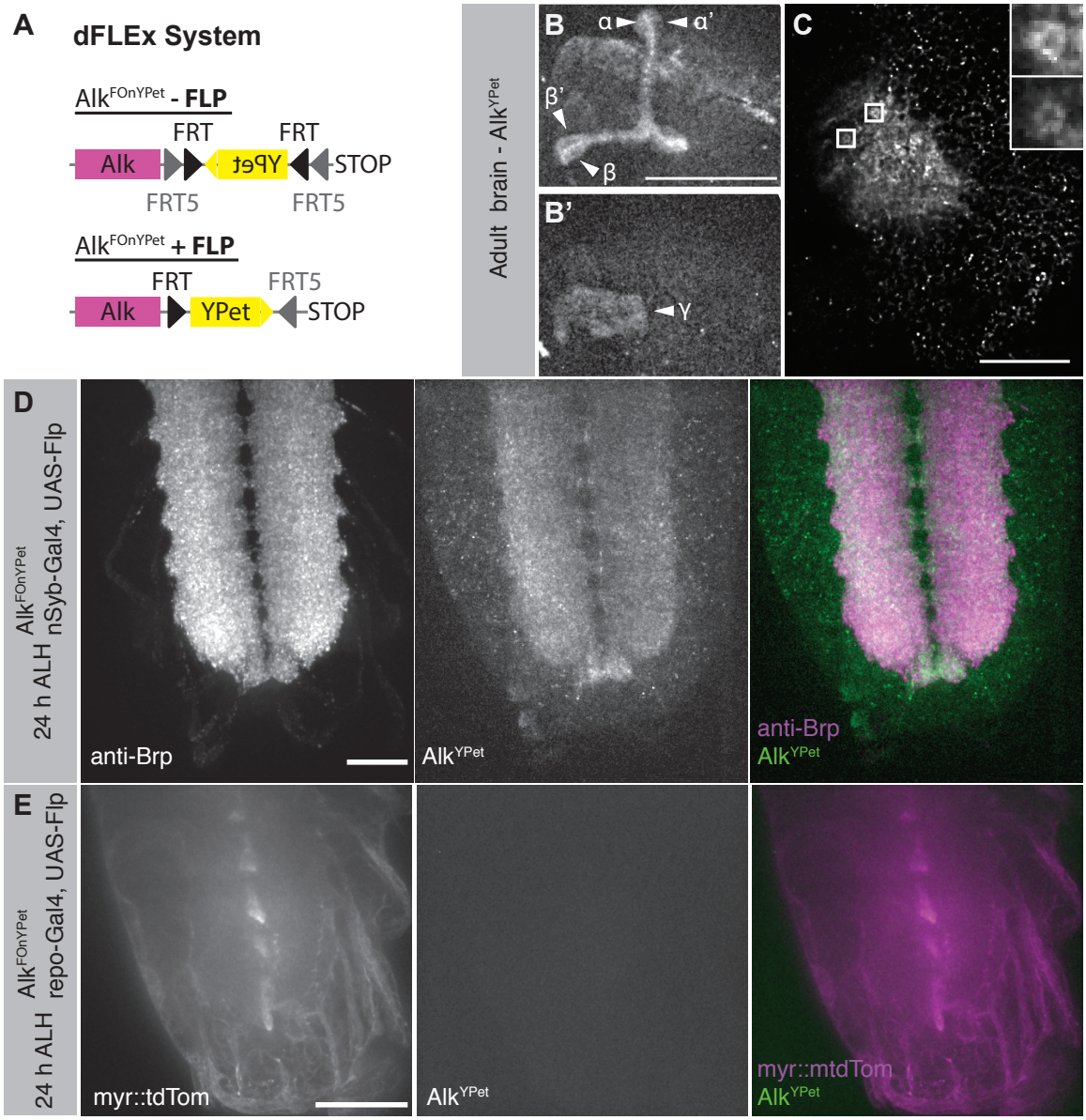

FIGURE 2.1. **Expression of Alk^{YPet} in adult and larval CNS. A**- Schematic of the conditional Alk^{FOnYPet} allele to label endogenous Alk. The cassette between FRT and FRT5 will be inverted in cells expressing FLP recombinase leading to expression of a YPet tagged Alk protein. **B-C**- Constitutive, endogenously Alk^{YPet} expression in the adult CNS. Alk^{YPet} localizes to the alpha, beta (B) and gamma (B) lobes in the mushroom body and to postsynaptic structures of microglumeruli in the mushroom body calyx. Scale bars 100 μm . **D-E**- Expression of Alk in the larval VNC. Induction of Alk in all neurons (nSyb-Gal4, D) shows strong Alk^{YPet} signal (green) in the neuropil (anti-Brp, magenta) and weak signal in the CNS, while no signal was found under FLP expression glial cells (repo-Gal4, E). Scale bars 20 μm .
Panels A, C, D, E are adapted from Gärtig et al. (2019).

2.2 Targeted mutation of jeb alters neuronal morphology

2.2.1 jeb^{BOnSTOP} is able to induce targeted mutations within cells and tissues

The expression data on Alk as well as Jeb (see section 1.4.1.1, figure 1.2) clearly suggests that Jeb-Alk is an anterograde, trans-synaptic signaling pathway in the larval motor system. These findings are in line with results from the larval neuromuscular junction and the visual system in adults (Rohrbough et al., 2013b; Bazigou et al., 2007). Next, I set out to determine the role of Jeb-Alk signaling on neuronal development in the larval CNS. However, neither jeb nor alk mutant animals are viable as removal of Jeb-Alk signaling impedes the visceral muscle differentiation. As these visceral muscle cells line the gut, jeb and alk mutant animals do not form a gut leading to lethality shortly after embryonic development (Loren et al., 2001; Englund et al., 2003; Lee et al., 2003).
Thus, I aimed to selectively removed Jeb from neurons using a new conditional mutant allele constructed in the dFLEx system. In this case, instead of two orthogonal FRT sites, attBx/attPx sites where introduced, which are recognized by the Bxb1 integrase (Huang et al., 2011). The conditional mutant allele cassette introduces an inducible translational stop codon as well as a transcription termination sequence (figure 2.2 A) (Gärtig et al., 2019). This Bxb1-inducible cassette was designed and cloned by Linda Manhart and Aaron Ostrovsky. With the help of the Microinjetion Service at the Fly Faciliy in Cambridge the construct was introduced to the endogenous jeb locus at MiMIC03124 (Gärtig et al., 2019). The MiMIC site and thus the inducible premature STOP codon is located within the open reading frame shortly before the LDL receptor domain of Jeb (Lee et al., 2003). The created new allele is termed jeb^{BOnSTOP}, where **BOnSTOP** stands for **B**xb1 turns **On** the **STOP**.
First, I confirmed that the jeb^{BOnSTOP} allele is functional as jeb$^{(BOnSTOP)}$/jeb^{2} animals are fully viable and fertile (figure 2.2 B). Next, I made use of the reported gut phenotype of jeb mutants: Induction of jeb^{BOnSTOP} in the mesoderm of jeb^{BOnSTOP}/ jeb^{2} animals using the early mesodermal driver mef2-Gal4 expressing UAS-Bxb1 led to animals that failed to form a gut, leaving only a few escapees (figure 2.2 C). This phenocopies the developmental effects observed in alk or jeb mutant animals (Lorén et al., 2003). Thus, I was able to demonstrate the effectiveness of the conditional jeb^{BOnSTOP} loss of function allele. Using this construct, cell- and tissue-specific loss of function of Jeb can be induced via targeted Bxb1 integrase expression.

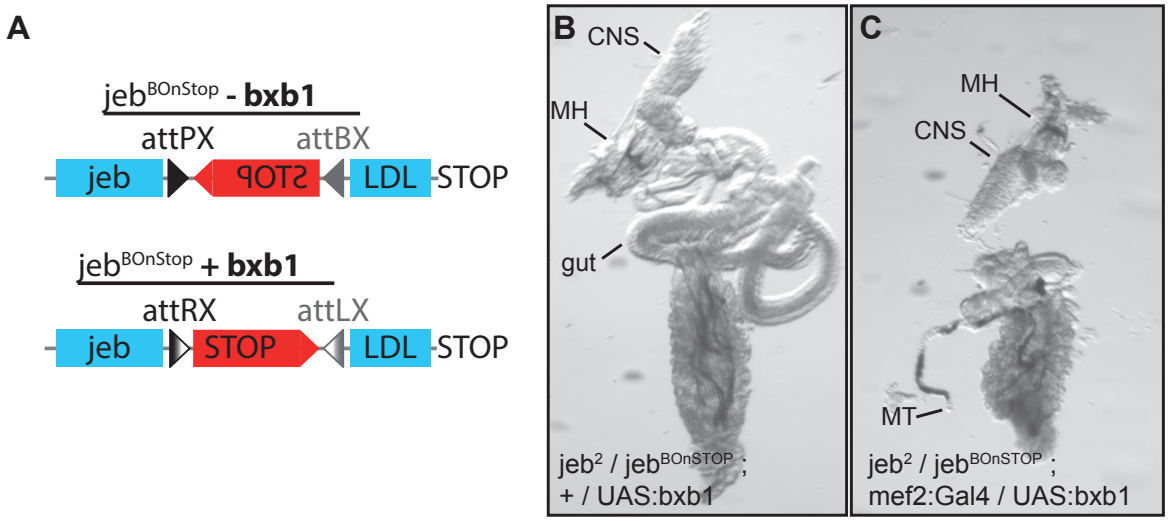

FIGURE 2.2. **jeb^{BOnSTOP} is a loss of function allele.** **A**- Schematic of the conditional Bxb1 integrase induced jeb^{BOnSTOP} allele. Targeted Bxb1 expression leads to Jeb mutation by inversion of an inserted exon that terminates and translation and transcription upstream of the type A LDL receptor domain. **B,C**- Uninduced jeb^{BOnSTOP} allele over the mutant jeb^{2} allele does not impede gut development (B). After induction of jeb^{BOnSTOP} in the early mesoderm (mef2-Gal4, UAS-Bxb1) gut formation is disrupted.
Panels are adapted from Gärtig et al. (2019).

2.2.2 Axonal filopodia number is regulated by Jeb-Alk signaling

Having established that jeb^{BOnSTOP} is suitable for cell- or tissue targeted experiments, I set out to investigate the role of Jeb-Alk signaling on the connectivity within the locomotor system of larva in detail. Previous data implicated a functional importance of Alk activity in the dendritic growth of RP2 motoneurons. In order to provide a model for the development of neuronal connectivity I aimed to investigate an interneuron presynaptic to RP2 motoneurons within the developing VNC. The cholinergic lateral interneuron (IN$_{lat}$) has previously described to form synapses onto the dendritic arbor of RP2 as well as aCC motoneuron (aCC) (Couton et al., 2015). The genetic accessibility of both IN$_{lat}$ and RP2, where for both very specific driver lines are available, creates a promising tool to describe the influence of anterograde, trans-synaptic Jeb-Alk signaling on a pair of identified synaptic partners. Therefore, this study focuses on the axonal morphology of the cholinergic lateral interneurons.
First, I investigated the morphological phenotypes of IN$_{lat}$ under pan-neuronal loss of function of jeb (jeb^{BOnSTOP}/ jeb^{2}; nSyb-Bxb1, 2.3). Expression of a membrane-targeted (myristolation tag) mTurquoise reveals the structure of IN$_{lat}$. Along the entire axon of IN$_{lat}$ that projects from the suboesophageal ganglion, where the cell body is located, laterally through all segments of the VNC (figure 1.4), axonal filopodia can be found. From image z-stacks of the abdominal region of the VNC a 3D reconstruction (Evers, 2004) was created to measure the lengths of all axonal structures. Interestingly, the

number of axonal filopodia increased significantly under abrogation of Jeb-Alk signaling (control: 0.59 ± 0.05 filopodia/ μm , n=8; pan-neuronal: 0.78 ± 0.04 filopodia/ μm , n=12, figure 2.3 E). The increase of filopodia number seemed to be equal in all abdominal segments, but the overall morphology of these filopodia was unaffected (figure 2.3 F)). A similar structural increase in axonal protrusions has previously been shown at the axon terminals in photoreceptor axons (Bazigou et al., 2007) suggesting a common neuronal answer to loss of Jeb. The observed increase in cellular presynaptic structure is opposite of the observed decrease in postsynaptic dendritic length under single cell Alk knock-down in RP2 motoneurons (Gärtig, 2016).

Pan-neuronal inhibition of Jeb-Alk signaling could potentially alter the overall development of the CNS and phenotypic variations in neuronal structure might not directly be attributed to missing Jeb activity or inhibited Jeb-Alk signaling in IN_{lat}. Usage of the eyesgone (eyg) promoter allowed for targeted expression of Bxb1 integrase in IN_{lat} leading to a cell-autonomous loss of function ($jeb^{BOnSTOP}$/ jeb^{2}; eyg-Gal4, UAS-Bxb1, figure 2.3 E). Such a cell-autonomous loss of jeb produces a similarly strong increase in filopodial branches as observed under pan-neuronal Jeb-Alk inhibition (0.98 ± 0.04 filopodia/ μm , n=19, figure 2.3 A). Hence, filopodial number in single axons is regulated through cell-autonomous processes rather than network wide Jeb-Alk activity; Jeb secretion by IN_{lat} is required to limit filopodial growth.

To further verify specificity of the phenotype to Jeb secretion I aimed to rescue Jeb activity in pan-neuronal and single cell mutant by overexpressing full length Jeb ($jeb^{BOnSTOP}$, UAS-jeb/ jeb^{2}; nSyb-Bxb1, eyg-Gal4, and $jeb^{BOnSTOP}$, UAS-jeb/ jeb^{2}; eyg-Gal4, UAS-Bxb1). The average number of filopodia was in both cases reduced an showed no significant difference to controls, but also not to pan-neuronal and single cell mutants. Interestingly, loss of Alk signaling within the interneuron, induced via the expression of a dominant-negative allele of alk (Alk^{DN}, Bazigou et al. (2007)), did not induce a filopodial phenotype (0.65 ±0.03 filopodia μm , n=12, igure 2.3 E). This result demonstrates that Jeb does not act as an autocrine signal and suggests that Alk itself is not presynaptically active within the analyzed system. This is further supported by the finding, that no Alk expression was detected under targeted expression in IN_{lat} (eyg-Gal4, UAS-Flp, ($Alk^{FOnYPet}$).

To further demonstrate that (1) the Alk^{DN} is sufficient to knock-down Alk activity and (2) support the notion that inhibition of Jeb-Alk signaling is essential for proper morphological development of IN_{lat}, I expressed Alk^{DN} pan-neuronally using the promotor elav-Gal4. Here, a new set of reagents was necessary in order to combine single cell labeling with pan-neuronal Alk^{DN} expression. A previously published LexA-Operon based expression system under the promotor BF29 was used (Couton et al., 2015) to label the membrane of IN_{lat} with a YPet fluorophore (myr:YPet), which was cloned and crossed by Jan Felix Evers. As expected, pan-neuronal knock-down of Alk recapitulates the increased filopodial growth seen under pan-neuronal mutation of jeb further

proving the direct link of Jeb-Alk activity and filopodial growth (control: +, 0.87 ± 0.19 filopodia / μm , n=13; elav> Alk^{DN}: 1.19 ±0.26 filopodia / μm , n=7; figure 2.4 A). Furthermore, the similarity of the cellular response of IN$_{lat}$ demonstrates that jeb directly acts through Alk and most likely no other receptors to regulate morphological development within the VNC.

Filopodia have been attributed with synaptogenic properties in e.g. the fly visual system (Özel et al., 2019). Therefore, I analyzed whether structural alterations in IN$_{lat}$ morphology affect the connectivity of the axon by quantifying release site along the axons using the active zone marker BrpShort^{Strawberry} (Banovic et al., 2010). BrpShort is a truncated version of the active zone marker Bruchpilot (Brp) that does not induce synapse formation by itself, but reliable locates to synaptic contacts and is therefore an ideal marker of functional synapses (Fouquet et al., 2009).

Regarding synaptic contacts, neither pan-neuronal nor cell-autonomous loss of function affected the number of release sites (control: +, 4.26 ± 0.42 number Brp/10 μm ; pan-neuronal : 4.35 ±0.26 Brp puncta/10 μm , n=11 ; cell-autonomous: 4.42 ±0.09 Brp puncta/10 μm , n=8, figure 2.5 A, E). Thus, Jeb-Alk regulates morphological development of the axon independent of presynaptic synaptogenesis.

Additionally, BrpShort^{straw} revealed that filopodia are largely devoid of Brp and active zones are mostly located at bouton-like swellings, where filopodia originate. Thus, these axonal filopodia of IN$_{lat}$ do not carrying synapses and that more filopodia do not result in more release sites is therefore reasonable. As a consequence, filopodia are not instrumental for the neuron to expand its territory to directly synapse onto a different set of potential partners.

In this context, the filopodia do not carry synapses themselves but it is conceivable that they could possibly attract postsynaptic partners to the bouton-like swellings at the base of the filopodia where Brp-marked active zones are located. A similar theory has been provided by Özel et al. (2015) and Özel et al. (2019). However, they demonstrate the presence of early synaptic markers, namely Syd-1 and Liprin alpha within filopodia, which could not be reproduced in IN$_{lat}$ neurons even with Syd-1-GFP overexpression (Gärtig et al., 2019; Banovic et al., 2010).

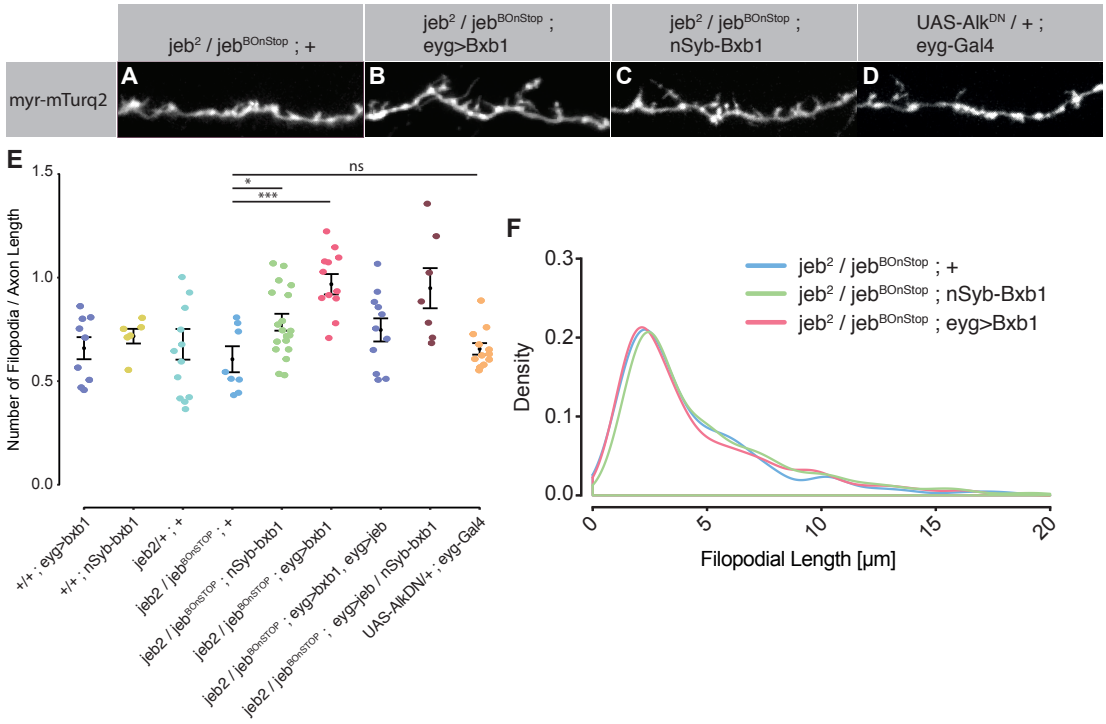

FIGURE 2.3. **Jeb secretion limits number of axonal filopodia in IN_{lat}. A-D**- Confocal images showing the axonal structure (myr::mTurq2) of cholinergic IN_{lat} at 48 h ALH with its filopodia. **E**- Number of filopodia along IN_{lat}axons normalized to axonal length as filopodia per 1 μm axonal length. Various controls demonstrate the specificity of the phenotype to loss of jeb. Attempted rescue with Jeb expression had no effect. **F**- Density plot detailing the frequency of lengths of individual filopodia.
Panels A, B, C, D, F are adapted from Gärtig et al. (2019) *=p<0.05; **=p<0.01; ***=p<0.001; ns not significant

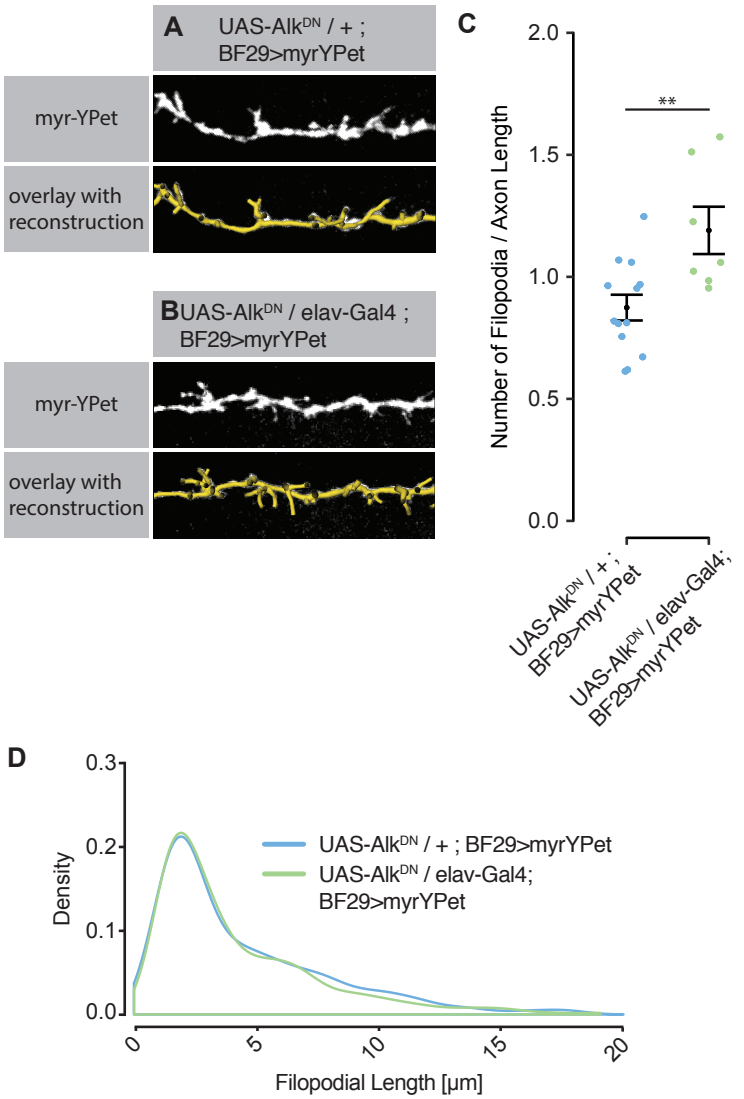

FIGURE 2.4. **Pan-neuronal knockdown of Alk recapitulates pan-neuronal jeb IN$_{lat}$ phenotype. A-B**- Confocal images showing the axonal structure (myr::YPet) of cholinergic IN$_{lat}$ including filopodia at 48 h after larval hatching. Compared to wild type animals (A) pan-neuronal knock-down of Alk (B) leads to increased formation of filopodia. **C**- Number of filopodia normalized to axonal length. **D**- Density plot detailing the frequency of filopodial lengths reveals no change in the structure of individual filopodia. *=p>0.05; **=p>0.01; ***=p>0.001; ns not significant.Data produced jointly with Jan Felix Evers.

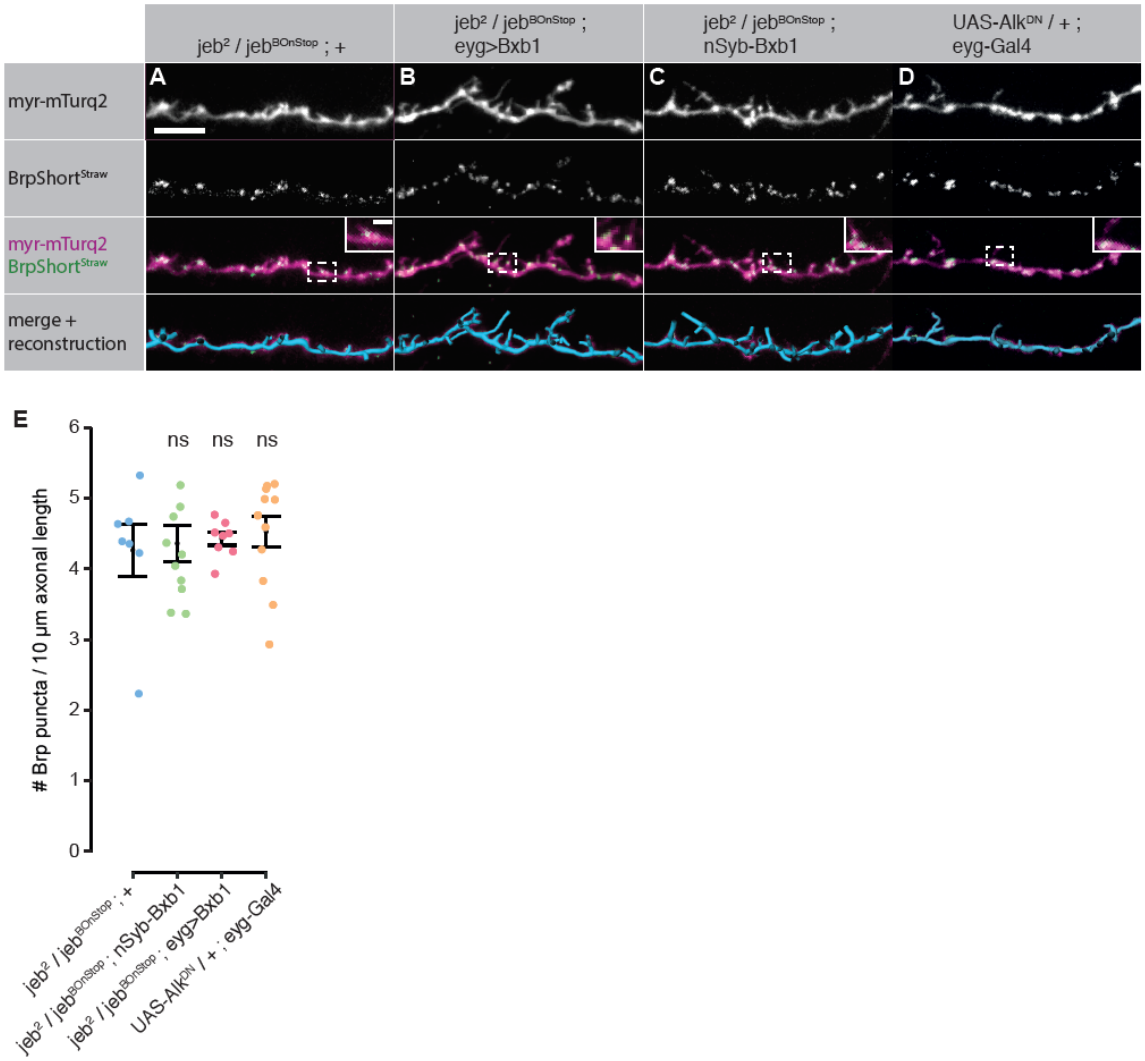

FIGURE 2.5. **Jeb has no effect on release site number or filopodial characteristics. A-D**- Confocal images showing the axonal structure (myr::mTurq2) of cholinergic IN_{lat} at 48 h ALH with its filopodia. Release sites ($BrpShort^{Strawberry}$) are often located at the base of filopodia. Compared to wild type animals (A) targeted Jeb loss of function in IN_{lat} (B) or pan-neuronally (C) has no effect on release site number but increases number of filopodia. Knock-down of Alk in IN_{lat} via $Alk^{(DN)}$ (D) expression has no effect. **E**- Density of presynaptic release sites marked by $BrpShort^{Strawberry}$ along the $IN_{(lat)}$axons.
Panels are adapted from Gärtig et al. (2019)

2.2.3 Collateral filopodia along the IN_{lat} axon are highly dynamic

Presynaptic filopodia across various species have been shown to be short-lived and highly dynamic (Meyer and Smith, 2006; Li et al., 2011; Sheng et al., 2018; Moradi et al., 2017). However, most of these studies have been in the context of growth and exploration at the growth cone or in axonal arborizations (Gallo, 2011; Moradi et al., 2017). Hence, the physiological role and function of collateral filopodia not leading to stable branch formation is unclear. Therefore, I set out to describe the dynamics of IN_{lat} collateral filopodia. Monitoring neuronal growth dynamics in larval VNCs

directly should allow to elucidate the role of these filopodia for growth and connectivity. Therefore I imaged IN_{lat} axons at 24 h ALH **in vivo** and after dissection at 48 h ALH. Methodological details on *in vivo* imaging of larva can be found in the Materials and Method section (section 4.2.2).

Because the available membrane marker myr::mTurquoise2 under eyg-Gal4 control showed relatively low expression levels during early larval development, *in vivo* imaging at 0 h ALH was not feasible. However, data were obtained at 24 h ALH and the same animals were subsequently sacrificed; at 48h ALH at which point the VNC was acutely dissected and imaged. This method allowed me to follow single branches over time and detect filopodia formation or retraction. For this analysis, distinctive filopodia that were clearly present at both stages were selected (see figure 2.6 A, B branches marked with asteriks). Subsequently. the reconstruction from 24 hours was stretched to overlay with the 48h-old neuron at those distinctive branches simplifying the identification of changes. This analysis revealed that around 65.5% ± 0.05% (n=4) of filopodia retract between 24 and 48h ALH in control animals (red in figure 2.6) , and during the same period 70.0% ± 0.01% (n=4) of all filopodia emerge as new (new at 48h, blue in figure 2.6). These results offer a first description of the growth dynamics of axonal filopodia during postembryonic circuit expansion *in vivo* in the *Drosophila* motor system. The high dynamic turnover of these filopodia also support the argument that no functional synapses form on these cellular protrusion.

As the data on filopodial numbers demonstrated (see previous section) cell-autonomous loss of jeb is sufficient to induce a measurable phenotypic variation. Therefore, due to the high death rate among animals (technical challenge of animal handling) and the low throughput of *in vivo* imaging and data analysis, this analysis was restricted to single-cell manipulation in order to determine how Jeb-Alk signaling affects axonal dynamics. In this genetic context, filopodial stability was unaffected by loss of jeb (28.9 ± 0.01%, n=4, red in figure 2.6 C). At the same time the proportion of newly formed filopodia first visible at 48 hours increased (77.8 ± 0.02%, n=4, Fig. 3D). This observation is in line with the increase in filopodia number under jeb mutation described above (see section 2.2.2). It further suggests that an increase in filopodial number is more likely due to increased filopodia outgrowth rather than an increased stability by an unchanged rate of filopodia formation.

In summary, general axonal targeting and pathfinding of IN_{lat} is not dependent on anterograde, trans-synaptic Jeb-Alk signaling as placing of the axons is unaltered. Additionally, the morphology and stability of individual filopodia are not affected by loss of Jeb. However, Jeb-Alk limits the formation of new filopodia during larval development as suggested by the increased number of collateral filopodia at 48 h ALH under singe-cell or pan.neuronal Jeb abrogation. The physiological role of the dynamics of filopodia could not be clarified completely As they do not carry mature synapses, as similarly shown in **Xenopus** citepLi2011, they could still act as attractants or guides for potential

postsynaptic partners: Explorative contacts on these filopodia might results in first cell-cell-adhesion and the induction of dendritic growth toward the active zone carrying axon swellings where mature synapses form in a next step. This theory would be in line with e.g. Özel et al. (2015) and Özel et al. (2019), except in regards to the protein composition in the filopodia, since Syd-1 is not present in IN_{lat} filopodia (Gärtig et al., 2019).

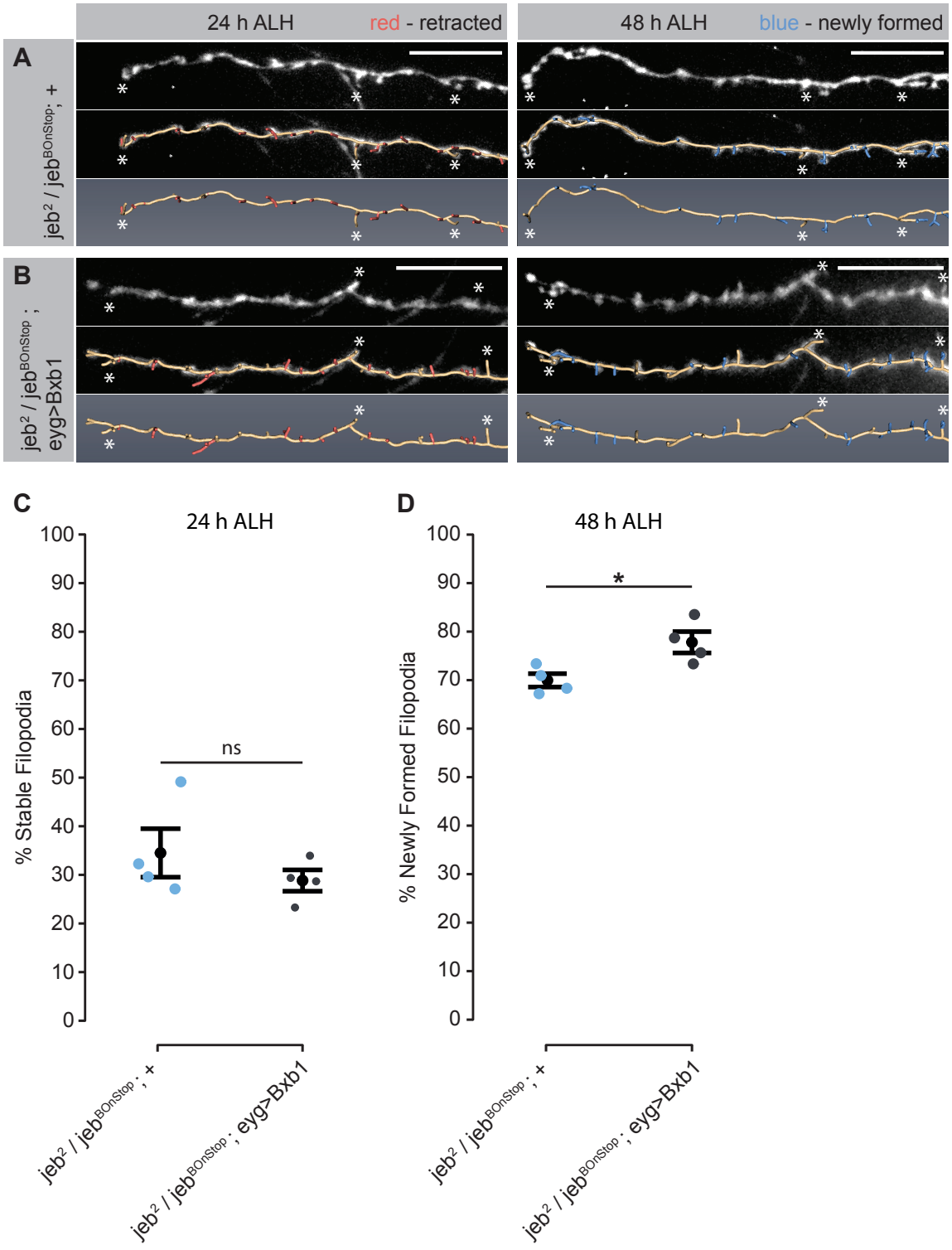

FIGURE 2.6. **Filopodial dynamics of IN$_{lat}$ are slightly affected by cell-autonomous loss of jeb. A, B**- In vivo imaging of IN$_{lat}$ (myr::mtdTom) in anesthetized larvae at 24 h ALH (A) and acutely dissected VNCs at 48 h ALH (B) with the overlayed reconstructions (lower panels) of control (top row) and single cell jeb mutant animals (bottom row). Sequential imaging allows tracking of filopodia removed after 24 h ALH (red in A) and new branches at 48 h ALH (blue in B). Asteriks mark persisting filopodia as landmarks. **C**- Percentage of filopodia removed from 24 to 48 h ALH (red in A) is not affected by loss of jeb. **D**- Percentage of newly formed branches after 24 h ALH (blue in B) is slightly increased by cell-autonomous loss of jeb. *=p>0.05; ns not significant. Scale bar - 20 μm .
Panels are adapted from Gartig et al. 2019

2.3 Jeb-Alk signaling regulates postsynaptic connectivity

Structural changes of neuronal morphology have traditionally been suggested to be accompanied by changes in neuronal connectivity. Importantly, especially synaptic plasticity and synaptogenesis have have been associated with an increase in number of dynamic filopodia in various systems (Özel et al., 2019; Sheng et al., 2018). Additionally, results from the neuromuscular junction (NMJ) have demonstrated that loss of Jeb-Alk signaling results in the strengthening of synaptic coupling (Rohrbough and Broadie, 2010; Rohrbough et al., 2013b). How do synaptic coupling at the NMJ and structural changes in the CNS relate? To address this question, I aimed to further investigate potential changes in connectivity in response to alteration of Jeb-Alk signaling. Does in the increased number of presynaptic filopodia affect synapse formation of IN_{lat} and its partner neurons within the central motor neuropil? If the number of active zones is unaffected, are amount or density of postsynaptic specializations impacted by missing Jeb-Alk signaling and the subsequently increased filopodial activity?

2.3.1 Drep2 is a marker of cholinergic postsynaptic specializations in the motor circuit

To address this problem, it was crucial to establish a reliable, non-toxic postsynaptic marker, that could allow for single cell synapse quantification. Previously, DNA fragmentation factor related protein 2 (Drep2) has been detected as a synaptically localized protein in the adult *Drosophila* CNS (Andlauer et al., 2014; Hussain et al., 2018). Within the mushroom body calyx Drep2 was detected to overlap with postsynaptic acetylcholine receptors of kenyon cells, but not presynaptic Brp providing evidence that Drep2 is a postsynaptic protein localizing to cholinergic synapses.
First, I verified the postsynaptic localization of Drep2 using expansion microscopy (ExM) (Chen et al., 2015; Chozinski et al., 2016) in collaboration with a Bachelor student under my supervision, Franz Bauer. We employed a conditional active zone marker at endogenous $Brp^{FOnYPet}$, that has been previously established in the Evers lab (figure 2.7 A) (Manhart, 2019; Gärtig et al., 2019). Targeted, neurotransmitter specific activation of $Brp^{FOnYPet}$ (using Trojan-Gal4 lines from Diao et al. (2015)) combined with immunohistochemistry against YPet (anti-GFP), Drep2 and Brp (to mark all synapses) and followed by ExM allowed a detailed analysis of relative localization of these synaptic markers. This analysis included the restriction of tagged Brp expression to cholinergic neurons (ChAT-T2A-Gal4, UAS-Flp; Diao et al. (2015)) as acetyl-choline is the main excitatory neurotransmitter in insects, as well as neurons expressing the inhibitory neurotransmitter gamma-aminobutyric acid (GABA; using gad1-T2A-Gal4, UAS-Flp) ((Diao et al., 2015; Pitman, 1971; Gerschenfeld, 1973)). Within the VNC

of 48 h old larvae we detected Drep2 signal reliably juxtaposed to Brp^{FOnYPet} marked cholinergic neurons (figure 2.7 B). GABAergic active zones, on the other hand, were devoid of Drep2 signal within the motor neuropil (figure 2.7 C). Consequently, I confirmed that Drep2 is a reliable marker of excitatory, cholinergic postsynaptic specifications also in the CNS of *Drosophila* larva.

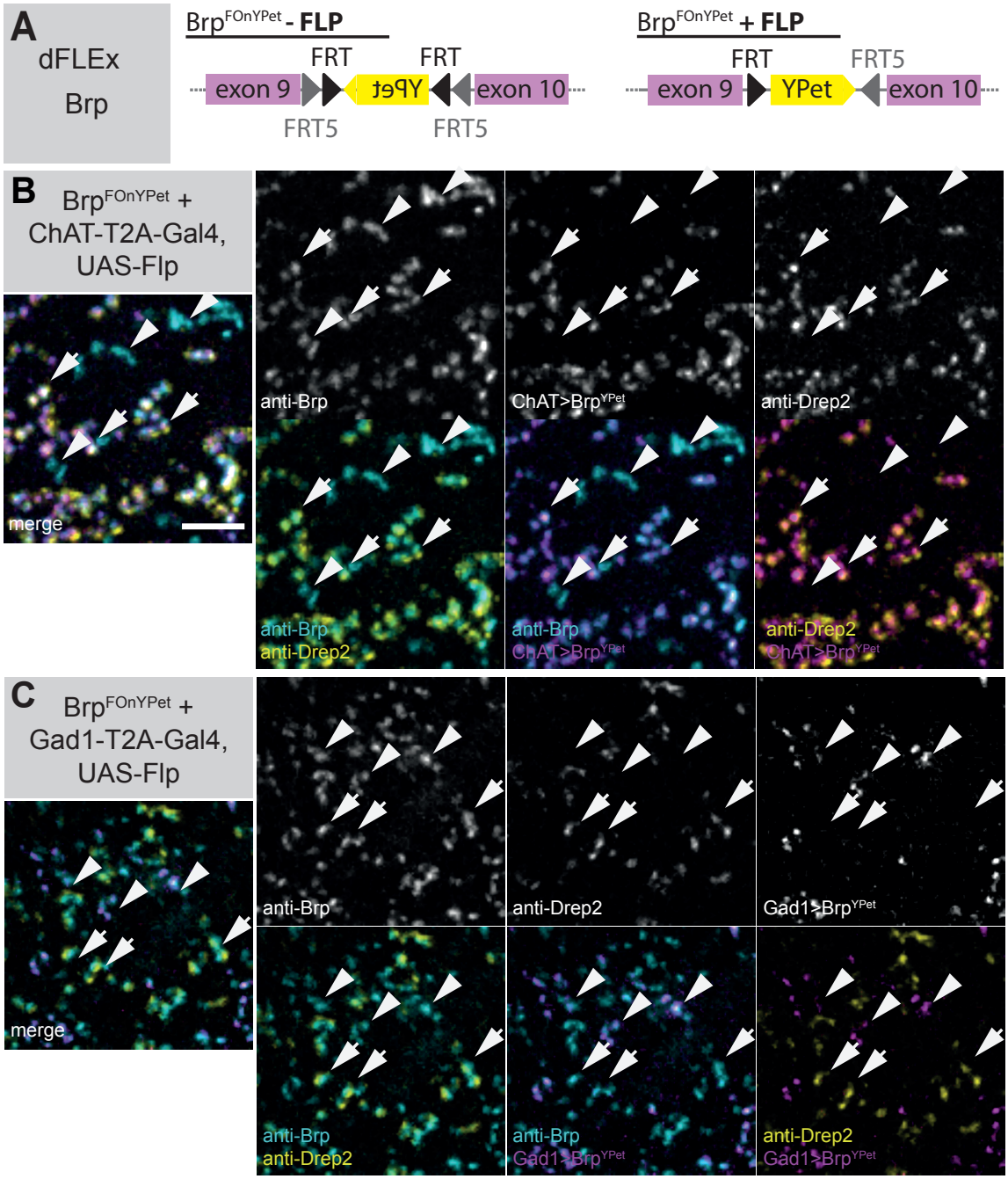

FIGURE 2.7. **Drep2 localizes to cholinergic but nor GABAergic synapses A**- Schematic of the conditional Brp^{FOnYPet} allele to label endogenous Brp between exons 9 and 10. The cassette between FRT and FRT5 will be inverted in cells expressing FLP recombinase leading to expression of a YPet tagged Brp protein (Manhart2019). **B, C**- Expansion microscopy reveals that cholinergic specializations (B, arrows, magenta) are juxtaposed to Drep2 (anti-Drep2, yellow). Other presynaptic release sites (arrowheads, anti-Brp, cyan) lack Drep2 and so do Brp^{YPet} marked GABAergic synapses (C, arrowheads, magenta)
Panels are adapted from Gärtig et al. 2019. Data produced jointly with Franz Bauer.

2.3.2 An endogenous, conditional and postsynaptic marker for the quantification of cholinergic synapses

Next, I aimed to employ Drep2 as a measure for the quantification of excitatory synapses on single motoneurons under modifications to the Jeb-Alk signaling pathway. Using the dFlex system Linda Manhart introduced a N-terminal, conditional YPet tag to the endogenous Drep2 locus via MiMIC15481 and homologous recombination (termed Drep2^{FOnYPet}) (figure 2.8 A) (Venken et al., 2011; Manhart, 2019).
First, I verified the functionality of the induced Drep2^{YPet} by comparing its relative localization to the presynaptic marker Brp with a published antibody against Drep2 (Andlauer et al., 2014). After immunohistochemistry followed by ExM no obvious differences were observable between the Drep2 antibody and Drep2^{YPet} (figure 2.8 A and B). A similar density of Drep2 puncta was observed resembling the pattern of cholinergic synapses also visible in figure 2.7. Additionally, for the Drep2 antibody as well as Drep2^{YPet}, Brp and Drep2 mostly occur in ribbon-like structures with Drep2 forming a slightly larger structure along the outside of the curve. Taken together, endogenously labeled Drep2^{YPet} recapitulates the localization of established Drep2 reagents.
Lastly, labeled Drep2^{YPet} could potentially interfere with physiological Drep2 function despite proper localization. Therefore, I verified that Drep2^{YPet} has no toxic effect on expressing neurons by looking at their dendritic growth. Previously a GFP-tagged version of Drep2 (UAS-Drep2^{GFP}) has been used, e.g. for the analysis of Drep2 interaction partners (Andlauer et al., 2014). However, regarding dendritic growth, the overexpression of Drep2^{GFP} (figure 2.8 E) results in stunted dendritic arbors in RP2 motoneurons as they show a smaller total dendritic length (TDL) at 48 h ALH (RN2FlpOut: 847.42 ± 27.19 μm ; RP2>Drep2^{GFP}: 669.95 ± 21.86 μm , figure 2.8 F). Importantly, the induction of the YPet-tag specifically in motoneurons (RP2-Flp, tubC-FRT-STOP-FRT-Gal4) (Ou et al., 2008) showed no significant changes in TDL (RP2>Drep2^{FOnYPet}: 792.09 ± 18.56 μm , figure 2.8 D and F). Additionally, Drep2^{GFP} shows a significantly denser Drep2 localization pattern within dendrites compared to Drep2^{FOnYPet}. Especially GFP signal in the primary neurite and soma is more pronounced (figure 2.8 D). This pattern is likely a direct consequence of the overexpression, but has the potential to significantly falsify the analysis of synaptic localization of Drep2 in addition to the demonstrated toxic effect on dendritic growth.

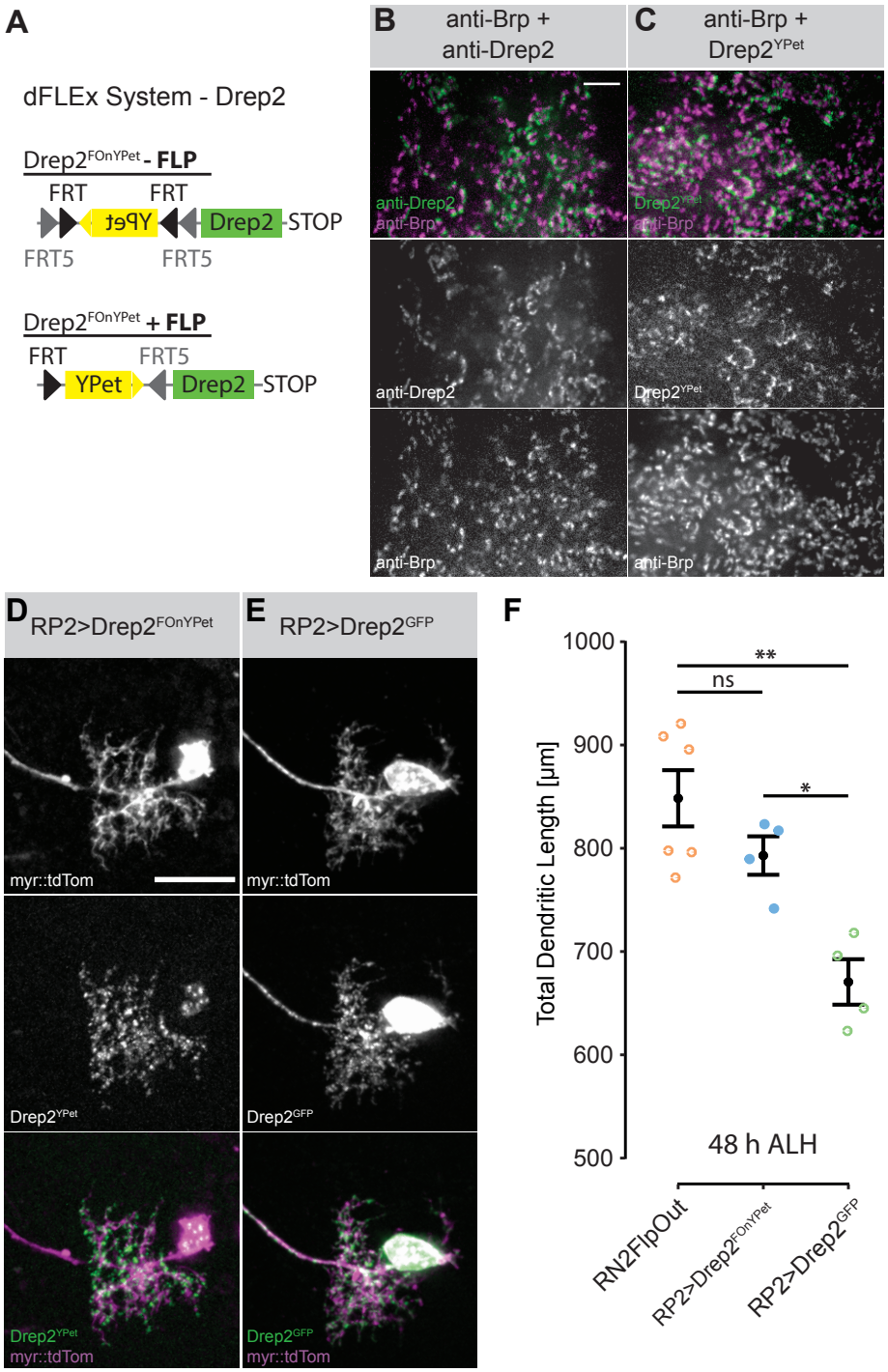

FIGURE 2.8. **Drep2^{YPet} confirms Drep2 antibody staining and is not toxic for dendritic development. A**- Schematic of the conditional Drep2^{YPet} allele to label endogenous Drep2 N-terminally. The cassette between FRT and FRT5 will be inverted in cells expressing FLP recombinase leading to expression of a YPet tagged Drep2 protein (Manhart2019). **B, C**- Expansion microscopy of larval VNCs shows that constitutive, endogenously Drep2^{YPet} (C, green) reproduces localization of Drep2 antibody (B, green) juxtaposed to anti-Brp (magenta). Scale bar 10 um **D**- Comparison of the localization of two Drep2 constructs, endogenously labeled Drep2^{YPet} or overexpression of Drep2^{GFP}, in the dendritic arbors of RP2 motoneurons (myr::mtdTom). Significantly higher localization of Drep2^{GFP} was detected in the cell body, primary neurite, axon and even throughout the dendritic arbor. Scale bar 20 um **E**- Total dendritic length of arbors expressing Drep2^{GFP} are significantly shorter than wild-type arbors, while endogenously tagged Drep2^{YPet} has no significant effect on dendritic length. *=p<0.05; **=p<0.01; ***=p>0.001; ns not significant. Data produced jointly with Franz Bauer.

2.3.3 Postsynaptic specifications regulated by Jeb-Alk

Having established the proper localization and functionality of Drep2^{YPet} I set out to introduce this tool as a measure of quantifying synaptic contacts in single cells. Through targeted induction of YPet-tag expression in motoneurons (RP2-Flp, tubC-FRT-STOP-FRT-Gal4, Ou et al. (2008), figure 2.9 A) the synaptic contacts in single aCC or RP2 neurons could be visualized. To achieve sufficient resolution, I used immunohistochemistry and ExM allowing me to resolve single postsynaptic specializations. Subsequently, likely functional synapses were verified by their juxtaposition to Brp (labeled using anti-Brp antibody). Additionally, flourescence of the membrane marker myr::mtdTomato2 was amplified (anti-RFP) (figure 2.9 B and C) to simplify identification of the neuron-of-interest during imaging and to allow correlation of Drep2 with the presence of expressing neuron. Through this technical approach I achieved a reliable quantification method for Drep2 puncta single cells within the CNS of larval *Drosophila*.
Drep2 was detected in the soma, the primary neurite and in the proximal part of the axon. These Drep2 puncta did not appose Brp and thus are not synaptically-localized proteins, but rather newly synthesized or currently trafficking Drep2 proteins. Nonetheless, most Drep2 puncta were localized throughout the dendritic arbor, where the great majority apposed Brp and was overlapping with the membrane marker. An average distance of 180 nm between Brp and Drep2 peak intensities was measured (2.9 D, n=15 synapses across three dendritic arbors), which closely agrees with the published distance of the Brp C-Terminus (marked by nc82) across the synaptic cleft to the postsynaptic receptor GluRIID (Liu et al., 2011). Hence, due to both location and correlation with Brp appropriate, post-synaptically localized Drep2 puncta were quantified representing excitatory, cholinergic synapses of the Drep2^{FOnYPet} expressing motoneurons.

This new tool for the quantification of excitatory synapses now allows to analyze the connectivity of single cells of the larval motor circuit. Therefore, I applied Drep2^{FOnYPet} to elucidate the role of Jeb-Alk signaling on synaptic connectivity within the motor system. First, I analyzed the effect of cell-autonomous inhibition of Alk signaling through the expression of Alk^{DN}. If activation of Alk by Jeb affects connectivity, we would expect changes in the number of synaptic contacts upon signal inhibition. Indeed, knock-down of Alk signaling in RP2 motoneurons resulted in an increased number of synapses along the dendritic arbors at 48 h ALH (Alk^{DN}: 484.00 ± 30.58 Drep2 puncta, n=4; control: 364.67 ± 4.82, n=3, figure 2.10 A to D). Therefore, this data allows the assumption that Alk activation is cell-autonomously inhibitory to the formation of postsynaptic specifications and thus, Jeb-Alk effectively limits the number of synaptic contacts along the dendrites of RP2 motorneurons.
Next, I aimed to analyze the effect of pan-neuronal abrogation of Jeb-Alk signaling.

To achieve this aim, I created a model that combined the two dFLEx systems that I have introduced above: (1) the inducible jeb mutation controlled by Bxb1 activity (section 2.2.2) and (2) the conditional YPet tag on Drep2 controlled by flippase activity. The use of those orthogonal systems allowed for targeted, neuron specific abrogation of Jeb signaling ($jeb^{BOnSTOP}$) combined with the targeted expression of postsynaptic marker $Drep2^{FOnYPet}$ with the FlpOut system (Ou et al. (2008)). Pan-neuronal activation of $jeb^{BOnSTOP}$ lead to an even higher increase in postsynaptic synapse number ($jeb^{BOnStop}/jeb^{2}$; nSyb-Bxb1: 616.40 ± 46.68, n=5) than single cell Alk knock-down. Taken together, cell-autonomous as well as pan-neuronal removal of Jeb-Alk signaling lead to an increase in the amount of postsynaptic specializations providing strong evidence that activation of Alk functions to limit the proliferation of postsynaptic specifications.

In order to describe the developmental of neuronal connectivity depending on Jeb-Alk signaling, the dendritic arbors of animals directly after hatching, at 0h ALH, were also analyzed for their connectivity. Interestingly, no significant effect was detected in first instar animals when quantifying the numbe rof postsynaptic specializations (control: 72.67 ±4.81 Drep2 puncta; Alk^{DN}:: 73.67 ± 4.48 Drep2 puncta, figure 2.10 E). This results suggests that the regulation of postsynaptic specializations through Jeb-Alk is effective primarily during larval life.

So far, I demonstrated that Jeb-Alk signaling (1) limits the amount of presynaptic filopodia, (2) has no effect on presynaptic release site numbers and (3) also limits the number of postsynaptic specializations. Surprisingly, the expectation that an increase in postsynaptic sites should correlate with an adequate increase in presynaptic active zones is not the case in the motor system of *Drosophila* larva. However, is has been shown, especially via electron microscopy, that the insect nervous system consists of mostly, if not exclusively, polyadic synapses, where one presynaptic release site is accessed by multiple postsynaptic specifications (Cardona et al., 2009). EM analysis has further demonstrated that the number can be vary greatly (2 or 20), with four postsynaptic sites connecting onto a single release site on average (Cardona et al., 2009, 2010; Meinertzhagen and Neil, 1991; Schneider-Mizell et al., 2016). Hence a possible explanation for the discrepancy between IN_{lat} and RP2 synapses is, that the increase in postsynaptic sites is offset not by more presynaptic release sites, but by an increase in the post-presynapse ratio meaning more connections onto a single release site. Following this thought Jeb-Alk would act as a regulator of the connectivity matrix of the motor neuropil significantly affecting the divergence of signal.

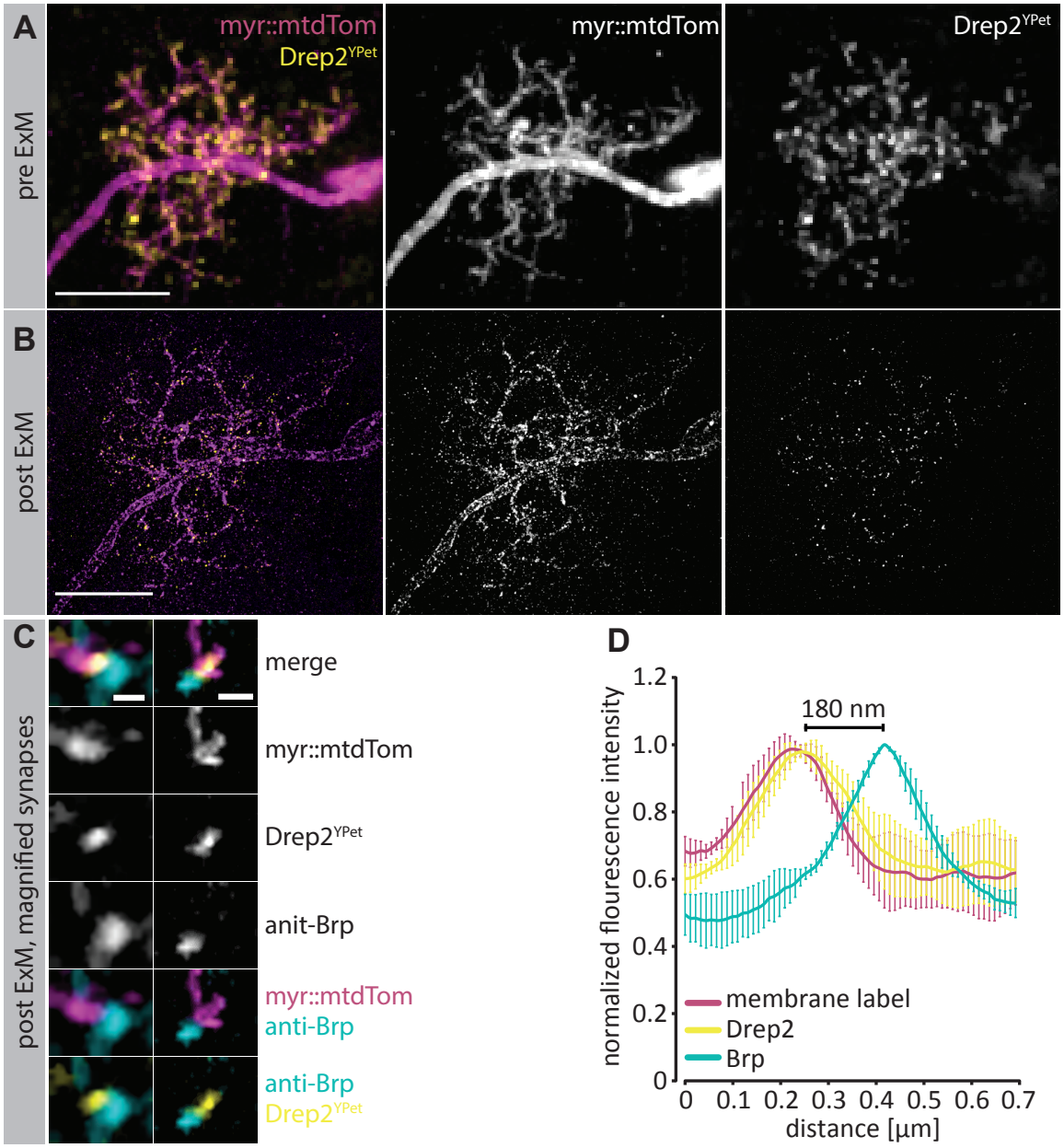

FIGURE 2.9. **Single cell expression of Drep2^{FOnYPet} allows for the quantification of post-synaptic sites. A**- RP2 motoneuron (myr::mtdTom) imaged before expansion microscopy (ExM) demonstrating dendritic localization of induced Drep2^{FOnYPet}. Scale bar 10 um. **B**- The same RP2 motorneuron as in (A) imaged after ExM revealing individual postsynaptic specializations within the dendritic arbor enabling their quantification. Scale bar 10 um. **C**- Two representative synapses along dendritic tree seen in (B). Co-staining with anti-Brp (cyan) allows the identification of mature synapses labeled with Drep2^{YPet} (anti-GFP, yellow). Membrane is labeled with myr:mtdTom (anti-RFP, magenta). Scale bar 500 nm. **D**- Normalized mean intensity profile of Drep2^{YPet} (yellow), Brp cyan) and dendritic membrane label (myr:mtdTom, magenta) across synaptic contacts imaged after ExM. Drep2^{YPet} signal recites within the dendritic membrane and the maximum locates in a mean distance of 180 nm to the maximum of anti-Brp. n=15 synapses across three RP2 dendritic arbors, five each.
Panels are adapted from Gärtig et al. 2019

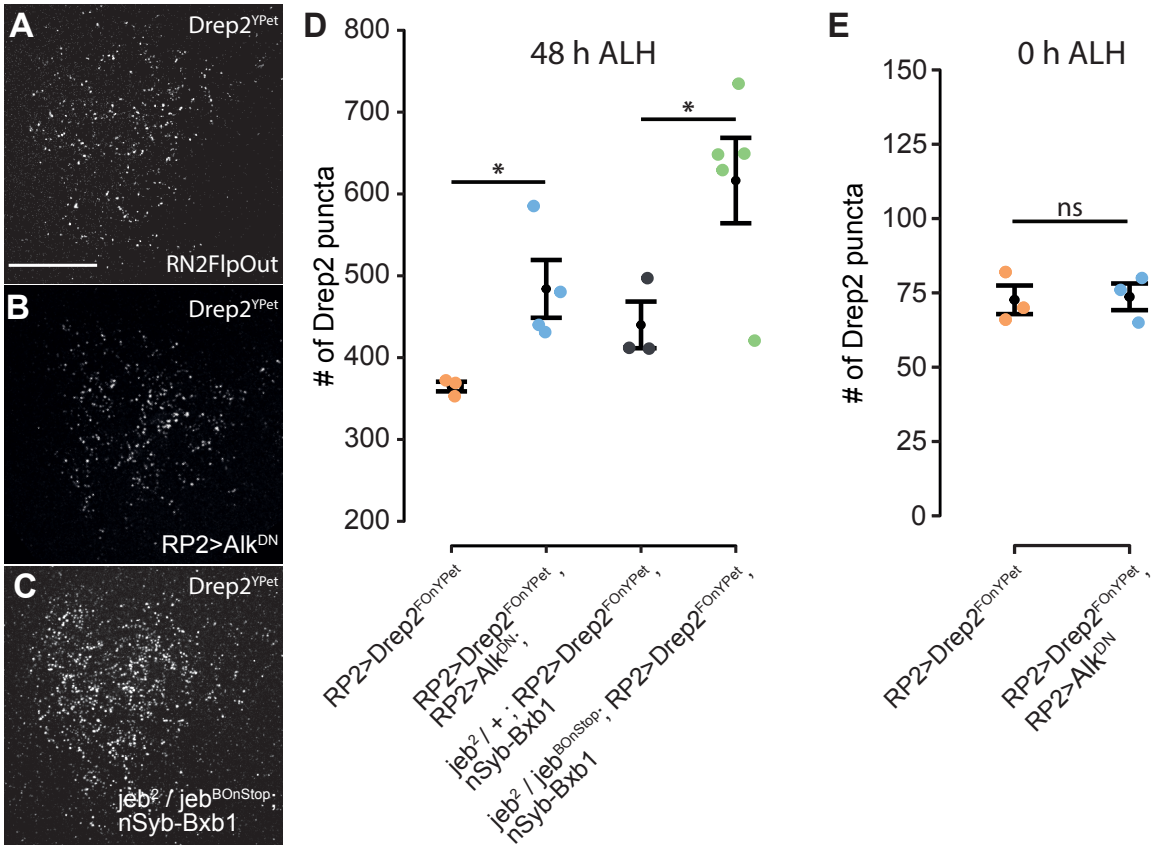

FIGURE 2.10. **Jeb-Alk Signaling is an inhibitor of postembryonic synaptogenesis.**
A-C- Images after ExM show max. intensity z-projections of endogenous Drep2YPet in RP2 dendrites in (A) control, (B) pan-neuronal jeb mutant, and (C) single cell Alk knockdown. **D**- Quantification of the number Drep2$^{(YPet)}$ juxtaposed with anti-Brp along the dendrites of RP2 motoneurons at 48 h ALH. Inhibition of Jeb-Alk signaling increases the number of postsynaptic specializations. Welch two-sample t-test *p¡0.05. **E**- Quantification of the number Drep2$^{(YPet)}$ juxtaposed with anti-Brp along the dendrites of RP2 motoneurons at 0 h ALH. Embryonic development of postsynaptic specialization is not affected by inhibition of Jeb-Alk signaling. Welch two-sample t-test.
Panels are adapted from Gärtig et al. 2019. Data produced jointly with Tatjana Kovacevic.

2.4 Dendritic growth is regulated by Jeb-Alk signaling

In 1976 Peters and Feldman related the probability of synaptic contacts to the availability of neuronal structures and the relative proximity of them (Peters and Feldman, 1976). According to the Peters rule it can be said that: [], the number of synapses increases with the coincident availability of axons and dendrites, [] (Rees et al., 2017). Transferring this hypothesis to our current model within the larval motor system allows the presumption that there is a high probability for the observed increase in postsynaptic contacts to be accompanied by an increase in dendritic structures, hence, total dendritic length. Additionally, it is intuitive, that larger dendritic arbors provide more potential space for the assembly of synapses.
In the same notion, it is accepted that the formation of synapses on neuronal out-

growth leads to a stabilization of axonal or dendritic structures, as proposed by the synaptotrophic growth theory (Cline and Haas, 2008; Niell et al., 2004). It has further been demonstrated that alterations in the synaptic input of different neurotransmitters positively affects local dendritic growth within the relevant input domains in flight motoneurons of adult *Drosophila* (Ryglewski et al., 2017). In the larval motor circuit, published data and the above described increase in Drep2 puncta demonstrate an increase RP2 synapses correlating with the growth of the dendritic arbor over the first 48 hours of larval development (Zwart et al., 2013; Couton et al., 2015). Here, of course, we are observing the growth of an entire organism with a growth of the CNS and the neurons within.
Nevertheless, there have also been experiments demonstrating a limiting effect of synpatogenesis on the growth of dendrites. Increased synaptic input during network formation through the experimental induction of additional presynaptic sites can reduce dendritic arbor size during network formation (Tripodi et al., 2008). These results contradict the basis of a solely positive trophic feedback from synaptogenesis. Furthermore, it should be noted, that an exclusively growth-enhancing effect of synapse formation cannot be unlimited, but must be confined by a upper boundary and a mechanisms enforcing said boundary.

Previous work by Jan Felix Evers has demonstrated that knock-down of Alk in single RP2 motoneurons leads to a reduction in TDL at 48 h ALh but not directly after hatching (unpublished data, Gärtig et al. (2019)). Taken together with the above established increase in Drep2 count under Alk^{DN} expression, there is no positive correlation between dendritic length and amount of postsynaptic specializations in this context. However, it remains elusive, how a pan-neuronal abrogation of Jeb-Alk signaling affects single cell development and morphology. How do possible competitive effects or effects due to changes in a neurons environment (meaning surrounding neurons) manifest in dendritic morphology?

2.4.1 Cell-autonomous and pan-neuronal Jeb-Alk inhibition regulate dendritic growth distinctively

Consequently, I set out to investigate the hypothesis that Jeb-Alk promotes dendritic growth as previously suggested by Alk knock down experiments (unpublished data, Gärtig et al. (2019)). I used the inducible jeb mutant $jeb^{BOnSTOP}$ under UAS-Bxb1 control and visualized dendritic arbor morphology using a Flpout-LexA system (RN2-Flp, tub84b-FRT-STOP-FRT-LexA.VP16, 13xLexAOp-myr::YPet; =RP2>myr::YPet). Interestingly, pan-neuronal removal of the ligand Jeb lead to an overall increase in TDL compared to the heterozygous mutant (control: + / jeb^2; nSyb-Bxb1 948.90 ±25.01

μm ; jeb^{BOnSTOP}/ jeb^{2}; nSyb-Bxb1: 1099.49 ± 26.20 μm ; figure 2.11 B, C, E). Hence, pan-neuronal abrogation of Jeb-Alk signaling results in increased dendritic growth in third instar larvae.

To verify this phenotype I aimed to knock-down Alk activity directly by expressing Alk^{DN} throughout the CNS as done for the analysis of IN$_{lat}$ morphology in section 2.2.2. Indeed, inhibition of the receptor Alk resulted in an increase in TDL, that was even more pronounced (elav-Gal4, UAS- Alk^{DN} 1186.83 ±54.02 μm , figure 2.11 D, E). The similarity of the effect of pan-neuronal Jeb and Alk inhibition again demonstrates that Alk acts directly downstream of Jeb and is the main, most likely even the only, receptor of Jeb in the developing *Drosophila* CNS. The data further confirm the direct effect of Jeb-Alk signaling on neuronal growth and morphology also on the dendritic arbor of motoneurons as for the interneurons, promoting the concept that Jeb-Alk regulates connectivity within the central motor circuit of *Drosophila*.

Importantly, neither knockdown of Alk nor targeted mutation of Jeb significantly affected dendritic targeting or the gross morphology of the RP2 dendritic arbors as seen by the localization of the entire arbor within the tissue (see figure 2.11 microscope images). Hence, I could determine an opposing phenotypic manifestation of cell-autonomous and pan-neuronal Jeb-Alk abrogation.

To further demonstrate the biological relevance of a direct Jeb-Alk signaling I analyzed the cell-autonomous dendritic arbor phenotype in more detail. First, I verified the direct effect of the knock-down Alk signaling described above (unpublished data, Gärtig et al. (2019)) by performing a rescue experiment using the RN2FlpOut-Gal4/UAS system and visualizing dendritic membranes with the marker myr::mtdtTom (Ou et al., 2008; Gärtig et al., 2019). I co-overexpressed functional Alk^{FL} with Alk^{DN}, which was able to partially rescue the growth reducing effect (control: 847.42 ± 27.19 μm , n=6; RP2>Alk^{DN}: 589.09 ±24.84 μm , n=4; RP2>Alk^{DN}, >Alk^{FL}: 695.25 ±25.56 μm , figure 2.11 F, G, H, J). Thus, physiological growth of dendritic arbors is directly depended on the cell-autonomous activation of Alk.

Lastly, I confirmed the anterograde signaling direction of Jeb-Alk by targeted activation of the conditional jeb^{BOnSTOP} mutant in RP2 motoneurons through the combination of the RN2FlpOut-Gal4/UAS with UAS-Bxb1. Here, no significant effect on arbor gross morphology or TDL was detected demonstrating that Jeb secretion by motoneurons plays no role in the activation of dendritic Alk (jeb^{BOnSTOP} / jeb^{2}; RP2>Bxb1, 859.93 ± 24.10 μm , figure 2.11).

A study on the function of Jeb-Alk signaling in adults has shown an increased body size of pupae in animals with inhibited Jeb-Alk signaling (Gouzi et al., 2011). Additionally, dendritic arbor size has been proven to correlate with larval body size measured as surface are (Zwart et al., 2013). To exclude the possibility that the observed increase in TDL at 48 h is solely a secondary effect of increased organismal growth I measured the effect of pan-neuronal Alk knock-down on the body surface of 48 h larva (figure 2.12).

There was no significant difference in body size upon Alk inhibition (control: 5.71 ± 0.21 mm^2, elav>Alk^{DN}: 5.18 ± 0.39). Therefore, the measured changes in TDL are more likely due to neuronal Jeb-Alk signaling rather than being a secondary effect of increased organismal growth.

In summary, we can make several conclusions: First, we see a correlation between the structural increases on both sides of the analyzed synaptic partners under pan-neuronal Jeb-Alk abrogation; axonal as well as dendritic structures extend. Therefore, Jeb.Alk activity non-cellautonomously limits neuronal expansion. Secondly, under pan-neuronal jeb mutation the increase in postsynaptic specializations is accompanied by a relative increase in dendritic length, thus, this correlation an example that supports the synaptotropic theory (Vaughn et al., 1974).

Third, the obvious, contradictory effect of single cell and pan-neuronal Jeb-Alk abrogation argues that postsynaptic overgrowth might be regulated at the tissue level through intercellular interaction and is less likely a cell-autonomous reaction to missing Alk activation. Here, the crucial observation is the increased presynaptic filopodia formation. Filopodia have been shown to be capable of forming early, explorative contacts as an efficient way for target discovery (Li et al., 2011; Özel et al., 2015, 2019) and it may be hypothesized that these events induce signaling to promote further dendritic growth. The correlation between axonal filopodia overgrowth and increased dendritic growth therefore provides the possibility that supernumerous axonal filopodia entice additional growth of dendritic structures.

Stunted growth under cell-autonomous knock-down of Alk could be explained as an indirect effect. Here, an ihibitory effect on dendritic elaboration might originate from increased excitatory synapse formation that occurs under reduced Alk activity. A negative effect of synaptogenesis on dendritic growth has been previously described (Tripodi et al., 2008). This hypothesis argues against the possibility that Alk activation directly promotes dendrite growth, which is supported by the dendritic overgrowth measured under pan-neuronal jeb mutants or Alk knock-down.

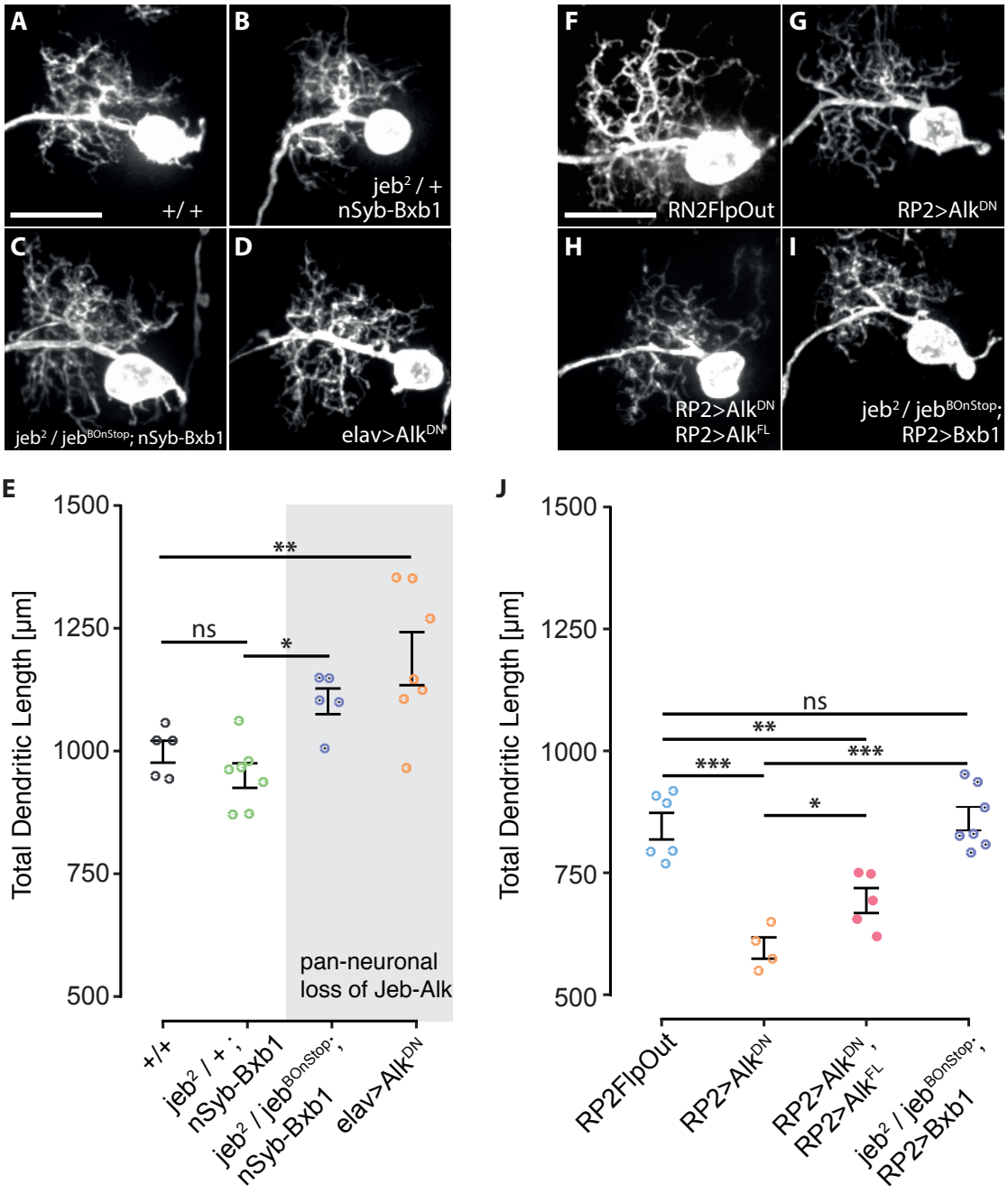

FIGURE 2.11. **Jeb-Alk signaling regulates growth of RP2 motoneuron dendrites. A-E**- Pan-neuronal abrogation of Jeb-Alk signaling via neuron-specfic mutation of jeb (C, nSyb>jeb*BOnSTOP*) or knock-down of Alk (D, elav>Alk*DN*) result in larger dendritic arbors compared to controls (A). RP2>myr::YPet was used to visualize dendrites and quantified (E) after reconstruction. **F-J**- Cell-autonomous Alk activity is required for normal dendritic growth as control arbor (F) are larger than cell-autonomous Alk inhibition (G, RP2>Alk*DN*). This effect can partially be rescued by co-overexpression of *Alk*DN*and*Alk*FL* (H). Removal of Jeb from motoneurons has no effect (I, RP2>jeb*BOnSTOP*). RP2>myr::mtTomato2 was used to visualize dendrites and quantified (J) after reconstruction
Data on control in (F) and RP2>Alk*DN* (G) produced by Barbara Chwalla. Panels are adapted from Gärtig et al. 2019

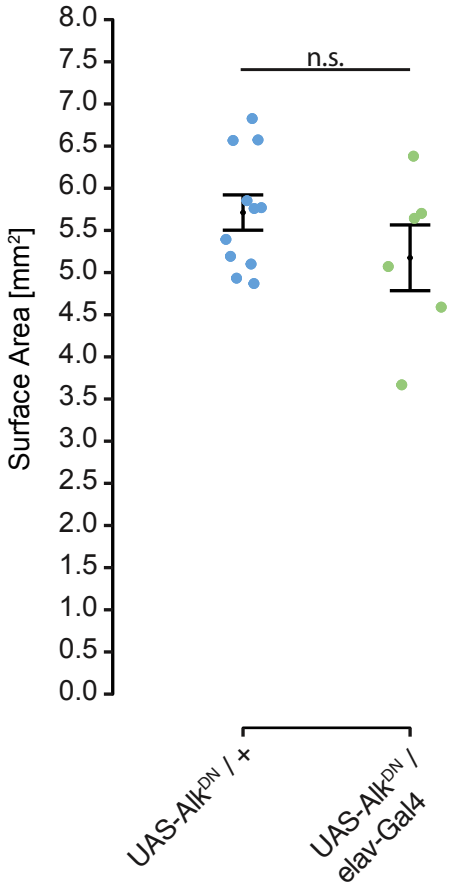

FIGURE 2.12. **Body size is not affected by pan-neuronal Alk^{DN} expression.** Larva at 48 h ALH were imaged and their body surface area was calculated from body length and width using the formula described in method section 4.2.7.3.
Panels are adapted from Gärtig et al. 2019. Data produced jointly with Jan Felix Evers.

2.4.2 *in vivo* imaging demonstrates exclusively postembryonic action of Alk for dendritic growth

The analysis of dendritic arbors at 48 h gives limited information on the postembryonic growth and development of these arbors. While this analysis clearly shows the final effect on overall tree length and structure no conclusions on early arbor development, growth dynamics or branch stability can be obtained. Additionally, the analysis of postsynaptic connectivity described above revealed an exclusively postembryonic effect of Jeb-Alk manipulations (figure 2.10) highlighting the importance of an analysis over time.

Therefore, I made use of *in vivo* imaging to describe the developmental aspects of of Jeb-Alk dependent dendritic growth (see section 4.2.2). During my Master thesis I was able to establish this technique as an effective way to perform quantitative analysis of dendritic growth dynamics at 24-hour intervals (Gärtig, 2016). Using this method, I provided a description of ta reduction of dendritic growth dynamics under cell-autonomous

Alk knock-down. However this analysis was preliminary and included only a small number of observations. Furthermore, any analysis on pan-neuronal Jeb-Alk abrogations were missing completely.

From the stated differences in pan-neuronal and single cell abrogation of Jeb-Alk signaling (reduced vs. increased total dendritic length) the question logically arises how dendritic growth dynamics are distinctively affected under these conditions. Since the smaller, Alk^{DN} expressing RP2 dendritic arbors exhibit a reduced formation of new branches, do larger arbors form new branches at a faster rate? Do stable branches elongate at a similar rate or more quickly? Do newly formed branches have a higher probability of stabilization? How does the changed environment (more presynaptic filopodia) affect branch dynamics?

To investigate the growth dynamics under pan-neuronal Jeb abrogation I combined the conditional $jeb^{BOnSTOP}$ mutant under nSyb-bxb1 control with the membrane marker myr:mtdTom. This was necessary in order to get a sufficient image quality when imaging through the animals cuticle (as compared to myr:YPet used for acutely dissected imaging) and to ensure comparability with the results previously obtained (Gärtig, 2016).

To determine the developmental importance of Jeb-Alk signaling in neuronal growth I firstly analyzed the dendritic growth curve from 0 h ALH over 24 h to 48 h ALH. Pan-neuronal loss of Jeb had no detectable effect on RP2 TDL at 0 h ALH, similarly to single cell Alk knock-down. (control: 184.99 ± 6.77 m; RP2> Alk^{DN}: 193.48 ± 8.29 μm ; $jeb^{BOnSTOP}$/ jeb^{2}; nSyb-Bxb1: 186.25 ± 4.58 μm ; figure 2.13). Hence, Jeb-Alk seems to not act during initial circuit formation as neither manipulations elicit a measurable dendritic phenotype at 0 h ALH. However, it has to be noted, that a successful activation of $jeb^{BOnSTOP}$ cannot be visualized. Therefore, it cannot be ruled our, that un-truncated Jeb is present in some neurons, however data from the Evers lab has demonstrated the high penetration of a Bxb1-induced flourophore already in first instar animals (unpublished data).

During the first 24 hours of postembryonic development the pan-neuronal loss of jeb causes a slightly quicker increase in dendritic length resulting in an 8% larger TDL at 24 h ALH compared to control neurons, while Alk^{DN} expression reduces arbor size (control: 404.40 ± 23.58 μm ; RP2>Alk^{DN}: 334.91 ± 16.35 μm ; $jeb^{BOnSTOP}$/ jeb^{2}; nSyb-Bxb1: 435.2 ± 20.77 μm ; figure 2.13) This difference increases in the subsequent 24 hours to a significantly 31%-longer mean TDL under pan-neuronal jeb loss when compared to control (control: 807.95 ± 36.52 m; RP2>Alk^{DN}: 673.81 ± 42.774 μm ; $jeb^{BOnSTOP}$/ jeb^{2}; nSyb-Bxb1: 1056.91 ± 69.18 μm ; figure 2.13). These results indicate the relevance of Jeb-Alk signaling during postembryonic circuit expansion and maintenance. They further demonstrate that the distinct effects of single cell knock-down and pan-neuronal jeb mutation already manifest before 48 h ALH but increase during larval development.

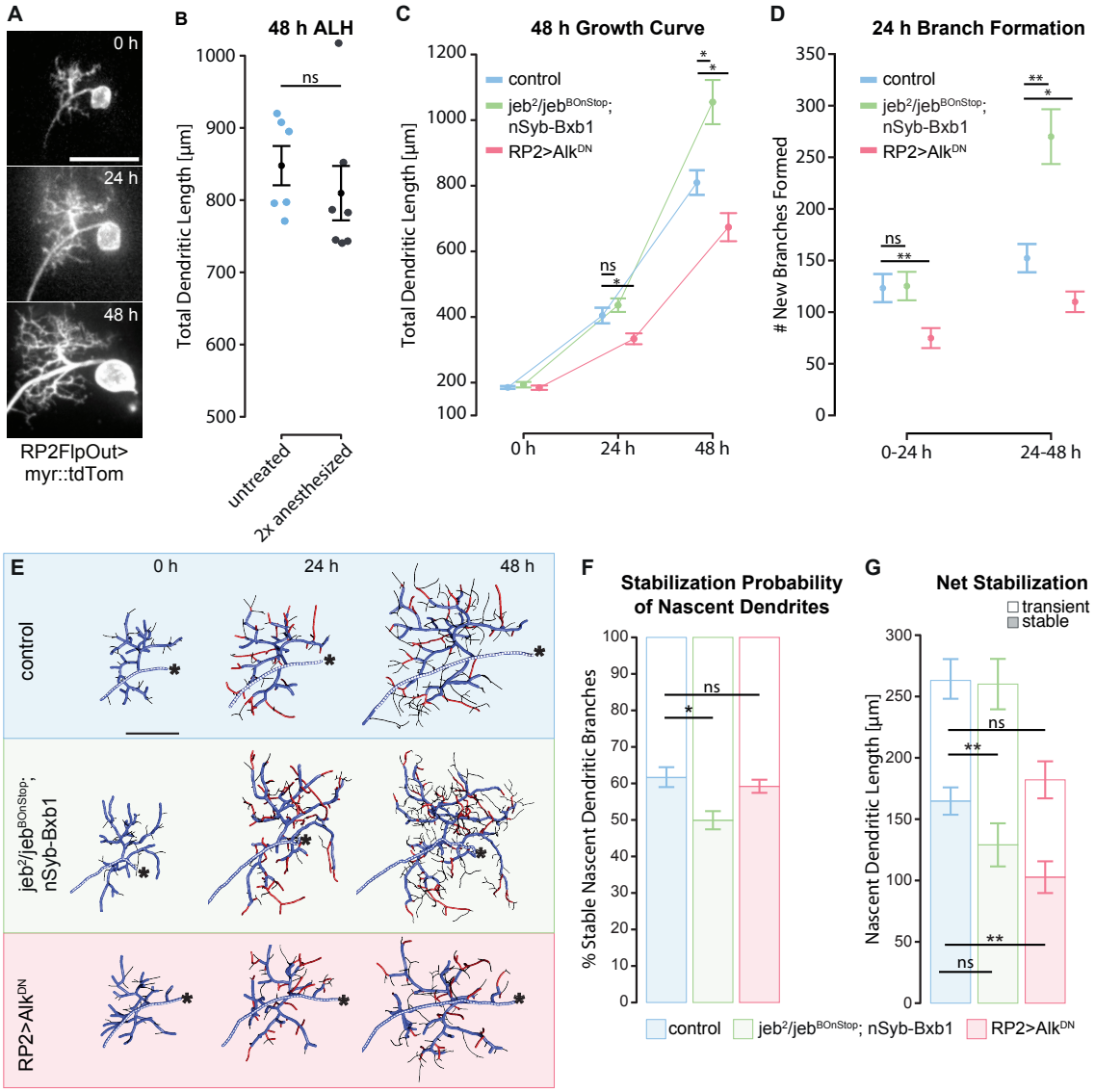

FIGURE 2.13. ***In vivo* imaging of RP2 dendritic turnover dynamics during postembryonic development. A**- Timeline of the dendritic arbor of an RP2 motoneuron from *in vivo* imaging. Image stacks at 0 an 24 h ALH were acquired from anesthetized larvae, the 48 htime point was from an acutely dissected VNC. Scale bar 20μm **B**- Comparison of the total dendritic length of anesthetized and untreated larvae. Anesthetization does not alter dendritic growth significantly. **C**- Growth curve of total dendritic length of RP2 motoneurons obtained from *in vivo* imaging. From 24 hours on manipulations on eb-Alk signaling affect dendritic length. Expression of Alk^{DN} (red) reduces postembryonic dendritic growth and pan-neuronal jeb mutation (green; jeb^{BOnSTOP}/ jeb^{2}; nSyb-Bxb1) increases dendritic length in comparison to control (blue). **D**- Analysis of branch formation from the tracing of single branches (see section 4.2.7.1). Expression of Alk^{DN} (red) reduces branch formation. Pan-neuronal induction of jeb mutation (green; jeb^{BOnSTOP}/ jeb^{2}; nSyb-Bxb1) causes an increase in branch formation after 24 hours when compared to control (blue). Caption continued on next page.

FIGURE 2.13. Caption continued from previous page.

E- 3D reconstructions of RP2 arbors demonstrate the dynamic branches of the arbors. Dendritic branches that persisted over 48 h (blue), branches that formed after the first image, and stabilized until 48 h ALH (red) and the remaining, more motile or younger branches (black). Asterisk marks the cell body. **F**- Stabilization probability. Barplots show the percentage of dendritic branches that newly formed after 0h ALH, and persisted (filled). Expression of Alk^{DN} (red) has no effect, while pan-neuronal loss of Jeb (green) reduces stabilization probability of dendritic branches when compared to control (blue). **G**- Summed length of dendritic branches that newly formed from 0 to 24h ALH and remained stable until 48 h ALH (filled), or retracted later (clear).

Blue control (RP2>myr::mtdTom); Green - pan-neuronal loss of Jeb (nSyb>jeb$^{(BOnStop)}$); red RP2 cell-autonomous knockdown of Alk (RP2>Alk^{DN}). *p<0.05, **p<0.01, ***p<0.001, ns not significant.

Panels are adapted from Gärtig et al. 2019. Some data points of control and Alk^{DN} originates from Gärtig (2016) and was reanalyzed for this thesis. Data on untreated animals was produced by Barabra Chwalla.

2.4.3 Dendritic dynamics correlate with presynaptic branch formation

In order to address the dynamics of dendritic growth, I identified individual branches across the entire arbor and tracked these individual branches during arbor growth over the time frame of *in vivo* imaging described above, the first 48 h of postembryonic development (for detail regarding the analysis see section 4.2.7.1). This tracking allowed quantitative analysis of branch formation, branch retraction and branch stabilization. Importantly, the 24-hour intervals are sufficiently short so that enough structure of the dendritic arbor persists allowing motile branches to be discerned as I showed previously (Gärtig, 2016).

In general, there is a substantial difference in the stabilization probability of dendrites versus presynaptic filopodia: 61.74 ± 2.72 % of newly formed branches in the first 24 hour interval (0h-24h ALH) persist until 48h ALH (2.13), while only 34.5% of presynaptic filopodia of IN_{lat} were detected twice (figure 2.6).

Previously, I described that cell-autonomous knock-down of Alk in single RP2 motoneurons reduces the formation of new branches (Gärtig, 2016), which I confirmed with more data points and a more stringent analysis (RP2>Alk^{DN}: 0-24h: 74.86 ± 25.62 branches, n=7; 24-48 h: 110.00 ± 26.36 branches, n=7, figure 2.13), without significant effect on their stabilization probability (RP2>Alk^{DN}: 59.24 ± 1.80%, n=7, figure 2.13). These results described a specific, cell-autonomous and negative effect of lacking Alk activity on the formation of new branches and hence dendritic elaboration.

The larger arbors under the pan-neuronal mutation of jeb observed at 48 h ALH introduced the hypothesis, that branch formation, branch stability or both should be increased. The analysis of dendritic arbors under pan-neuronal jeb loss in detail revealed that the significantly more elaborate trees at the same time exhibit a significantly increased rate of branch formation from 24 h to 48 h ALH (control: 123.29 ±

36.07 branches, n=7; jeb^{BOnSTOP}/ jeb^{2}; nSyb-Bxb1: 270.00 ± 53.08 branches, n=4). Surprisingly, this genotype was also associated with a reduced probability of stabilization for newly formed branches when compared to dendrites of control neurons (control: 61.74 ± 2.72%, n=7; jeb^{BOnSTOP}/ jeb^{2}; nSyb-Bxb1: 49.95 ± 2.49%, n=4, figure 2.13). As a consequence, although branch formation is increased, the total length of new structures at 24 h ALH persisting until 48 h ALH is around 20% less demonstrating that pan-neuronal removal of jeb destabilizes postsynaptic structures *in vivo* (control: 167.36 ± 11.38 μm , n=7; jeb^{BOnSTOP}/ jeb^{2}; nSyb-Bxb1: 134.60 ± 16.67 μm , n=4, figure 2.13). Nonetheless, this reduced branch stabilization is off-set by the striking increased branch formation leading to an overall larger dendritic arbor.

To put this into a tissue-wide context, it is to be noted that the increased dendritic branch formation correlates with the observed increased formation of presynaptic filopodia under pan-neuronal loss of jeb (figure 2.13). Thus, a possible explanation summarizing the opposite phenotypes of single cell Alk^{DN} and pan-neuronal jeb loss as well as the correlation of added pre- and postsynaptic structures could be the following: It is conceivable that presynaptic filopodia are capable of inducing dendritic branches leading to an increased possibility that existing release sites along the axon are contacted by a dendrite. Subsequently, synaptogenesis may occur increasing both dendritic length and the number of postsynaptic specializations. Physiologically, undisturbed Jeb-Alk signaling at an established synapse potentially inhibits the formation of further presynaptic filopodia, also reducing the probability of dendritic branch formation. Therefore, Jeb-Alk signaling functions to induce a negative feedback on neuronal expansion and addition of intercellular connections. In coherence with this, an increased formation of postsynaptic specializations as marked by Drep2 (single-cell Alk knock-down) at the same time reduces the sensitivity to branch induction by filopodia. This logic nicely explains the observed smaller dendritic arbor in light of a normal density of presynaptic filopodia.

Consequently, Jeb-Alk signaling prevents an uncontrolled expansion of dendritic growth and synapse formation by providing a negative feedback to the presynaptic cells. The nature of this negative signal, which is induced by Alk activation, is however not yet identified and should be the subject of future work to elucidate a critical mechanism regulating neuronal expansion in order to produce stable circuits.

2.5 Correlation between network activity and neuronal mophology an connectivity

In the previous sections I described Jeb-Alk as a novel molecular mechanism that orchestrates the expansion of neuronal circuits. Growth curve analysis of dendritic

trees as well as quantification of postsynaptic specializations in early and later larval life suggest a specificity of Jeb-Alk signaling for postembryonic development. The amount of synaptic contacts decreases when Alk signaling is inhibited demonstrating as negatively regulatory function of the pathway. Carlo Giachello and Richard Baines at the University of Manchester analyzed the electrophysiological and behavioral characteristics of animals devoid of Jeb-Alk signaling (pan-neuronal jeb^{BOnSTOP}) (Gärtig et al., 2019). They demonstrated that at 48 h ALH but not 0 h ALH excitatory spontaneous rhythmic currents in RP2 show increased durations. This observation is reminiscent of the electrophysiological properties of epilepsy-like models in *Drosophila* Giachello and Baines (2015); Giachello et al. (2019). Larvae of epilepsy-like *Drosophila* models have been described to be less resilient to network manipulations: After an electroshock they show extended recovery times until the animal takes up normal crawling again. Similarly, animals with jeb -/- CNSs exhibit such a drawn-out recovery time (Gärtig et al., 2019).

Taken together these observations imply several points. For one, these measurements further strengthen the argument that Jeb-Alk is specific for postembryonic development, a period of massive organismal growth accompanied by correlating neuronal growth and thus expansion of neural circuits and their connectivity. Jeb-Alk further limits synaptogenesis of motoneurons in the CNS and thus apparently synaptic excitation, too. Secondly, they demonstrate a functional relevance of neurodevelopmental processes depending on Jeb-Alk signaling. The observed morphological phenotypes of the synaptic partner analyzed result in altered electrical properties of motoneurons. Consequently, under pan-neuronal manipulations the properties of the motor circuit exhibits changed and the nervous system is less resilient to disturbances. Taken together, my detailed morphological analysis constitutes a first correlation of epilepsy-like behavior and altered neuronal connectivity, which is clearly true for a nervous system devoid of jeb.

On the grounds of these conclusion, I followed up with the question whether the observed morphological alterations regarding dendritic length and synaptic input, represent a common feature of epilepsy-like models in *Drosophila* or, more generally, is hyperconnectivity a characteristic of a less stable network. The next sections are therefore dedicated to a detailed morphological analysis of motoneurons in a pharmacologically induced epilepsy-like model based on exposure to the neurotoxin picrotoxin during embryonic development.

2.5.1 Embryonic overactivation of the central nervous system during a sensitive period

Nervous system development occurs in distinct steps. Neurons have certain developmental capabilities at various developmental stages. For example, the cellular response to

neural activity can be greater or smaller depending on the age of an organism (Hensch, 2005, see). Possible cellular responses include the outgrowth of neural protrusion or adjustments in synaptic connectivity. These plastic capabilities of neurons are limited in later development. This closure of periods of heightened plasticity likely ensures a balance between stability and plasticity. These periods of increased plasticity have been termed critical periods, as they greatly influence the development of the CNS long-term (Hensch, 2005). They have therefore been implied to be important during the emergence of mental disorders like epilepsy, schizophrenia or autism spectrum disorders. Critical periods highlight the pivotal role of neural activity for the adequate development of neuronal networks.

Intrinsic, spontaneous activity is for example crucial for activity-dependent developmental processes during the assembly of neuronal circuits (Blankenship and Feller, 2010; Kirkby et al., 2013). The presence and the importance of spontaneous activity has been demonstrated in motor circuits of vertebrates and invertebrates (Nishimaru et al., 1996; O'Donovan et al., 1998; Warp et al., 2012; Crisp et al., 2008, 2011; Myers et al., 2005; Borodinsky et al., 2004). For the development of the *Drosophila* larval motor circuit a critical period exist from 17 to 19 h after egg laying, where neural activity greatly influences the further development the animal. Here, proper activity is necessary for the onset of peristaltic movement of the embryo (Crisp et al., 2011) and activity manipulation change the excitability of motoneurons (Giachello and Baines, 2015; Giachello et al., 2019).

These activity manipulations of Giachello and colleagues result in behavioral phenotypes reminiscent of epilepsy-like models. Both genetic and chemical-induced epilepsy-like models exist in *Drosophila*. Genetically, a set of mutations, the so-called bang-sensitive mutants result in nervous systems that are less resilient to overexcitation. More importantly, it has been demonstrated that non-physiological activity levels during the critical period create a network that is more susceptible to destabilizing manipulations (Giachello and Baines, 2015, 2017): wild-type animals treated with the known proconvulsant picrotoxin (PTX) explicitly during embryogenesis show a larval seizure phenotype. This seizure phenotype is measured as the recovery time post electroshock and resembles the published epilepsy-like models of the bang-sensitive mutant family (Giachello and Baines, 2015). Both, genetic precondition as well as acute, drug-induced activity manipulations, produce less stable networks. Therefore, intrinsic activity during the critical period is pivotal for the emergence of proper network function. How or whether at all network stability, or lack thereof, is manifested in anatomical properties (cell morphology, synaptogenesis; similar to Jeb-Alk manipulations) and to what degree this is set by embryonic neural activity is unclear.

Plasticity of a neuronal circuit in response to activity can manifest via in changes in neuronal connectivity. Plastic connectivity itself can be achieved in different ways. On the one hand synaptic plasticity, which is the strengthening or weakening of single

synapses, on the other hand structural plasticity, meaning the formation and degradation of cellular outgrowths (axon, dendrite) or synapses (Fauth and Tetzlaff, 2016). Jeb-Alk manipulations resulted in an increase in the number of postsynaptic sites in single motoneurons, but no matching increase in release site number presynaptically. This variability demonstrates a postsynaptic, structural plasticity in response to a developmental perturbation but no matching presynaptic structural plasticity. Consequently, I aimed to elucidate whether postsynaptic plasticity is a way of expressing network properties under different manipulations. Here, we build on the data from Giachello et al. (2019) (preprint), where they demonstrated that (1) activity manipulation during embryogenesis (e.g. PTX feeding) affects synaptic excitation of motoneurons, (2) manipulation of a specific, cholinergic premotor interneuron (A27h) is sufficient to induce network instability and (3) pan-neuronal activity perturbation reduces synaptic transmission of A27h interneuron (A27h) to aCC motoneurons. Together with the presented findings on postsynaptic specializations under Jeb-Alk manipulation and the simultaneous epileptic phenotype we set out to find biological correlations. Do epilepsy-like behaviors correlate with increased postsynaptic proliferation? Are presynaptic release site numbers affected by the treatment with a proconvulsant?

2.5.2 Dendritic structure is unaffected by embryonic activity manipulations

Across various organisms it has been shown that activity affects neuronal growth (Fauth and Tetzlaff, 2016). Therefore, I investigated in collaboration with bachelor students Franz Bauer and Eunchan Lee whether dendritic development is affected by overexcitation of the CNS during embryonic development. Over-excitaiton was achieved by feeding the gamma-aminobutric acid (GABA) receptor inhibitor PTX to gravid females. As mentioned above, we focused on the dendritic arbors of aCC and RP2 motoneurons across larval development by analyzing total dendritic length (TDL) at 0 h as well as 48 h ALH (figures 2.14 and 2.15). Interestingly, neither aCC nor RP2 motoneurons showed a significant difference in TDL in first instar larvae (0h ALH: RP2 control: 231.00 $\pm$ 13.04 μm ; RP2 +PTX: 218.00 $\pm$ 4.98 μm ; aCC control: 326.40 $\pm$ 11.68 μm ; aCC +PTX: 331 $\pm$ 12.72 μm). Dendritic arbors show no changes in overall dendritic structure and are normally localized within the VNC. Further, dendritic growth is also unaffected as TDL in third instar animals was not significantly changed (48 h ALH: RP2 control: 722 $\pm$ 42.07 μm ; RP2 +PTX: 845 $\pm$ 51.09 μm ; aCC control: 1001.56 $\pm$ 64.43 μm ; aCC +PTX: 1034.33 $\pm$ 48.43 μm). In conclusion, dendritic growth of RP2 and aCC motoneurons is unaffected by increased embryonic activity levels. Neither an acute affect shortly after hatching nor a long-term change in dendritic length (48 h ALH) was detected.

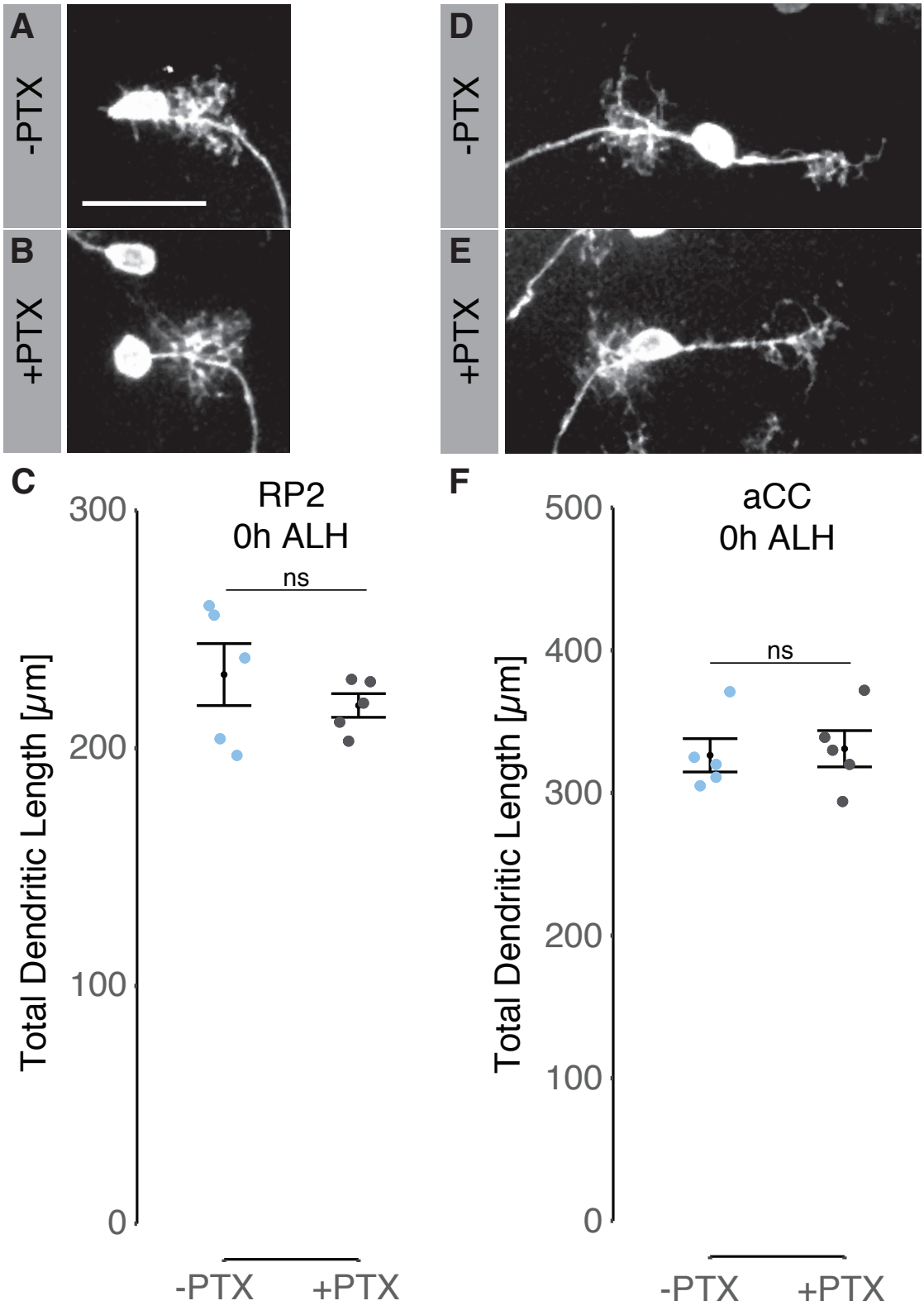

FIGURE 2.14. **Exposure to picrotoxin does not affect embryonic dendritic growth. A,B**- Max. intensity z-projections of RP2 dendrites labeled with myr::mtdTomato2 in (A) control animals and (B) offspring of PTX fed adults at 0h ALH.. **C**- Total dendritic tree length of RP2 motoneurons at 0 h ALH reveals no effect of picrotoxin treatment. **D,E**- ax. intensity z-projections of aCC dendrites labeled with myr::mtdTomato2 in (A) control animals and (B) offspring of PTX fed adults at 0h ALH. **F**- otal dendritic tree length of aCC motoneurons at 0 h ALH reveals no effect of picrotoxin treatment.
Scale bar 20 μm . *=p<0.05; ns not significant. Data produced jointly with Franz Bauer.

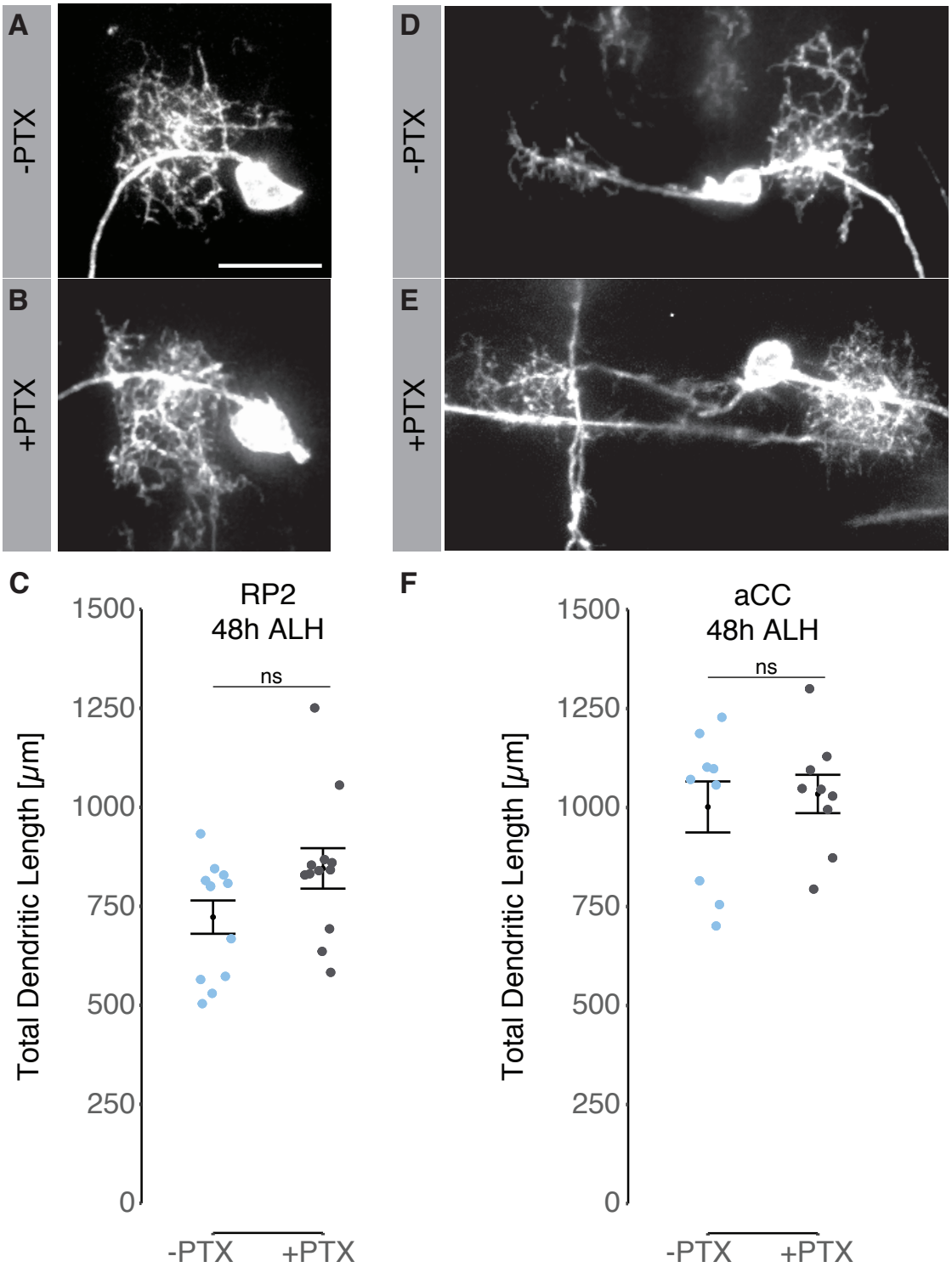

FIGURE 2.15. **Activity manipulations during embryogenesis by exposure to picrotoxin have no effect on the dendritic development of motoneurons at 48 h ALH. A,B-** Max. intensity z-projections of RP2 dendrites labeled with myr::mtdTomato2 in (A) control animals and (B) offspring of PTX fed adults at 48 h ALH. **C-** Total dendritic tree length of RP2 motoneurons at 48 h ALH reveals no effect of picrotoxin treatment. **D,E-** Max. intensity z-projections of aCC dendrites labeled with myr::mtdTomato2 in (A) control animals and (B) offspring of PTX fed adults at 48 h ALH. **F-** Total dendritic tree length of aCC motoneurons at 48 h ALH reveals no effect of picrotoxin treatment.
Scale bar 20 μm . *=p<0.05; **=p<0.01. Data produced jointly with Franz Bauer.

2.5.3 Embryonic activity manipulations affects connectivity of RP2 but not aCC long-term

Above (see sections 2.3.3 and 2.4.1) I demonstrated that dendritic growth and the number of synaptic sites in dendritic arbors can be regulated independently; a smaller arbor (RP2>Alk^{DN} in figure 2.11) can carry more postsynaptic sites (RP2>Alk^{DN} in figure 2.10). Therefore, I set out to investigate whether embryonic activity manipulations can translate to changes in the number of postsynaptic specializations as measured by Drep2 puncta despite unaffected dendritic length.

For this experiment, I expressed the endogenous, conditionally YPet-tagged Drep2 protein as described earlier (figures 2.9 and 2.10). Similarly, the number of Drep2 puncta in RP2 and aCC arbors was quantified after immunohistochemistry and expansion microscopy. First, we evaluated motoneurons at 48 h ALH. For RP2 motoneurons a clear increase in the number postsynaptic specializations of around 30% on average was detected (48 h ALH: RP2 control: 589.00 ± 37.38 Drep2 puncta, n = 7 neurons; RP2 +PTX: 763.71 ± 19.98 Drep2 puncta, n = 7; figure 2.16 A, B, C).

At the same time, aCC motoneurons, show a significant increase in the number of postsynaptic sites, too (aCC control: 988.89 ± 48.50, n = 9; aCC +PTX: 1170.00 ± 48.04, n = 8; figure 2.16 D, E, F). Here, the relative increase from control to PTX treated animals was a marginally lower with around 20%. The absolute increase was however slightly higher, which relates to the overall higher number of synapses along the larger dendritic arbors of aCC neurons compared to RP2.

Taken together, a CNS-wide over-activation during a sensitive period of neural development results in a significant increase in the number of postsynaptic specializations that form during larval development. It should be noted, that this data only includes two types of motoneurons, and we observed a difference in the strength of the effect between RP2 and aCC. Hence, the impact on other neurons was not studied and could potentially differ. To what degree the observed effect is a direct consequence of increased embryonic activity or the relative reduction activity post PTX exposure is unclear.

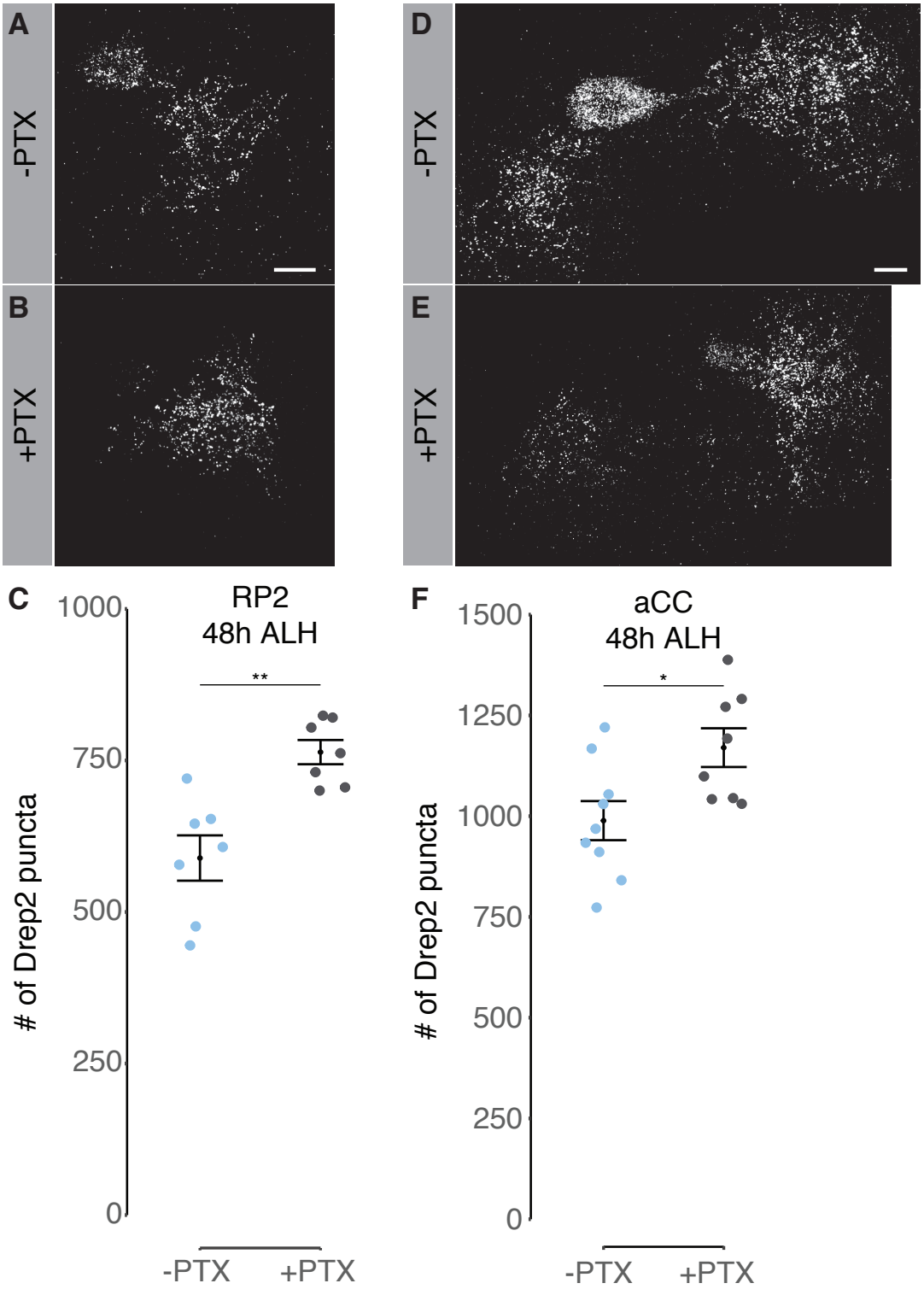

FIGURE 2.16. **Exposure to picrotoxin significantly increases number of synapses onto motoneurons at 48h ALH. A,B**- Images after ExM show max. intensity z-projections of endogenous Drep2^{YPet} in RP2 dendrites in (A) control animals and (B) offspring of PTX fed adults at 48h ALH. **C**- Quantification of the number Drep2^{YPet} juxtaposed with anti-Brp along the dendrites of RP2 motoneurons at 48 h ALH shows significant increase in the mean number of postsynaptic specializations. **D,E**- Images after ExM show max. intensity z-projections of endogenous Drep2^{YPet} in aCC dendrites in (A) control animals and (B) offspring of PTX fed adults at 48h ALH. **F**- Quantification of the number Drep2^{YPet} juxtaposed with anti-Brp along the dendrites of aCC motoneurons at 48 h ALH shows a significant increase in the mean number of postsynaptic specializations.
Scale bar 20 μm . *=p<0.05; ns not significant. Data produced jointly with Franz Bauer and Eunchan Lee.

2.5.4 Already early synaptogenesis is affected by picrotoxin manipulations

Next, I aimed to investigate the developmental aspect of embryonic activity manipulations in more detail. I demonstrated earlier that the input onto RP2 motoneurons increases several-fold over the first 48 hours of development (figure 2.10). Is the increased input onto motoneurons already manifested early on or rather an adaption during postembryonic development?

In regards to electrophysiological properties of motoneurons Richard Baines has elaborated on the set-point theory, which states that a certain level of excitation is established during a sensitive period of development, which will then be maintained throughout the animals life. Thus, an altered activity level during this sensitive period has a sustained effect on cellular properties (Giachello and Baines, 2017).

In coherence with this theory, the following scenario for the number of synaptic contacts along dendritic arbors of motoneurons is feasible: RP2 motoneurons could adapt their connectivity throughout larval life to the absence of the hyper-excitation that we induced during the sensitive period by increasing synaptogenesis. Here an effect on synapse number directly after hatching would not necessarily be expected. Another scenario would be that an increased number of synaptic contacts is already induced during embryogenesis as a direct effect of increased neural activity on single cell connectivity. Therefore, more synaptic input would be seen at 0 h ALH. This difference could subsequently be maintained or even amplified by circuit expansion during larval development.

In electrophysiological measurements Giachello and Baines (2015) described aberrant synaptic excitation of aCC and RP2 motoneurons in larvae after an over-excitation during the sensitive period using optogenetic tools. They show an increased duration, but decreased frequency of spontaneous rhythmic currents, which constitute cholinergic excitatory input (Giachello and Baines, 2015). Importantly, this is true for third instar as well as first instar larvae. Accordingly, they describe an early adaptation of neuronal characteristics that is maintained throughout larval life and cannot be adjusted despite normal neural activity. Above, I demonstrated that late larval seizure phenotype is accompanied by an increase in synaptic input. Are early electrophysiological changes also accompanied by a corresponding increase in the number of postsynaptic specializations marked by Drep2?

Indeed, we find that the number of postsynaptic specializations is affected by embryonic PTX treatment already at 0h ALH. On the one hand, RP2 motoneurons exhibit approximately 13% increase in synaptic input on average, however this increase in not statistically significant (0 h ALH: RP2 control: 93.50 $\pm$ 5.12; RP2 +PTX: 105.40 $\pm$ 5.91; figure 2.17 A-C). On the other hand, aCC motoneurons increase their postsynaptic specializations by a significant 22% (aCC control: 151.63 $\pm$ 8.95; aCC +PTX: 185.17

± 11.03; figure 2.17 D-F). As for the data on synaptic connectivity at 48 h ALH, we observe a difference in the strength of the effect on RP2 and aCC motoneurons. A reduction in inhibitory activity due to the blocking of GABA-receptors with PTX increases the amount of excitatory, cholinergic input onto RP2 and aCC motoneurons during embryogenesis. Consequently, this results supports the theory that increased neural activity promotes excitatory synaptogenesis during embryonic development.
Taken together, I demonstrated that over-excitation of the entire nervous system results in an increased synaptogenesis along the dendritic arbors of RP2 and aCC motoneurons during development. This increase occurs already during embryogenesis as Drep2 puncta count was elevated at 0 h ALH. Subsequently the relative increase is maintained in a comparable strength over the next 48h of development. Therefore, the depletion of PTX over time, that should result in a return of GABAergic inhibition and a subsequent reduction in overall neural activity, might have limited influence of synaptogenesis. It rather seems that the amount of excitatory input is set by neural activity during embryogenesis and scales with organismal and neuronal growth afterwards.

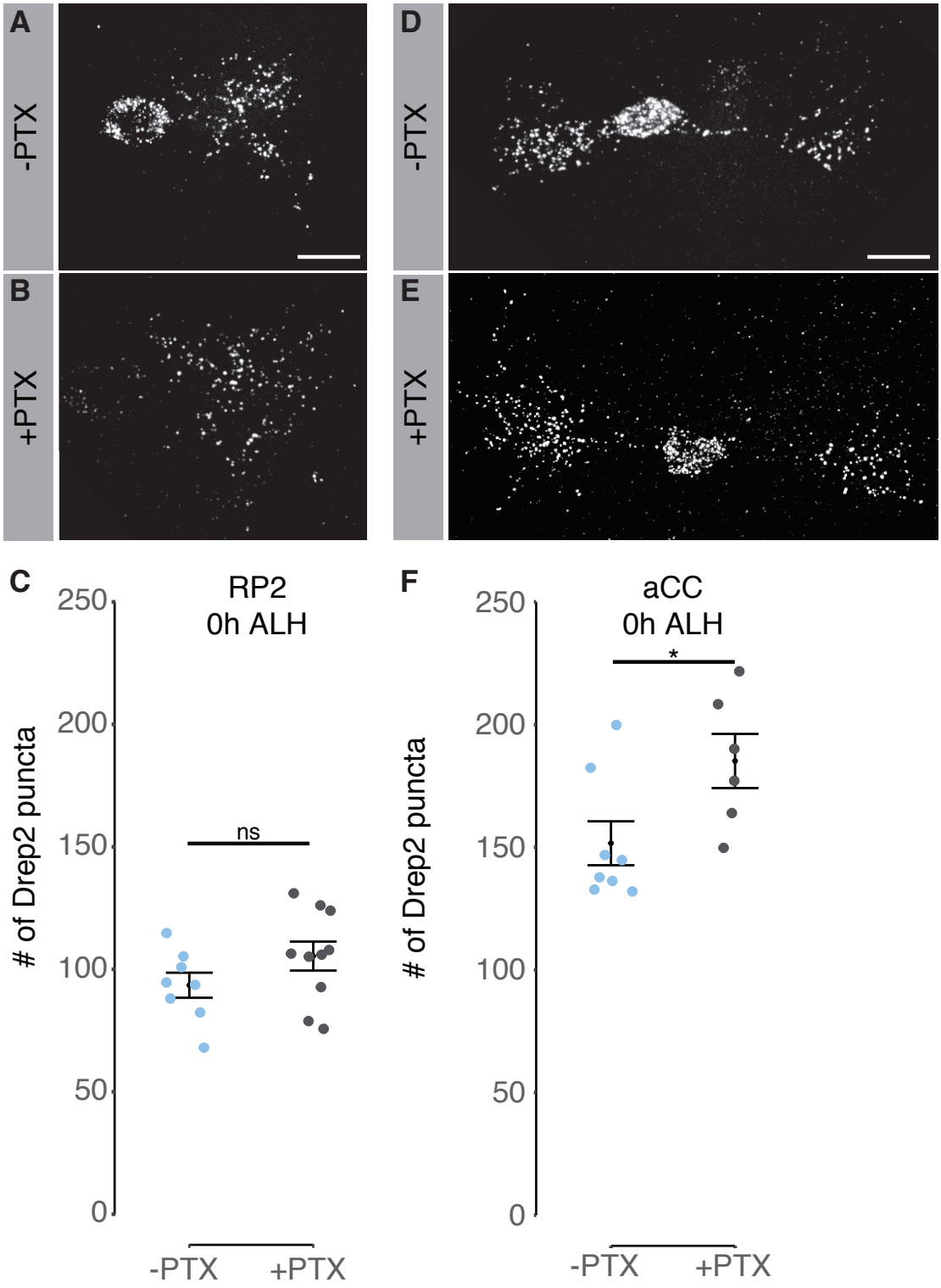

FIGURE 2.17. **Exposure to picrotoxin during embryogenesis affects synaptogenesis in motoneurons. A,B**- Images after ExM show max. intensity z-projections of endogenous Drep2^{YPet} in RP2 dendrites in (A) control animals and (B) offspring of PTX fed adults at 0h ALH. **C**- Quantification of the number Drep2^{YPet} juxtaposed with anti-Brp along the dendrites of RP2 motoneurons at 0 h ALH shows a slight but insignificant increase in the mean number of postsynaptic specializations. **D,E**- Images after ExM show max. intensity z-projections of endogenous Drep2^{YPet} in aCC dendrites in (A) control animals and (B) offspring of PTX fed adults at 0h ALH. **F**- Quantification of the number Drep2^{YPet} juxtaposed with anti-Brp along the dendrites of aCC motoneurons at 0 h ALH shows a significant increase in the mean number of postsynaptic specializations.
Scale bar 20 μm . *=p<0.05; ns not significant. Data produced jointly with Franz Bauer and Eunchan Lee.

2.5.5 A27h release site number is unaffected by embryonic picrotoxin treatment

Next, I set out to investigate the influence of network activity on the upstream interneurons of motoneurons. Giachello and colleagues studied the effect of embryonic PTX treatment on specific neurons of the motor circuit (Giachello et al., 2019). They discovered that embryonic optogenetic activity manipulations of a cholinergic A27h is sufficient to create a less resilient circuit. Using electron microscopy, the A27h has been shown to form synapses onto aCC motoneurons (Fushiki et al., 2016) (figure 1.4). Therefore, these cholinergic interneurons are of special interest to this study as they of critical relevance to the stability of the network and directly synapse onto the above analyzed aCC motoneurons, for which an increase in synaptic input was demonstrated (figures 2.16 and 2.17).

In order to elucidate neuronal adaption to network activity more broadly, I investigated the synaptic connectivity of A27h interneurons by quantifying its release sites marked by the presynaptic protein Brp. Here, I employed a dFLEx construct with the fluorophore mRuby2 (Lam et al., 2012) surrounded by an attP and an attB site, which are targets for Bxb1 recombinase ($Brp^{BOnmRuby2}$, see). Limiting the expression of Bxb1 with the Gal4/UAS-System and a driver line that is expressed in A27h interneurons (R36G02-Gal4, Fushiki et al. (2016), figure 2.18 A) I was able to quantify release sites in A27h neurons specifically (figure 2.18 B). Here it is to be noted, that the used driver line expresses not only in A27h cells but also in three other neurons per hemisegment (Fushiki et al., 2016) (unpublished data from the Evers lab by Franz Bauer). However, the only neuron in this driver line projecting through the commissure, the connection of both hemisegments, is the interneuron A27h (Fushiki et al., 2016).

For the experiment, we used and antibody against Brp to label the neuropil of VNC and all presynaptic release site, while for the mRuby2 flourescence in A27h no antibodies for signal amplification were used, because the endogenous mRuby2 flourescence was sufficiently bright after ExM. At 48h ALH, quantification of all Brp^{mRuby2} labeled puncta in the commissure revealed no significant difference upon treatment with PTX (figure 2.18 C, Ctrl: 67.6 ± 6.26 Brp^{mRuby2} puncta ; n=5; +PTX: 68.6 ± 5.20 Brp^{mRuby2} puncta ; n=8). Therefore, the critical role of A27h neurons in the stability of the motor circuit seems to not be reflected in the connectivity as measured by the number of active zones. Furthermroe, the PTX experiments demonstrate an adaption of postsynaptic connectivity, while presynaptic sites do not change in number or density. Noteably, this observation relates to the effects seen under manipulations of Jeb-Alk signaling in the previous sections (figure 2.5 and figure 2.10).

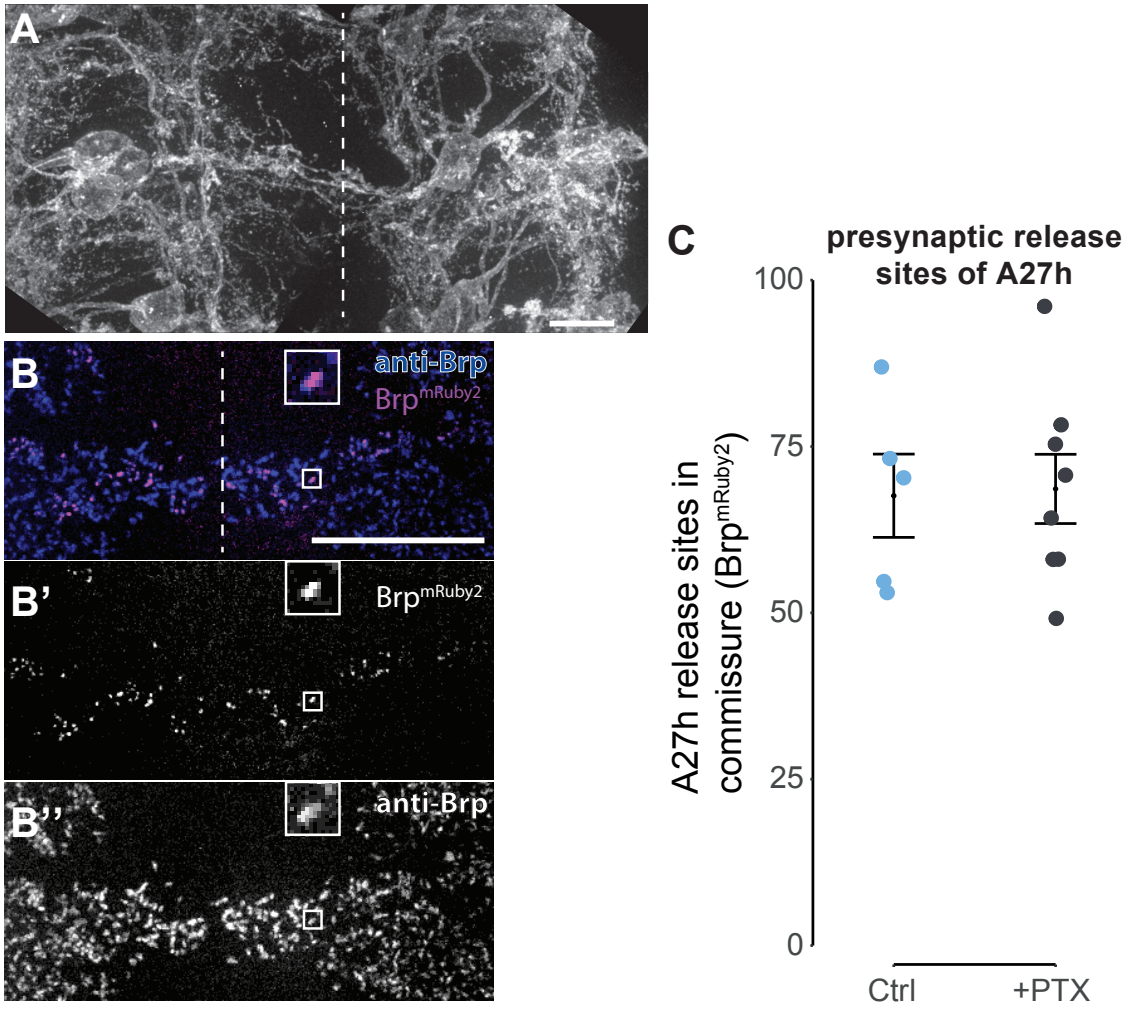

FIGURE 2.18. **Release site number in A27h interneurons is unaffected by PTX treatment.** **A**- Expression pattern of R36G02-Gal4, UAS-myr-mTurquoise2 amplified with anti-GFP visualized after ExM as max. intensity z-projections at 48h ALH. Several cell bodies are visible as well as neuronal branching patters. Two axons crossing the midline are assigned to A27h interneuron. Dashed line indicates midline. **B**- Brp^{mRuby2} (magenta, B') expression pattern after ExM as partial max. z-projection. Additional staining for all active zones (anti Brp, blue, B") helps cognition of commissure. Inset shows overlap of Brp^{mRuby2} and anti-Brp of an exemplary synapse at 3x magnification to whole image. Dashed line indicates midline. **C**- Number of presynaptic release sites at 48h ALH measured as Brp^{mRuby2} overlapping with anti-Brp is not affected by embryonic PTX exposure.
Scale bar 20 μm . *=p<0.05; ns not significant. Data produced jointly with Eunchan Lee.

2.5.6 Direct connectivity of interneurons onto motoneurons is affected in diverging ways

Finally, I analyzed specific contacts between RP2/aCC motoneurons and neurons activated by the A27h driver line, which was possible via the correlation of Brp^{mRuby2} and $Drep2^{YPet}$ (figure 2.19). Only synapses marked by the motoneuron specific $Drep2^{YPet}$ as well as the interneuron specific Brp^{mRuby2} could be quantified. Here, YPet signal was amplified for ExM using antibodies, while mRuby2 signal stable enough and thus sufficiently strong after ExM. The stochastic labeling with the RN2-FlpOut system (Ou

et al., 2008) that expresses in aCC as well as RP2 motoneurons allowed for the analysis of both synaptic input onto RP2 and aCC in the same genotype. Here it is important to consider, that while aCC input can specifically attributed to A27h, by limiting the analysis to synapses in the commissure (figure 2.19 A-G), which of the neurons included in the employed driver line (R36G02-Gal4) synapse onto RP2 is unclear. Fluorescent labeling with R36G02-Gal4 marks several neurons and their cellular structures are ovelapping within the neuropil to a degree that makes it impossible to assign all structures to a specific neuron even after expansion (figure 2.18 A). Electron microscopy data on these unidentified neurons have also not been published yet.

Regarding the connections onto RP2, the number of detected co-labeled synapses in the neuropil at 48 h ALH was decreased by embryonic PTX treatment, although RP2 cholinergic synapses overall had increased (figure 2.19 H, RP2-A27h Ctrl: 9.0 $\pm$ 0.82 synapses ; n=6; RP2-A27h +PTX: 5.6 $\pm$ 0.75 synapses ; n=5). This result would argue that the analyzed presynaptic neurons contribute a smaller proportion of the total synaptic input of RP2 after an over-activation of the nervous system during embryogenesis.

Next, I analyzed the effect of PTX on A27h-aCC specific synapses. Here, no significant effect on the number of A27h-aCC synapses was detected (figure 2.19 I, aCC-a27h Ctrl: 12.4 $\pm$ 0.51 synapses ; n=5; aCC-a27h +PTX: 18.5 $\pm$ 3.12 synapses ; n=4) . Again, this results differs from the overall increase of cholinergic input onto aCC demonstrated above. This difference suggests that the relative input from A27h to aCC is affected by PTX treatment. Interestingly, a change in relative connectivity could relate to observed changes in synaptic transmission from A27h to aCC as observed by Giachello et al. (2019).

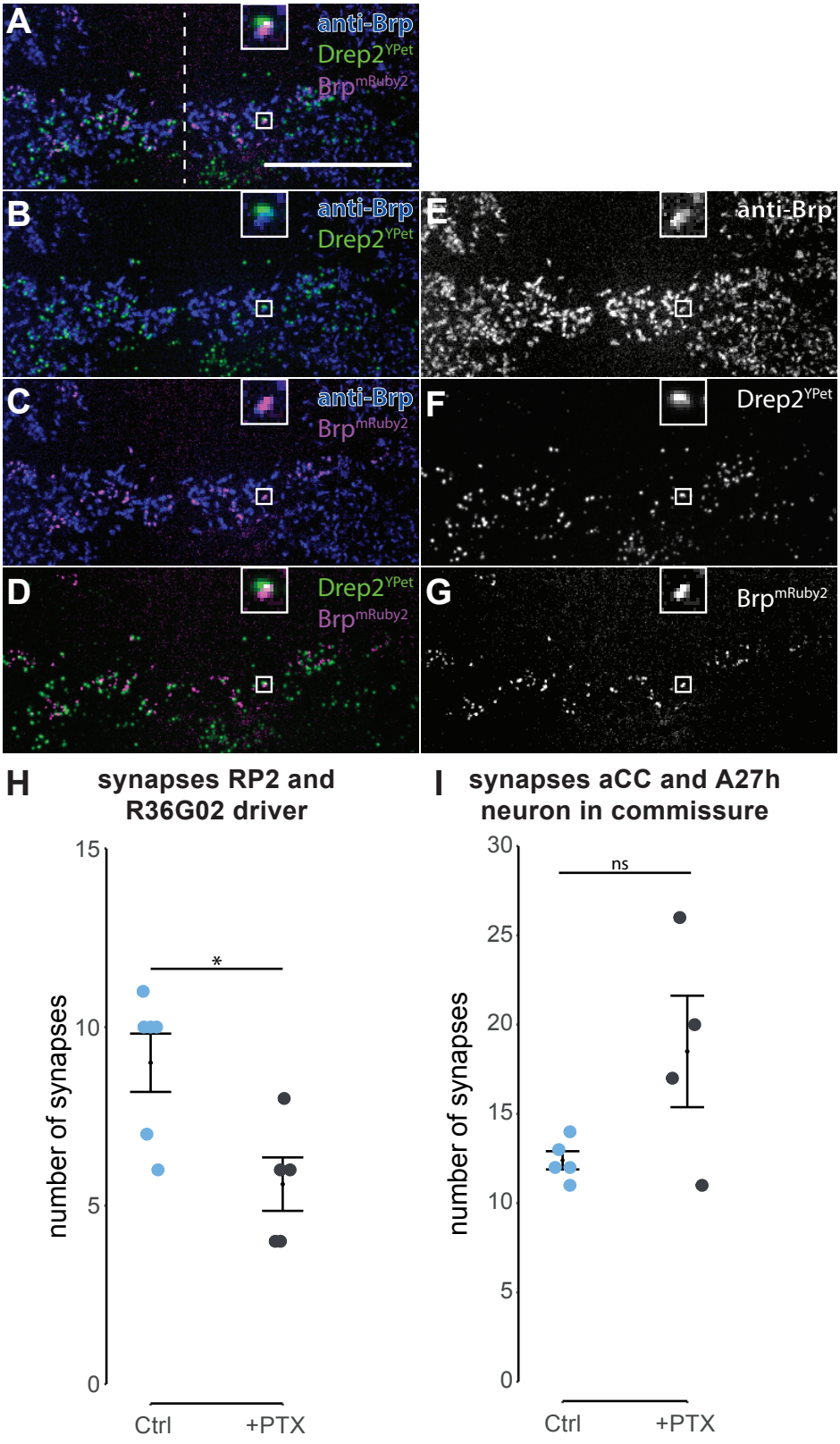

FIGURE 2.19. **Embryonic PTX exposure affects synaptic connectivity of individual neurons differentially. A-G**- Images after ExM show partial max. intensity z-projections of endogenous Brp^{mRuby2} (magenta, G) under R36G02-Gal4, endogenous Drep2^{YPet} (green, F) in an aCC motoneuron, and anti-Brp (blue, E). Inset shows an exemplary synapse at 3x magnification to whole image. Dashed line indicates midline. Same sample as fig 2.18 B. **H**- Quantification of the number Drep2^{YPet} juxtaposed with Brp^{mRuby2} (R36G02-Gal4) along the dendrites of RP2 motoneurons at 48 h ALH shows a slight decrease after PTX treatment. **I**- Quantification of the number Drep2^{YPet} in aCC juxtaposed with Brp^{mRuby2} (R36G02-Gal4) specifically located within the commissure at 48 h ALH reveals no effect of PTX treatment.
Scale bar 20 μm . *=p<0.05; ns not significant. Data produced jointly with Eunchan Lee.

CHAPTER 3

Discussion

Adequate connectivity of circuits needs to be established during the development of the nervous system to ensure function. During this process every single neuron needs to acquire proper connections. Neural activity has been described as a factor that helps to regulate the establishment of adequate connections, most prominently during critical periods of development. These connections create functional circuits that are conserved during later development and, at the same time, these circuits maintain a certain plasticity that allows adaptations to body growth, to environmental changes or during learning and memory formation. Intercellular, molecular signaling must play an essential role during the balancing of stability and plasticity, especially in form of trans-synaptic signaling directly affecting the connectivity of synaptic partners.
Extensive work has aimed to understand the mechanisms underlying initial circuit formation. While it has been described that neural activity changes circuit properties, how the cell biology of single neurons is affected remained unclear. Furthermore, it was largely unknown how the later postembryonic circuit expansion during massive organismal growth is regulated on a molecular level. Lastly, it has also been subject to debate which components of neuronal circuits are plastic and to what extent adjustments occur on the presynaptic or postsynaptic side.
With this study, I was able to shed light on the effects of both genetic prerequisites as well neuronal activity in the context of circuit development. Using novel genetic techniques, I analyze neuronal morphology *in vivo* and quantify single cell connectivity in *Drosophila* larvae using light microscopy. As a consequence, I establish Jeb-Alk signaling as a regulator during postembryonic circuit expansion. Neuronal growth as well as synaptogenesis are limited by nervous system-wide Jeb-Alk activity during larval development. Additionally, I demonstrate that manipulations of neuronal activity during embryonic development are sufficient to alter the connectivity of motoneurons

long-term. I provide a detailed anatomical study of cellular morphology and synaptic connectivity that reveals significant changes in the number of synaptic contacts in motoneurons. Across these experiments it is a common theme that postsynaptic, but not presynaptic, synapse formation is altered.

3.1 Postembryonic development of motor neurons dendrites and synaptic input

The requirements for cellular growth processes during initial circuit formation and postembryonic development are significantly different. Postembryonic growth requires the maintenance of function while the circuits must adapt to a growing body size. I demonstrate that Jeb-Alk activity regulates the growth of motoneurons (figure 2.11) and limits the number of their postsynaptic specializations during larval development (figure 2.10). Hence, Jeb-Alk acts during this later phase of neuronal development. As such Jeb-Alk is a negative regulator of connectivity within the larval motor circuit which seems to ensure adequate scaling of the circuit and proper function as well as its resilience to external manipulations.

I provide the first light microcopy-based analysis of the development of connectivity of single neurons within the growing central nervous system (CNS) of larval animals. The number of postsynaptic specifications in RP2 motoneuron (RP2) motoneurons increases five-fold from 0 h to 48 h ALH (figure 2.10). This is accompanied by a correlating increase in dendritic length (4.4 fold, figure 2.13). These measurements agree with the previously reported increase in dendritic length (Zwart et al., 2013). Therefore, these results suggest that synapse number correlates with dendritic as well as body growth over the first 48 h of development (Zwart et al., 2013).

In the larval sensory system, a lower growth rate has been reported with electron microscopy (EM) (Gerhard et al., 2017). In a 96 h age difference they report a five-fold difference in dendritic length as well as synaptic input, an observation they make for various neuron-types in the nociceptive system. This reported development of connectivity has also been verified with light microscopy (Tenedini et al., 2019). In comparison to the data in this thesis, connectivity of these sensory circuits seems to develop slower than connectivity of the motor circuit. A scaling of motor circuits directly with body size is relevant in order to maintain the excitation of larval muscles that necessarily scale with a growing body size. It is however surprising, that the nociceptive circuit of which the sensory neurons line the body wall does not need the same speed of development. It should be further investigated, why these circuits exhibit distinct rates of development. Possibly different degrees of synaptic plasticity may be the reason for differences in structural scaling.

Interestingly, a previous analysis of the synaptic input onto RP2 motoneurons specifically by the presynaptic lateral interneuron (IN_{lat}) showed a ten-fold increase in direct connectivity using GFP reconstitution across synaptic partners (GRASP, Couton et al. (2015)). Together with the observed five-fold increase in total cholinergic input onto RP2 dendrites in this thesis, a change in the relative connectivity of IN_{lat}-RP2 can be implied, which was not yet directly confirmed. My data regarding the connectivity of aCC motoneuron (aCC) and A27h interneuron (A27h) suggests a change in the connectivity of these synaptic partners as well (figures 2.16 and 2.19). However, a change in relative connectivity contradicts findings from the sensory system where a preservation of the relative synaptic input of individual synaptic partners was reported (Gerhard et al., 2017; Tenedini et al., 2019) as well as a recent study suggesting that relative connectivity is a more reliable measure for the development of circuit connectivity than absolute synaptic input of a single neuron (Aleman et al., 2019). Indeed, a maintenance of relative input seems intuitive as a simple way of maintaining circuit connectivity constant, when no new neurons are added. What governs connectivity of identified partners is largely unknown especially in the motor circuit. A role of neuronal activity has been implied to adjust relative connectivity within the visual circuit (Penn et al., 1998). Studying the factors regulating the relative connectivity within central circuits might boost our understanding of circuit formation and maintenance *in vivo*.

In general, a correlation between the outgrowth of neuronal protrusion and synaptogenesis has been seen as a central concept of circuit formation formulated in the synaptotrophic theory (Vaughn, 1989; Cline and Haas, 2008). The underlying concept is that the formation of synapses affects the subsequent growth of dendrites or axons. The growth characteristics of larval motoneuron supports this basic theory in neuroscience; healthy development is characterized by a correlation of dendritic length and synaptogenesis. While this correlation was detected in control animals, I could further demonstrate an independent regulation of dendrite length and synaptogenesis under genetic and pharmacological manipulations. Single-cell knockdown of *Drosophila* anaplastic lymphoma kinase (Alk) resulted in smaller dendritic arbors but higher synaptic input (figures 2.10 and 2.13). Similarly, picrotoxin (PTX) experiments demonstrated an increase in cholinergic postsynaptic specializations without changes in total dendritic length (figures 2.15 and 2.16). These findings contradict the basic assumption that dendritic length is a reliable approximation of synapse number, at least when molecular signaling or neural activity is disturbed. In coherence with this, various mental disorders show abnormal dendritic morphology and altered spine numbers (as a proxy for synapses) that can occur in any combination and are not necessarily correlated (Kulkarni and Firestein, 2012). Hence, in the context of the known work, my data can be interpreted as an indication, that healthy neuronal development during organismal growth is characterized by a direct correlation of dendritic length and synapse number, but genetic predisposition or pharmacological perturbation unhinge this correlation.

In a broader sense in regards to development, dendrite-synapse growth correlation seems to be a simple mechanism that evolved to ensure adequate connectivity levels within the CNS and optimize an animals fitness. This idea also resembles the concept of the Peters rule, which states that the number of synapses correlates with neuronal morphology as more overlap of axon and dendrites of synaptic partners increases the number of their synapses (Peters and Feldman, 1976; Rees et al., 2017). Further research may investigate the mechanisms connecting dendritic growth and synaptogenesis to understand disorders originating from a developmental disruption of this link. I provide an example of *Drosophila* as a model to address this question. In this context, it might also be valuable to study the trajectory of dendrite growth and synapse formation in more detail and over a longer period of time.

3.1.1 Jeb-Alk as a regulator of postembryonic neuronal development

Jeb-Alk signaling in mesodermal development was demonstrated to be essential for animal survival (Loren et al., 2001; Lorén et al., 2003; Englund et al., 2003). Building on previous studies on Jeb-Alk in the *Drosophila* CNS (Bazigou et al., 2007; Gouzi et al., 2011, 2018) and their localization within the embryonic and larval ventral nerve chord (VNC) (Rohrbough and Broadie, 2010) I investigated the role of ligand and receptor in central neurons. In order to specifically analyze neuronal effects of Alk, I expressed a previously published dominant negative allele (Alk^{DN}) in single neurons or pan-neuronally (Bazigou et al., 2007).

For Jeb, on the other hand, the Evers lab developed a new tool that allowed for targeted mutation of jeb. A combination of a construct containing a conditional STOP-codon with bxb1 integrase target sites and bxb1 integrase under an early pan-neuronal driver (nSyb-bxb1) was chosen (figure 2.2 A). A strength of the bxb1 integrase based approach is that the sequences of both attPX and attBX sites are changed after enzymatic activity and cassette inversion (to attRX and attLX, respectively) (Huang et al., 2011; Manhart, 2019). The target sites are thus rendered unrecognizable for the bxb1 integrase. Consequently, no second inversion can take place, a strength when compared to another commonly used FPL-FRT system (flippase, flippase recognition target). In this system, The FRT sites remain unchanged after enzymatic activity. Therefore, the combination of two incompatible FRT sites is required to prevent further inversions of the same cassette (Fisher et al., 2017; Gärtig et al., 2019; Williams et al., 2019). Alternatively, the widely used technique of RNA interference (RNAi) to knock down gene expression could have been used for spatial control of Jeb function (Perrimon et al., 2010). Technical difficulties with RNAi have been shown in regards to incomplete knock down of gene function and the possibility of off-target effects (Ma et al., 2006; Perkins

et al., 2015). Both effects would limit the power of experiments and therefore require stringent test for these technical limitations. Lastly, a comparison of FLP-induced genetic mutation and RNAi knock down clearly demonstrated the advantage of conditional mutations in regards to penetrance (Frickenhaus et al., 2015). Recent advances with the CRISPR/Cas9 have also been applied to induce spatially restricted gene mutations on a large scale (Port et al., 2020). This method could allow powerful experiments for the analysis of molecular pathway in neurons during development. However, one strength of the dFLEx system applied in this thesis remains in regards to the versatile application of a single construct at various loci by insertion in existing landing sites (Venken et al., 2011).

Taken together, this thesis provides they first systematic use of a bxb1-based conditional mutant allele for the analysis of molecular pathways (results also published in Gärtig et al. (2019)). I used this approach to specifically described postembryonic processes of neuronal development. I could proof the principle function of the jeb^{BOnSTOP} allele *in vivo*, that is activated by bxb1 integrase together with (2.2). Effectiveness of the molecular tool was further confirmed by significant phenotopic variation in four independent experiments on presynaptic filopodia (2.3), number of DNA fragmentation factor related protein 2 (Drep2) puncta (2.10) and dendritic growth in dissections (2.11) and *in vivo* (2.13) using four different genotypes. Lastly, pan-neuronal jeb mutation is phenotypically similar to pan-neuronal knock-down of Alk, which is in line with the previously reported activation of Alk by Jeb.

Based on the results presented in this thesis, I hypothesize a largely postembryonic action of Jeb-Alk signaling based on the altered connectivity and neuronal morphologies at 48 h ALH and the lack of significant changes in these regards at 0 h ALH. Here, a strong argument for the postembryonic specificity of Jeb-Alk signaling is the dendritic growth phenotype in Alk^{DN} expressing motoneurons (2.13). Expression of Alk^{DN} is driven by the RN2 promoter, which was shown to be active early after neuronal specification using flourophores (Ou et al., 2008), and by the overexpression with the Gal4/UAS-system. Therefore, we can confidently assume that normal dendritic length at 0 h ALH (2.13) occurs despite inhibition of Alk activity. However, I could not clarify the dynamics and penetrance of the nSyb-bxb1 expression based activation of jeb^{BOnSTOP}.

Regarding the effectiveness of nSyb-bxb1 driven conditional jeb mutation in early development I do not provide evidence for the time point at which jeb mutation was successful in all neurons. However, preliminary results from Jan Felix Evers using a combination of nSyb-bxb1 and a conditionally fluorophore-tagged allele of Bruchpilot (Brp) with the attPX/attBX sites (Manhart, 2019) showed that while not all synaptic sites are labeled at 0 h ALH, tagged Brp locates to all synaptic sites within the first instar stage (personal communication). Together with the relatively long life time of the Brp protein (Manhart, 2019) this suggests early inversion events. Therefore, we

assumed a similar penetrance for the jeb locus. A possibility to approach the question of inversion event coverage could be the combination of conditional mutation with the conditional expression of a fluorophore that labels mutated cell as done by Fisher et al. (2017). However, this technique introduces a significantly larger sequence (additional UAS and fluorophore, almost 1.5k bp) to the target locus. This large sequence could affect gene expression, and a longer cassette can also reduce inversion rate. Furthermore, we have evidence that overexpression of fluorophores influences development, e.g. the dendritic length of mtTomato2 and YPet expressing RP2 motoneurons is different (2.11). Lastly, it remains to be clarified what concentrations of Jeb protein remain for what period of time. This is a question of the protein life time Jeb in regards to synthesized protein before cassette inversion. This question may be addressed with western blots analyzing Jeb concentration throughout development. Here, an antibody could detect a shift to the truncated version of Jeb. This thesis does not provide this analysis of Jeb protein life time. Taken together, RN2>Alk^{DN} data strongly suggests postembryonic action of Jeb-Alk. Jeb experiments support this notion, but my results do not allow to rule out any embryonic action of Jeb.

3.2 Presynaptic filopodia regulate of postsynaptic dendritic growth but not necessarily formation of postsynaptic specializations

Cell-autonomous and pan-neuronal inhibition of Jeb-Alk signaling distinctively affects dendritic growth and the formation of postsynaptic specializations. While dendritic elaboration is decreased in cell-autonomous manipulations but increased under pan-neuronal jeb abrogation (2.11), both genetic conditions increase the number of synaptic contacts on RP2 dendritic arbors (figure 2.10). Therefore, we can observe two different phenotypic correlations. Under cell-autonomous knockdown of Alk the number of synaptic contacts increases despite a smaller dendritic arbor. More intuitive is the observation under pan-neuronal mutation of jeb, where both dendritic arbor size and the number of Drep2 puncta increase. These data suggest that Alk activity inhibits synaptogenesis postsynaptically. Dendritic growth, however, is regulated independently. Under pan-neuronal inhibition of Jeb-Alk signaling increased dendritic growth and synaptogenesis occur together with increased growth of presynaptic filopodia. Studies have shown that neurotrophic factors increase the number of filopodia while also promoting synaptic coupling in vertebrate neurons (Menna et al., 2009; Spillane et al., 2012). Furthermore, it has been demonstrated that increased filopodial dynamics occur during periods, where a higher rate of synapse formation can also be observed (Wu et al., 1999; Sheng et al., 2018). In line with this, I observe an increase in presynaptic filopodia

(figure 2.3) and the same manipulation causes an increase in dendritic turnover in RP2 motoneurons (figure 2.13). However, Alk^{DN} expressing neurons have significantly smaller dendritic arbors (figure 2.11) and we expect that the number of presynaptic filopodia is not increased in this context. Therefore, the correlation of increased pre- and postsynaptic structural elaboration and turn-over suggests that presynaptic filopodia are capable of inducing postsynaptic arbor growth.

Do these presynaptic filopodia also induce synaptogenesis onto RP2 dendritic arbors? Presynaptic filopodia of IN$_{lat}$ do not carry Brp-labeled active zones themselves and are dynamic (figure 2.6). Furthermore, Syd-1, a marker for more immature synapses, was also not detected in filopodia of IN$_{lat}$ (Gärtig et al., 2019). While Syd-1 was previously chown to be located in axonal filopodia in the visual system (Özel et al., 2019), there is also evidence from *Xenopus* that dynamic filopodia do themselves not carry any synaptic contacts (Li et al., 2011). Additionally, I detected an increase in the number of postsynaptic sites even on smaller Alk^{DN} expressing dendritic arbors (figure 2.10). Again, this occurs under the lack of supernumerary filopodia seen under pan-neuronal Jeb-Alk inhibition (figure 2.3). In summary, put into the context of published filopodial modes of action, my data support the notion, that axonal filopodia within the investigated circuit stimulate dendritic growth, but are not necessary for an increased synaptogenesis.

My data does not rule out the possibility that presynaptic presynaptic filopodia-dependent stimulation of dendritic growth increases synaptogenesis and promotes connectivity to some degree. Increased dendritic elaboration can increase the chance of contact between dendrites and presynaptic release sites and contact might ultimately induce synaptogenesis. Additionally, there is work in vertebrates and invertebrates showing that synapse formation is capable of stabilizing cellular protrusion (Niell et al., 2004; Constance et al., 2018). This also supports a synergy of growth induction by filopodia followed by synapse-dependent dendrite stabilization. Nonetheless, the results of single-cell Alk knockdown demonstrate that presynaptic filopodia are not necessary for increased postsynaptic synapse formation.

3.3 Jeb-Alk signaling correlates with the plasticity of circuits

Jeb-Alk signaling has previously been described as a negative regulator of short-term and long-term memory formation in the CNS of adult *Drosophila* (Gouzi et al., 2011, 2018). Across animal phyla, learning and memory is a process highly dependent on the plasticity of a network as experience, in the form of patterned neuronal activity, shapes the connectivity of neuronal circuits. Consequently, Gouzi et al. demonstrate that

Alk negatively regulates protein synthesis dependent long-term memory formation and locates to the sites of structural plasticity within the mushroom body during olfactory learning (Gouzi et al., 2018).
In the same vein, larval growth of neuronal circuits is a process that is based on the plasticity of circuits. As the animal grows so do neurons, their dendritic arbors elaborate, synaptic endings enlarge and new synapses form. While some synaptic connections are maintained, the connectivity of individual synaptic pairs scales with neuronal growth (Couton et al., 2015). Now, trans-synaptic Jeb-Alk in the larval motor circuits acts in two ways: (1) Alk activation limits the formation of postsynaptic sites on the dendritic arbor (figure 2.10) and (2) the secretion of Jeb seems to elicit a negative feedback that restricts the formation of presynaptic filopodia (figure 2.3). These two mechanisms of Jeb-Alk signaling act to limit the increase of synaptic input into RP2 motoneurons that occurs from 0h ALH to 48h ALH (figure 2.10). This observation implies a limitation of the structural plasticity occurring within the motor circuit, as the adaption to tissue growth is capped. In this context, I suggest that activation of Alk could also act in the adult mushroom body to limit the formation of new synaptic contacts upon conditioning.
Another point supporting the argument that Jeb-Alk limits plasticity is the observation that branch retraction and outgrowth of dendrites is increased upon inactivation of Jeb-Alk signaling; pan-neuronal abrogation of Jeb lead to a decrease in the dynamic of dendritic growth (figure 2.13). Increased dynamics of cellular protrusions have been correlated with synaptogenesis during experience-dependent plasticity (Maletic-Savatic et al., 1999; Zhou et al., 2016; Huang, 2017; Sheng et al., 2018). Therefore, I suggest that one mechanism by which Jeb-Alk signaling reduces structural plasticity of circuits is through the limitation cellular dynamics, meaning outgrowth of filopodia, dendritic and axonal.
Taken together, the effects of Jeb-Alk signaling on short- and long-term memory and on neuronal development in the larval motor circuit strongly imply this pathway to limit plasticity of the nervous system. It likely acts as an inhibitor of synaptogenesis, both in memory formation and learning, and during the process of larval growth. In this regard, my work was able to provide a new function of Jeb-Alk signaling that suggests a common mechanism of the pathway during plastic processes in larval and adult *Drosophila*. Additionally, inhibition of murine Anaplastic lymphoma kinase (ALK) also improves learning in Neurofibromatosis type 1 (NF1) -/- mice (Weiss et al., 2017) suggesting an evolutionary conserved role of ALK in learning and memory. Hence, I hypothesize ALK functions as an inhibitor of neuronal plasticity more generally, a role that might be evolutionary conserved as well. Therefore, it would be interesting to investigate ALK-dependent dendritic growth and synaptogenesis in the murine brain.
While it has been shown that Alk is upregulated by learning paradigms (Gouzi et al., 2018), structural plasticity based on Alk activity during memory formation has not been

addressed directly in *Drosophila* either. The larval mechanism of Alk as an inhibitor of synaptogenesis suggests that its activity in the mushroom body could similarly limit formation of new synaptic connections fulfilling its role as a memory filter (Gouzi et al., 2018). In fact, theories suggest that learning must be limited because hyper connectivity can be disadvantageous (Raman et al., 2019) and capacities for storing new experiences need to be conserved (Tetzlaff et al., 2012). Using expansion microscopy on the adult brain (as performed in Mosca et al. (2017)) would allow to test for Alk as a negative regulator of structural plasticity during learning and memory formation. It could further be investigated how initially increased learning performance could affect further learning paradigms, to see whether limitation of learning at a single instance is important to ensure long-term performance. Additionally, an expansion microscopy study could possibly demonstrate a direct link of synaptogenesis as a mechanism for memory formation in addition to synaptic plasticity.

A major difference between the mechanistic underlying Alk function in adult memory formation and larval neuronal growth regards Alk activation. Jeb is dispensable for memory formation and an upregulation has no effect of memory performance (Gouzi et al., 2018). Instead the authors suggest an auto activation of Alk due to a higher probability of random dimerization after increased expression. Normal dendritic growth and synaptic connectivity of larval motoneurons is however dependent on both Alk activity and Jeb expression (figures 2.10 and 2.11). One might argue that the observed contradicting phenotypes of RP2>AlkDN and pan-neuronal jeb mutation could be due to different mechanisms of Alk auto activation and Jeb-induced Alk activity. Here, a base level of auto activation is present and an additional Jeb-induced activation occurs. While RP2>AlkDN removes all Alk-activity, pan-neuronal jeb$BOnSTOP$ mutation blocks only the jeb-dependent Alk activation. However, the observation that pan-neuronal AlkDN recapitulates jeb mutation refutes this theory. Furthermore, Alk$^{full-length}$ expression in RP2 motoneurons has no effect on dendritic growth only with co-overexpression of Jeb (data not shown, personal communication with Jan Felix Evers). It still remains unclear to what extent the suggested Alk auto activation during memory formation affects synaptogenesis and dendritic growth. Again, this might be resolved by a detailed anatomical study of the mushroom body upon conditioning.

Another reported phenotype that underlines the possible general implications of ALK signaling in plastic processes is related to sleep. Bai and Sehgal (2015) showed that Alk mutation in adult flies also counteracts the reduced sleep phenotype of Neurofibromin 1 (Nf1) deficiency hence Alk limits sleep. Further Alk mutant flies show a higher sleep drive after sleep deprivation (Bai and Sehgal, 2015). Sleep itself has been linked to plastic processes as, behaviorally, sleep deprivation impairs memory formation (Stickgold et al., 2001). On a cellular level, experience-dependent accumulations of synaptic proteins that form during wake time, are reduced during sleep (Gilestro et al., 2009). Taken together with Alk being a negative regulator of learning (Gouzi et al., 2011, 2018),

we can hypothesize that Alk might limit experience-dependent changes during wake time reducing the need of a clean up during sleep, effectively reducing sleep drive. These findings suggest that Jeb-Alk signaling plays a role in the correlation of memory and sleep behaviors depending on the regulation of synaptic and dendritic growth by Jeb-Alk.

3.4 Possible relation of Alk signaling and mental disorders

3.4.1 Correlations of Alk and Nf1 suggest close genetic interaction

The plasticity of a brain largely relates to its cognitive capabilities. For example, the plastic circuits of young children are faster and better at learning new languages (Takesian and Hensch, 2013). In the previous section I demonstrated the implications of this thesis to the relevance of Jeb-Alk signaling for the growth-related plasticity of neuronal circuits based on the regulation of both dendritic growth and synaptogenesis. I further summarized studies implying antagonistic functions of Nf1 and Alk signaling especially during learning and memory formation (Gouzi et al., 2011; Weiss et al., 2017). In humans, mutation of Nf1 causes NF1, a genetic disorder resulting among other symptoms - in cognitive impairments. Affected children exhibit learning deficits (Brown et al., 2013). Similarly, learning deficits are also measurable in mice and fly models of NF1, where they can be rescued by Alk inhibition (Gouzi et al., 2011), Weiss et al. (2017)). Anatomically, mutation of Nf1 in rat and mice models show changes in the morphology (shorter neurites) and connectivity (less dendritic spines) of hippocampal neurons underlying cognitive impairments (Oliveira and Yasuda, 2014; Brown et al., 2013). Regarding Alk, this thesis clearly demonstrates the importance of proper Jeb-Alk signaling for the limitation of neuronal growth and connectivity in larval *Drosophila*. Given the interaction of Nf1 and Alk in learning and memory an analysis of their interaction during morphological development of motoneurons is promising. This idea is further support by the fact that Nf1 is expressed widely within the larval VNC similar to Alk (Walker et al., 2006) providing the possibility of a role of Nf1 in *Drosophila* neurodevelopment. Interestingly, NF1 patients also exhibit a higher prevalence of seizures (Santoro et al., 2018). However, to this point no studies have investigated seizure probability in the fly or mouse model of NF1. As jeb mutants show a seizure phenotype (Gärtig et al., 2019), seizure susceptibility might be an additional connection of Jeb-Alk signaling and Nf1 activity. This hypothesis should be investigated. Here, I see a promising project that could clarify the role of Jeb-Alk signaling in a fly mode of a cognitive disease. This project could further elucidate neurodevelopmental function

of Nf1 that results in adult phenotypes.
Taken together, the links and numerous correlations of Alk with Nf1 are a strong implication of the relevance of Alk signaling in the development of Nf1-dependent cognitive impairments. Therefore, investigating the genetic interaction of Nf1 and Alk during larval development in regards to resilience of the network and anatomical development of neurons could serve as a way to dissect the potential of Alk or downstream components of Nf1 and Alk (namely MAPK pathway (Rohrbough et al., 2013a; Gouzi et al., 2011; Weiss et al., 2017) as therapeutic targets.

3.4.2 Anatomical pathologies and seizure proneness are common to mental disorders

More generally, anatomical pathologies - observed as synapse density and dendrite architecture - are a common phenotype in various cognitive disorders (Kulkarni and Firestein, 2012). Similarly, many patients of various mental disorders show seizure proneness as a comorbidity (Besag, 2018; Berry-Kravis, 2002; Krajnc, 2015). Is it understood that seizures are one common malfunction of various developmental disorders that show an underlying altered circuit connectivity. While more crude behavioral output seems unaffected (crawling) and survival under laboratory conditions is verified, the more delicate functions of memory formation and electroshock resistance are impaired. Taken together, seizure susceptibility and the anatomical alterations of motoneuron connectivity provide strong arguments that imply a role of Jeb-Alk as a mechanism in the orchestration of connectivity to a finer level. Clarification of the role of Jeb-Alk in cognitive impairments more generally will be interesting. Another study has provided evidence, that Jeb-Alk is downregulated in a fly model for Fragile X syndrome, a genetic disorder resulting in cognitive impairments (Friedman et al., 2013). Studies on the role of Alk in other fly and ALK in mouse models of mental diseases, for example autism spectrum disorders, might demonstrate further potential of ALK as a therapeutic target for neurodevelopment diseases.

3.5 A correlation of reduced network resilience and increased neuronal connectivity

A phenotypic analysis of pan-neuronal jeb mutants revealed that RP2 motoneurons show altered electrophysiological properties and that larvae are more susceptible to electrical manipulations (Gärtig et al., 2019). The longer recovery time after electroshock resembles a behavior observed in *Drosophila* epilepsy-like models (Giachello and Baines,

2015) that can also be generated by PTX exposure during embryogenesis (Giachello and Baines, 2015). Seizures are considered the output of inadequate activity - periods of increased, synchronized action potential firing - within the nervous system. These malfunctions may occur due to abnormal neurodevelopment in regards to the electrical properties of neurons, synaptic physiology or the connectivity of circuits. Consequently, these finding demonstrate the relevance of Jeb-Alk signaling for normal development leading to proper function of the *Drosophila* CNS.
Interestingly, I present two distinct manipulations creating less resilient networks which are both on an anatomical level characterized by increased synaptic input onto motoneurons at 48 h ALH (figure 2.10, 2.16) (Gärtig et al., 2019). Hence, pharmacological manipulations with PTX as well as genetic inhibition of Jeb-Alk signaling result in a hyperconnectivity of the analyzed neurons (RP2 and aCC). How other neurons are affected was not studied. So far, studies in mice have demonstrated a correlation of proneness to seizure with additional excitatory synapses (Chu et al., 2010; Chao et al., 2007) and with increased excitatory synaptic input measured with electrophysiology (Zhang et al., 2014). Epileptic patients show increased functional connectivity of brain regions (Vollmar et al., 2011) and increased neuronal connectivity has also been demonstrated as some brain regions display higher density of synaptic contacts compared to healthy individuals (Marco et al., 1997). Therefore, is has been suspected that synchronized activity of certain brain regions during seizure is based on increased excitatory connectivity of local circuits. In line with this, I provide evidence that seizures in *Drosophila* larvae are also characterized by motoneurons with increased excitatory which originates from an increase in cholinergic synapses. Hence, I demonstrate that hyperconnectivty is an anatomical commonality for various model of seizure-prone nervous systems. I further hypothesize that seizures occur in nervous system that exhibit abnormally high connectivity, or vice versa, neurodevelopmental impairments leading to increased connectivity of neuronal circuits can cause epilepsy-like conditions. Furthermore, my results also strengthen *Drosophila* as a model to study the underlying molecular mechanisms leading to epileptic phenotypes.

3.6 Neural activity during a critical period defines excitatory connectivity

Neural activity during critical periods affects neuronal development long term (Hensch, 2005). Synaptic coupling and neuronal excitability within the larval motor circuit is altered by manipulations of neuronal activity in the critical period during *Drosophila* embryogenesis (Giachello and Baines, 2015; Giachello et al., 2019). To understand this long-term change of network properties better, I investigated the effects of neural

activity on the morphology and connectivity of larval motor neurons.

3.6.1 Dendritic growth is independent of embryonic GABAergic activity

Exposing embryos to the gamma-aminobutric acid (GABA) receptor antagonist PTX during embryogenesis had no effect on dendritic elaboration of motoneurons shortly after or long-term throughout larval life(figures 2.14 and 2.15). Consequently, blocking of GABA-dependent inhibition has no acute effect on dendritic growth during embryogenesis. Several studies have demonstrated that a link between dendritic growth and GABA-receptor activation exists in vertebrates. GABA activity is involved in the stabilization of outgrowing dendrites in newly formed neurons within cultured olfactory bulb slices of rats (Gascon et al., 2006). Other studies showed opposite phenotypes as reduced levels of $GABA_A$ receptors in cultured rat hippocampal neurons leads to shorter dendritic arbors (Rui et al., 2013) and GABA receptor blockage in mice infant cultured neurons decreases dendritic length (Nishimura et al., 2008). In *Xenopus*, blockage of GABA transmission leads to less branched neurons in the optic tectum and prevents experience-dependent dendritic plasticity (Shen et al., 2009). Conclusively, I could not describe a similar role of GABA in the early growth of dendritic arbors in *Drosophila* for the neurons investigated. Instead, I show that the activation of GABA-receptors is not necessary for dendritic growth in *Drosophila*, which implies a different mechanism to vertebrate GABA receptors during dendritic growth. This adds to the observations of mechanistic differences in GABA-receptors between vertebrates and insects, like the distinct responsiveness to various GABA-receptor inhibitors (reviewed in Manev and Dzitoyeva (2010)). It remains unclear what molecular differences underlie these observations.

More generally, I demonstrate that chronic network hyperexcitability is without consequences on the structural development of the dendritic arbors of motoneurons in the central motor circuit. Neural activity is widely accepted to regulate dendritic growth (Mcallister, 2000, see). In *Drosophila*, embryonic aCC motoneurons respond homeostatically to missing cholinergic excitation with increased arbor growth, and this effect was further narrowed to a local effect of synapse formation on dendritic growth (Tripodi et al., 2008). In the same vein, chronic cell autonomous overexcitation of single motoneurons with the warmth-gated cation channel dTrpA1 reduces arbor size of aCC motoneurons in larvae (Oswald et al., 2018). However, this study further demonstrated that a network wide increase of activity by raising the ambient temperature had no effect on dendritic arbor growth (Oswald et al., 2018). This observation corresponds with my results (figures 2.14 and 2.15), suggesting that chronic network hyperexcitability is distinct from single neuron manipulations. Possibly, only the latter may affect morphological development of the dendritic arbors of larval motoneurons.

An explanation could be that an outlier neuron might need to adapt to its surrounding or is rather instructed to adapt. On the other hand, an overall increased activity allows single neurons to follow their genetic growth program.
Looking at activity-dependent dendritic growth beyond *Drosophila*, it has been reported that neural activity during a critical period affects dendritic elaboration across vertebrates, for example, both in the barrel cortex of rat and mice (Maravall et al., 2004; Chen et al., 2015) as well as the visual cortex of cats (Antonini and Stryker, 1996)). In *Xenopus*, activity-dependent dendritic growth occurs in the optic tectal neurons, which is directly dependent on the availability of e excitatory glutamate receptors within the dendrites, (Sin et al., 2002). Furthermore, reducing dendritic growth by GABA receptor blockage is dependent on glutamate receptor activity arguing that the effect is due to overall excitation of the neuron (Nishimura et al., 2008). The influence of neural activity on dendritic growth is a basic component of the synaptotrophic hypothesis (Vaughn, 1989) arguing that synapses, or exchange of information across them, including but not limited to neurotransmitter release, directs neuronal development. Contrary to this theory and the publications summarized here, my data suggests that early dendritic growth in the motor circuit of *Drosophila* is independent of network activity (figures 2.14 and 2.15). It will be interesting to investigate to what extent this principle holds true for other neurons in the larval CNS.

3.6.2 Neural activity regulates dendritic growth and synaptogenesis distinctively

In addition to morphological development, neural activity is further accepted to induce structural plasticity by influence synaptogenesis (Fauth and Tetzlaff, 2016). Studies on activity-dependent connectivity are more abundant in vertebrate than invertebrate models. Across model organisms, the precise regulation of connectivity in central circuits has been difficult to study as it requires visualization of synaptic contacts at a sufficiently high resolution. This thesis demonstrates that excitatory synaptic input onto motoneurons increases when network excitation is increased by PTX-dependent GABA inhibition using a light microscopy based approach (figure 2.17). As such this observation can be understood as a Hebbian style plasticity. Other observations in *Drosophila* have rather demonstrated homeostatic mechanisms (Yin and Yuan, 2015), where increased excitation leads to less synaptic contacts (Sheng et al., 2018; Yuan et al., 2011) or reducing activity promotes synaptogenesis (Kremer et al., 2010). However, one must consider that experiments presented in this thesis only indirectly increased excitation by blocking GABA-ergic inhibition. As such it remains unclear what effect direct manipulation of excitatory activity would have on excitatory synapse formation. Investigation of this relation will further elucidate whether reported homeostatic

mechanisms in sensory circuits transfer to the motor circuit (Sheng et al., 2018; Yuan et al., 2011; Kremer et al., 2010). First hints allowing this transfer are provided by analyses of excitation dependent, homeostatic dendritic growth of motoneuron arbors (Tripodi et al., 2008; Oswald et al., 2018). Oswald et al. (2018) further demonstrate the distinct effects of single cell versus organismal overexcitation, which should also be analyzed on the level of synaptic input. Expression of Trp1A in single RP2 or aCC motoneurons could possibly produce a distinct phenotype to PTX manipulations in regards to synaptic input, similar to the observations on dendritic length by Oswald and colleagues.

We might further relate increases in excitatory synapse numbers to intra-neuronal competition for dendritic growth observed in adult flight motoneurons (Ryglewski et al., 2017). The allocation of dendrites into neurotransmitter specific territories could possibly also increase the relative number of synaptic contacts of the corresponding neurotransmitter. Therefore, it might be possible that the increased number of excitatory synapses (figure 2.17) is accompanied by a decrease in the number of GABA-ergic synaptic input onto motoneurons. However, as no distinct neurotransmitter territories have been defined for larval motoneurons, this is not yet answered.

My study demonstrates a major technical advance for the field by providing a first quantitative analysis of single cell connectivity using synaptic labels at an endogenous level. Especially the establishment of an conditional, endogenous marker for cholinergic synapses, the most common excitatory neurotransmitter type, should advance further research. Only very recently endogenously, C-terminally tagged dopamine receptors have been used (Kondo et al., 2020) and endogenously tagged potassium channels Shaker (Sh) and ShaI that originated from the MIMiC library established by the Bellen lab (Nagarkar-Jaiswal et al., 2015) were used to study visual respone properties in the *Drosophila* visual lamina. A conditional synaptic marker has so far only been published for presynaptic vesicle proteins using a system that is based on the FLP recombinase as in this or the B2 recombinase (Williams et al., 2019) thesis. Despite the major advance the conditional drep2 allele brings, we are currently limited to one specific neurotransmitter type as Drep2^{YPet} is specific to cholinergic synapses (figure 2.7, Andlauer et al. (2014)). A neurotransmitter-independent quantification would require the endogenous tagging of a protein that locates to all synaptic contacts. However, for example the promising candidate for a postsynaptic marker Discs large (Dlg), that locates to postsynaptic sites in the muscle at the neuromuscular junction (NMJ), has been shown to be unreliable in the larval CNS (personal communication with Jan Felix Evers). Therefore, more research should be focused on finding an ubiquitous postsynaptic marker for the *Drosophila* CNS.

Nonetheless, I could demonstrate that endogenous expression of Drep2^{YPet} reliably marks excitatory, cholinergic synapses in the *Drosophila* CNS, which are the vast majority of all excitatory synapses. While leaving out other synaptic contacts, this tool

also opens the possibility of analyzing proportions of e.g. inhibitory and excitatory input once a marker specific for GABA-ergic postsynaptic specializations is established. Such a set of tools could allow studies that relate to the observation on dendritic building material by Ryglewski et al. (2017). Does the inhibitory-excitatory balance adapt within a maximum number of synaptic sites? How does this correlate with dendritic elaboration? This would be especially meaningful for the PTX experiments, that specifically inhibit GABA neurotransmission.

3.7 Do critical periods set homeostatic limits through structural adjustments?

In addition to the acute effects of GABA receptor inhibition during embryogenesis I analyzed the resulting synaptic input onto RP2 and aCC motoneurons at 48 h ALH (figure 2.16). The goal of following dendritic arbors over postembryonic development was to understand the long-term effects of acute hyperexcitability during a critical period. Here, the crucial point is the limitation of GABA inhibition to embryogenesis by feeding PTX to gravid females. Therefore, PTX is not present in the larval CNS resulting in a return of GABA inhibition and therefore a inhibition of the network or single neurons. Feeding of picrotoxin as well as optogenetic tools have been applied previously to demonstrate that manipulations of neural activity during a critical period are sufficient to induce or prevent epilepsy-like conditions in *Drosophila* larvae (Giachello and Baines, 2015). Conclusively, it has been hypothesized that neural activity during the critical period regulates neuronal excitability and sets homeostatic limits by defining a set-point (Giachello and Baines, 2017). Genetic predispositions or pharmacological treatments can tip this set point towards hyperexcitability resulting epilepsy-like conditions. It is unclear what the underlying mechanisms are by which the set-point is encoded. To what degree do my results on the synaptic input of aCC and RP2 motoneurons support the homeostatic set-point theory on the level of circuit connectivity?
In terms of structural plasticity, a homeostatic process regulating the excitability of a neuron can be the formation or degradation of synaptic contacts with either inhibitory or excitatory neurons (Fauth and Tetzlaff, 2016). Using Drep2, I analyzed the excitatory connections. According to the set-point theory it could be hypothesized that return of GABA dependent inhibition during larval life might be compensated by a homeostatic increase in excitatory input aiming to maintain neuron excitability within the homeostatic limits around the set-point (in coherence with Giachello and Baines (2017)). This should be detected as a relative increase of Drep2 puncta from 0 h to 48 h above the normal growth curve. Indeed, I observe an increase in synaptic input at 48 h ALH compared to controls (figure 2.16). However, this observation is already preceded

by an increase at 0h ALH (figure 2.17). The observed increase of mean synaptic input in RP2 and aCC motoneurons is 15-30% over both time points. Hence, I hypothesize that, if excitatory synaptogenesis increases excitability, structural homeostatic plasticity of excitatory synapses is not a mechanism that maintains an encoded set-point of excitability during larval development of motoneurons. It seems more likely that a relative amount of excitatory input set during embryogenesis is afterwards maintained by the neuron, this would constitute a "structural set-point". Basically, connectivity of larval motoneurons is characterized by a specific growth program, where neuronal growth and connectivity scale with organismal growth (Zwart et al., 2013) (section 3.1). A change of the starting point (synapses at 0 h ALH) results in a similar relative change at a later time point. This growth must be regulated by molecular mechanisms, and within this thesis I provide evidence that Jeb-Alk signaling is one component coordinating postembryonic growth and excitatory input of motoneurons (figure 2.10 and section 3.1). Therefore, I hypothesize, that Jeb-Alk maintains the increased excitatory input in the epilepsy-like model. This hypothesis should be tested by exposing jeb mutant animals to pircotoxin during embryogenesis and observing the postembryonic development of synaptic input.

3.8 High inter-individual variance and its implications

The analysis of the connectivity of circuits has been of interest for neuroscientist since the beginnings of the field. Visualizing, quantifying and understanding the connections between the myriads of neurons in the nervous system enables us to draw conclusions with respect to the way the CNS is set up and how it functions. In the last decade, electron microcopy has established the field of connectomics in *Drosophila* (Saalfeld et al., 2009; Cardona et al., 2009). Extensive work of groups at the Janelia research Campus produced a complete EM volume of an adult brain (Zheng et al., 2018)) as well as larval brains of which large portions have been reconstructed (Kohsaka et al., 2019; Gerhard et al., 2017; Schneider-Mizell et al., 2016). These technological advances enabled the description of connectivity of various circuits but also allowed first comparative studies describing differences between animals (Gerhard et al., 2017; Aleman et al., 2019). However, one major weakness of these studies is the number of observations: As EM data acquisition and analysis are time and resource consuming they include comparisons of only one or two neurons at a time. This study employs a combination of selective promotors, conditional endogenous tagging and expansion microscopy to reveal the connectivity of single cells in the central motor circuit. Using this approach, I quantify the connectivity of three to ten samples per treatment even

for larger 48 h dendritic arbors (2.16). Thus, studying connectivity of single cells in the larval *Drosophila* CNS with light microscopy with the resolution and detail presented in this thesis and applying this method to quantify developmental patterns is new.
One striking feature of my analysis it the detected spread of the data. Both at 0 h as well as 48 h ALH the number of excitatory input marked by Drep2 across animals was 50-70% different between the lowest and highest data points within an experiment (figures 2.10, 2.16, 2.17). While some variance may originate from the strong relation of age and synapse number and the methodological small age spread (2h), this cannot explain the entire range of Drep2 counts. Even more drastic is the spread of specific connections between RP2 and IN_{lat} previously quantified with GRASP (Couton et al., 2015), where almost 300% difference was measured. Taken together, the great spread of synaptic connectivity in my experiments questions the significance of comparing single data points obtained from EM. Indeed, EM studies of sensory circuits in the larval VNC showed a high variance (15 to 40% depending on the experiment) for the specific neurons within one animals (different hemispheres) and between animals (Ohyama et al., 2015; Jovanic et al., 2016). While connectomics provide essential information on the morphology of neurons and their synaptic partners to unravel circuit architecture, comparative studies based on single observations in EM should be looked at carefully as my study underlines. The successful application of expansion microscopy and endogenous labeling of synaptic proteins in this thesis constitutes a first quantitative light microscopy based study of single-cell connectivity in the developing CNS of *Drosophila*. This technical advance may promote future research on the role of various genetic and environmental factors on neuronal connectivity *in vivo*.
The high variability in connectivity also provides information of mechanisms governing circuit development. First, my data supports the notion that connectivity of the same circuit can vary greatly between individuals, an observation that has been made in animals and humans (Mueller et al., 2012; Marder, 2011). Previous work on *Drosophila* larval motoneurons also demonstrated a spread in dendritic length (Zwart et al., 2013). My work elaborates on this and adds the even higher percental range of synaptic connectivity. Despite inter-individual variation circuit function can remain the same as a study with a simple three neuron model demonstrated (Prinz et al., 2004). The authors further highlight that intrinsic and synaptic parameters of single neurons are most likely less strictly regulated than the overall network performance. Indeed, a detailed morphological analysis revealed a high variation in branching of the same neuron across animals despite similar circuit output (Otopalik et al., 2017). All things considered, variations in single neuron connectivity is likely compensated by other parameters. Thus, it can be concluded that the robustness of CNS function is ensured by developmental mechanisms of compensation allowing for adjustment of different parameters. In summary, my data adds a puzzle piece to our understanding of the sloppiness (Otopalik et al., 2017) underlying robust circuit formation.

Secondly, the variation also relates to the importance of intrinsic genetic developmental programs versus the extrinsic regulation. It is clear that the very similar genetic background still results in great inter-individual variability. Thus it might be argued that neuronal development is sensitive to finer genetic or epigenetic differences. However, the network is capable to adapt as single neurons adjust arbor size or synaptic connectivity, thus demonstrating plasticity of neuronal development to extrinsic factors. Vice versa, other neuronal parameters might be adapting to changes in dendritic growth or synaptic input. For example, mistargeting of certain neurons in *Drosophila* larvae can alter neuronal development and connectivity of their synaptic partners (Couton et al., 2015; Aleman et al., 2019). Of course, neural activity is crucial in directing neuronal and overall network connectivity (Fauth and Tetzlaff, 2016). Here, it is conceivable that environmental conditions as well as individual crawling and feeding behavior could affect motor circuit development through e.g. temperature variations, nutrient availability, or sensory input.

3.9 Conclusion and Outlook

The molecular mechanisms of initial circuit formation from pathfinding through targeting and synaptogenesis are well studied. What mechanisms maintain and scale synaptic connectivity in circuits of the CNS during growth are largely unknown. This thesis provides evidence of a molecular mechanism regulating coordinated growth of synaptic partners and demonstrates long-term effects of neural activity on the synaptic patterns in the larval motor circuit.

This thesis emphasizes the relevance of adequate neuronal Jeb-Alk signaling for robust network function. Analysis of synaptic input and dendritic growth of postsynaptic motoneurons and of axonal morphology of a presynaptic interneuron revealed the role of Jeb-Alk signaling in coordinated circuit expansion during larval growth. Without Jeb-Alk the number of postsynaptic input increases significantly, while presynaptic release sites are unaffected. Jeb-Alk as such constitutes a negative feedback for postsynaptic synaptogenesis. Negative feedback mechanisms are essential to keeping a homeostasis and keeping connectivity within a normal range. Consequently, the importance of Jeb-Alk for the stabilization of neural networks becomes clear. I further suggest possible implications of this finding for medical applications in cognitive impairments. Further research on the effects of manipulations of ALK in models of developmental cognitive disorders beyond NF1 - e.g. autism spectrum disorder or epilepsy - should elucidate its role in determining cognitive capabilities developmentally or acutely through the maintenance of adequate synaptic patterns.

Furthermore, I present two distinct manipulations creating less resilient networks. In both cases, we observe an increased synaptic input onto motoneurons. Here, the crucial

point is a one-sided, unbalanced increase in synaptic specializations as presynaptic release sites are unaffected. In coherence with observed increases in synaptic density in vertebrates it can be speculated that hyperconnectivity of networks is common to pathologies of the CNS and is basis for a malicious imbalance. It would be interesting to investigate how this hypothesis holds true in *Drosophila* of other mental retardations or cognitive impairment. How genetic manipulations, exposure to specific patterns of neural activity or pharmacological inhibition may then prevent hyperconnectivity or connective imbalance could open up new possibilities for the development of therapeutic targets. Here a focus should lie on the developmental timing of treatment considering the importance of critical periods for network tuning and the establishment of synaptic patterns.

I identified cellular and molecular mechanisms required for the establishment and maintenance of synaptic patterns for reliable circuit function. In this context, I view it as a promising project to study the correlation of the molecular and neural mechanisms in more detail. Alk limits cholinergic input onto motoneurons and increased neural activity during embryogenesis increases cholinergic input onto motoneurons. To test a correlation, it would be interesting to study the effect of increased Alk activity during a critical period. Can Alk activation limit the activity-dependent addition of synaptic contacts? And vice versa, can we change the development of synaptic input in epilepsy-like models if postembryonic Jeb-Alk signaling is inhibited. A description of ALK as a molecular regulator maintaining established synaptic patterns more globally would promote *Drosophila* models for developmental mechanisms creating stable circuits. Our anatomic knowledge of fly circuits and the genetic accessibility of flies would allow for detailed *in vivo* studies on the role molecular and activity-dependent processes. This could add valuable mechanistic insight to the findings from vertebrate models to create a more comprehensive picture of the cellular and molecular mechanisms that create and maintain functionally stable circuits.

CHAPTER 4

Material and Methods

4.1 Materials

4.1.1 Fly Stocks

For this study the following stocks were used. Flies were either obtained from Bloomington Drosophila Stock Center (BDSC), by generous donations from colleagues as indicated or produced in the group of Jan Felix Evers.

GENOTYPE	SOURCE	IDENTIFIER
Oregon R	BDSC	RRID_BDSC_5
w; if/CyO, WgZ ; TM2 Ubx/TM6 Hu Tb,	BDSC	
w; elav-Gal4[w^+]/CyO wgZ;	BDSC	RRID_BDSC_51941
yw;;nSyb-GAL4	BDSC	RRID_BDSC_51941
w;; repo-Gal4 / TM6b, Hu, Tb	BDSC	RRID_BDSC_64349
w;; mef2-Gal4	BDSC	RRID_BDSC_27390
w;; R36G02-Gal4	Fushiki et al. (2016)	RRID_BDSC_49939
w; Apxa/CyO, DGY; TM6 Sb DGY	BDSC	
yw; alk^{MiMIC10448};	BDSC	RRID_BDSC_54555
yw; drep2^{MiMIC15483}/SM6a;	BDSC	RRID_BDSC_61067
yw; jeb^{MiMIC03124}/SM6a;	BDSC	RRID_BDSC_36200
yw; Brp^{MiMIC01987}/SM6a;	BDSC	RRID_BDSC_37043

GENOTYPE	SOURCE	IDENTIFIER
w; if/Cyo, WgZ ; EyG:Gal4,UAS:bxb1/TM6 Hu Tb	BDSC	RRID_BDSC_25574
w; df(j2R)BSC40/SM6a;	BDSC	RRID_BDSC_7146
w; jeb^{S}H0442/CyO, wgZ;	Oh et al. (2003)	FBal0144029
w;UAS:alk$^{EC.UAS.Tag:MYC}$;	Bazigou et al. (2007)	FBal0194692
w;UAS-Brp$_{Short}$::Strawberry / CyO, wgZ;	Fouquet et al. (2009)	FBal0265994
w;UAS-Drep2::GFP	Andlauer et al. (2014)	FBtp0097167
w;;10xUAS-IVS-myr::mtdTomato2 / TM6b, Hu, Tb	Pfeiffer et al. (2010)	RRID_BDSC_32221
w;;UAS-Bxb1 / TM6b, Hu, Tb	Sutcliffe et al. (2017)	RRID_BDSC_67628
w; UAS-Jeb	Varshney and Palmer (2006)	FBal0194694
w;; UAS-Alk^{FL}	Lorén et al. (2003)	FBal0125507
w; jeb$^{MI03124-TG4.1}$/CyO ;	Diao et al. (2015)	FBal0304213
w;; ChaT$^{MI04508-TG4.0}$/TM6B Tb;	Diao et al. (2015)	RRID_BDSC_60317
w;; jeb$^{MI09277-TG4.2}$/TM6B Tb	Diao et al. (2015)	FBal0304224
yw; jeb^{BOnSTOP}/CyO ,dfd-GMR-nvYFP ;	This thesis	
yw; alk^{FOnYPet}/CyO ,dfd-GMR-nvYFP ;	This thesis	
yw; Brp^{FOnYPet}/CyO ,dfd-GMR-nvYFP ;	Manhart 2019	
yw; Brp^{FOnmRuby2}/CyO ,dfd-GMR-nvYFP ;	Manhart 2019	
yw; Brp^{BOnmRuby2}/CyO ,dfd-GMR-nvYFP ;	Manhart 2019	
yw; drep2^{FOnYPet}/CyO ,dfd-GMR-nvYFP ;	Manhart 2019	
yw; alk^{YPet}/CyO ,dfd-GMR-nvYFP ;	This thesis	
w;;nSyb-bxb1 (inserted in VK00027)	Aaron Ostrovsky	
w;;10xUAS-IVS-myr::mTurquoise2 / TM6b, Hu, Tb (inserted in attP2)	Aaron Ostrovsky	
w;;13xLexAOp-myr::YPet / TM6b, Hu, Tb, dfd-GMR-nvYFP (inserted in attP2)	Aaron Ostrovsky	
w;;R36G02-Gal4,UAS-bxb1 /TM6b, Hu, Tb, dfd-GMR-nvYFP	This thesis	

GENOTYPE	SOURCE	IDENTIFIER
w;Brp^{BOnmRuby2} / CyO, dfd-GMR-nvYFP;R36G02-Gal4,UAS-bxb1 @ VK00027 /TM6b, Hu, Tb, dfd-GMR-nvYFP	This thesis	
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w;; RN2:FLP, Tub84b:FRT<STOP<FRT-LexA:VP16, 13xLexAOp-myr::YPet / TM6b, Hu, Tb, dfd-GMR-nvYFP ;	Jan Felix Evers	
w;; RN2:FLP, Tub84b:FRT<STOP<FRT-LexA:VP16/ TM6b, Hu, Tb, dfd-GMR-nvYFP ;	Jan Felix Evers	
w;drep2^{FOnYPet} / CyO, dfd-GMR-nvYFP; RN2:FLP, Tub84b:FRT<STOP<FRT-LexA:VP16/ TM6b, Hu, Tb, dfd-GMR-nvYFP ;	Jan Felix Evers	
w; UAS-Flp / CyO, dfd-GMR-nvYFP; eyg-Gal4, 10xUAS-IVS-myr::mtdTomato2 / / TM6b, Hu, Tb, Sb, dfd-GMR-nvYFP	This thesis	
w; jeb^2, UAS-Brp$_{Short}$::Strawberry / CyO, dfd-GMR-nvYFP; eyg-Gal4, 10xUAS-IVS-myr::mTurquoise2 / TM6b, Hu, Tb, Sb, dfd-GMR-nvYFP;	This thesis	
w; jeb^2, UAS-Brp$_{Short}$::Strawberry / CyO, dfd-GMR-nvYFP; eyg-Gal4, 10xUAS-IVS-myr::mTurquoise2 , UAS-Bxb1 / TM6b, Hu, Tb, Sb, dfd-GMR-nvYFP	This thesis	
w; jeb^{BOnSTOP}/ CyO, dfd-GMR-nvYFP; RN2-Flp, tub84B-FRT-STOP-FRT-LexA.VP16, 13xLexAOp2-IVS-myr::YPet/ TM6b, Hu, Tb, Sb, dfd-GMR-nvYFP	This thesis	
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GENOTYPE	SOURCE	IDENTIFIER
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w; jeb^2 / CyO, dfd-GMR-nvYFP; eyg-Gal4, 10xUAS-IVS-myr::mtdTomato2 / TM6b, Hu, Tb, Sb, dfd-GMR-nvYFP	This thesis	
w; jeb^2/ CyO, dfd-GMR-nvYFP; eyg-Gal4, 10xUAS-IVS-myr::mtdTomato2 , UAS-Bxb1 / TM6b, Hu, Tb, Sb, dfd-GMR-nvYFP	This thesis	
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w; UAS-Alk^{D}N ; RN2:FLP, Tub84b:FRT<STOP<FRT-LexA:VP16, 13xLexAOp2-IVS-myr::YPet / TM6b, Hu, Tb, Sb, dfd-GMR-nvYFP	This thesis	
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w; jeb^{2} / CyO, dfd-GMR-nvYFP; UAS-Bxb1 / TM6b, Hu, Tb, Sb, dfd-GMR-nvYFP	This thesis	
w; UAS-Flp, Brp^{FOnmRuby2} / CyO, dfd-GMR-nvYFP; eyg-Gal4, 10xUAS-IVS-myr::mTurquoise2 / TM6b, Hu, Tb, Sb, dfd-GMR-nvYFP	This thesis	
w; UAS-Flp, Brp^{FOnmRuby2} / CyO, dfd-GMR-nvYFP ;	Manhart 2019	
w; UAS-Flp, Brp^{FOnYPet} / CyO, dfd-GMR-nvYFP; ;	Manhart 2019	
w ; df(jeb), UAS-Jeb / CyO, dfd-GMR-nvYFP;	Jan Felix Evers	
w ; UAS-Jeb / CyO, dfd-GMR-nvYFP ; UAS-Alk^{FL} / TM6b, Hu, Tb	Jan Felix Evers	

4.1.2 Antibodies

Table 4.2 lists all antibodies used for immunohistochemistry experiments, their provider and the corresponding dilution used in this study.

Table 4.2. Antibodies for Immunohistochemistry

Antibody	Type	dilution	Company
rabbit anti-GFP	primary	1:10000	Gift from S. Sigrist
mouse anti-Brp (nc82)	primary	1:700	Developmental Studies Hybridoma Bank (DSHB)
rabbit anti-RFP	primary	1:2000	Clontech
rat anti-GFP	primary	1:1000	Nacalai Tesqe
Donkey anti-rat, Alexa Flour 488	secondary	1:500	Jackson ImmunoResearch
goat anti-rabbit, Atto 647N	secondary	1:1000	Sigma-Aldrich
goat anti-rabbit, Alexa Flour 568	secondary	1:1000	Molecular Probes
goat anti-mouse, Alexa Flour 568	secondary	1:200	Molecular Probes
goat anti-mouse, Atto 647Nn	secondary	1:2000	Sigma-Aldrich
goat anti-mouse, STAR RED	secondary	1:1000	aberrior
goat α-mouse	secondary	1:500	NEB
CutSmart Buffer	secondary	1:1000	NEB

4.1.3 Enzymes and Buffers

All enzymes and corresponding buffers used in this study are listed in table 4.3. The enzymes were diluted according to company specifications.

Table 4.3. Enzymes and buffers

Enzymes and Reaction Buffers	Company
5 High-Fidelity DNA Polymerase	NEB
Q5 Reaction Buffer	NEB
Taq DNA Polymerase	NEB
ThermoPol Buffer	NEB
DpnI	NEB
CutSmart Buffer	NEB
Proteinase K	NEB

4.1.4 Software

Software used in this study is listed in table 4.4.

Program	Company
Micro Manager	Edelstein et al. (2014)
Fiji	Schindelin et al. (2012)
FileMaker	FileMaker, Inc.
ApE	M. Wayne Davis (`http://biologylabs.utah.edu/jorgensen/wayned/ape/`)
ilastik	Sommer et al. 2011 `https://www.ilastik.org`
Illustrator	Adobe
R	R Core Team (2016) `https://www.R-project.org/`
R-Studio	RStudio Team (2015) `http://www.rstudio.com/`
NIS Elements 5.1	Nikon

Table 4.4. Software

4.1.5 Kits

All kits used for experiments in this study are listed in table 4.5

Instrument	Company	Lot/Charge
peqGOLD Cycle-Pure Kit	peqlab	022614
peqGOLD Plasmid Miniprep Kit	peqlab	012215
QIAGEN Plasmid Midi Kit	QIAGEN	151010699
PureLink Genomic DNA Mini Kit	Invitrogen	1351407

Table 4.5. Kits used in this study

4.1.6 Instruments

Instruments listed throughout the experiments in this study are listed in table 4.6.

Instrument	Company
Binocular SMZ800N	Nikon
Binocular SMZ1270	Nikon
Cold Light Source KL 1500 LCD	Zeiss Microscopy
Flourescent Stereoscope MZ FLIII	Leica
PCR cycler Biometra	Analytik Jena
Centrifuge 5417R	Eppendorf
Centrifuge Megafuge 40R	Thermo Scientific
Centrifuge Biofuge 13	heraeus Sepatech
Incubator Multitron	HT Infors
Incubator 'Function Line' B 6	Heraeus Instruments
Incubator HPP 750	Memmert GmbH + Co. KG
Power supply	Bio Rad
Precision Scale Kern	440-45
ISlide warmer XH-2005	TED PELLA, Inc
Anaesthetization chamber	custom built

Table 4.6. Instruments

4.1.7 Chemicals

Chemicals used for experiments are listed in table 4.7

Name	Company	Lot/Charge
acrylamide, 40%	Sigma Aldrich	193200104
agar	BioFroxx	2235GR100
agarose	Invitrogen	0000335337
ampicillin	Sigma Aldrich	121M4072
apple juice	REWE - Beste Wahl	
ammonium persulfate (APS) , 10% (w/v)	Carl Roth	202184501
Bis-Tris	Carl Roth	145227333
bleach	DanKlorix	
disodium hydrogen phosphate dihydrate	AppliChem	2H007054
Ethylenediaminetetraacetic acid (EDTA)	AppliChem	127381
ethanol, 96%	Zentrallager	
ethanol, 99%	Sigma Aldrich	SZBF1170V
hydrogen peroxide	AppliChem	0F003672
liquid blocker super pap pen	Daido Sangyo	
methacrylic acid N-hydroxysuccinimide ester (MA-NHS)	Sigma Aldrich	MKBT9943V
methanol	Sigma Aldrich	STBF4574V
nail polish	Dirk Rossmann GmbH	
4-Morpholinepropanesulfonic acid (MOPS)	Carl Roth	494209954
paraformaldehyde (PFA)	EMS	130201
picrotoxin	Sigma Aldrich	P1875
poly-L-lysine (PLL)	Sigma Aldrich	SLBQ5716V
sodium dodecyl sulfate (SDS)	Serva	130858
sodium acrylate	Sigma Aldrich	MKBV0031V
sodium bisulphite solution	Carl Roth	444220949
sodium chloride	AppliChem	2X006706
sodium dihydrogen phosphate hydrate	Grüussing	2156
sodium hydroxide	Sigma Aldrich	SZBC3460V

Name	Company	Lot/Charge
sucrose	Südzucker	
N,N,N',N'-Tetramethylethylenediamine (TEMED)	Sigma Aldrich	196239027
2,2,6,6-Tetramethylpiperidinyloxin (TEMPO)	Sigma Aldrich	BCBR1162V
tris base	Sigma Aldrich	SLBQ2142V
tris-HCl	Carl Roth	023192119
triton X-100	Sigma Aldrich	SLBD2441V
Tween20	Carl Roth	3252322226
yeast extract	Invitrogen	23195052

4.1.8 Recipes

The following recipes for buffers and for the production of plates were applied.

Name	Chemical	Volume/ Concentration
Sørensen's Buffer	Disodium hydrogen phosphate	40 mM
	Sodium dihydrogen phosphate hydrate	40 mM
PBT Buffer	Sørense's Buffer	500 mL
	TritonX-100	0.3%
Applejuice Plates	Agar	24 g/L
	Sugar	25 g/L
	Applejuice	250 mL/L
LB Medium and LB Agar	NaCl	10 g/L
	Trypton	10 g/L
	Yeast Extract	5 g/L
	Agar	15 g/L

4.2 Methods

4.2.1 Animal Rearing

All flies were kept on a standard Drosophila medium (for recipe and cooking instructions see homepage of Bloomington Drosophila Stock Center: `http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/bloomfood.htm`).
Fly stocks were kept at room temperature while crosses were kept in an incubator with regulated humidity (60%) at 25°C. Embryo collection for experimental staging was performed in laying pots with apple juice plates as lids. Flies were kept at 30°Cover night to increase egg yield speed up development.

4.2.2 *in vivo* Time Lapse Imaging

According to the method described in Gärtig (2016) and Gärtig et al. (2019). Animals for intra-vital live imaging were collected in laying pots on apple juice plates. The chorion was removed from the embryos by incubation with bleach for 2 minutes. Embryos at the trachea filled stage (19.5 hours after egg laying) were transferred onto apple juice plates with yeast and placed at 25°C. For imaging the larva was shortly cleaned from yeast in a H_2O bath. The animal was then placed with the ventral side down on the glass plate of the custom build anaesthetization chamber. The animal was held in place with a cover slip that pressed on to the dorsal side driven by a motor module. Once fixed in position the animal was immobilized by anaesthetization with desflurane (Füger et al., 2007). Subsequently, image stacks of RP2 motoneuron (RP2) were acquired. The same animal was imaged using intra-vital imaging at first and second instar stage. At third instar acute *ex vivo* dissections of the ventral nerve chord (VNC) were performed, as the accumulated tissue prevents a sufficient resolution.

4.2.3 Acute *ex vivo* Dissection

4.2.3.1 Larval Dissection

Animals for dissections were collected in laying pots on apple juice plates. The chorion was removed from the embryos by incubation with bleach for 2 minutes. Embryos at the trachea filled stage at least 19 h after egg laying were transferred onto apple juice plates with yeast and placed at 25°C. Time of hatching was recorded and acute *ex vivo* dissections of the central nervous system (CNS) were performed under the stereomicroscope at the second instar stage (24 h ALH). For the dissection larvae were washed in H_2O and placed into a 60 mm-petri dish containing Sørensen's phosphate buffer (pH 7.2, 0.075 M). Using a needle, the mouth including the mouth hooks was removed from the animal. By gently pushing inner organs out of the so created opening the VNC was moved outside the body. After disconnecting all remaining trachea and

nerves from the CNS, it was mounted onto a PLL coated cover slip and positioned with the ventral side down.

4.2.3.2 Adult Dissection

Animals were anesthetized with CO_2 and washed in 70% ethanol for at least 30s. The animal was placed in Sørensen's phosphate buffer (pH 7.2, 0.075 M) and the head was disconnected from the body. Using two pairs of forceps first the upper head chitin plate was removed. Then the mouth parts were removed. Lastly, both facette eyes were removed exposing the entire adult brain. Any remaining chitin pieces were removed and the brain was mounted on a PLL coated cover slip and positioned with the posterior side down

4.2.4 Immunohistochemistry

Larval VNC were dissected in Sørensen's phosphate buffer (pH 7.2, 0.075 M). Samples were mounted on a PLL (Sigma) coated coverslip and fixed for 15 minutes in 2% paraformaldehyde (v/v) (Electron Microscopy Sciences) and 3% sucrose (w/v) in Sorensen's (pH 7.2, 0.075 M). After 30 minutes of washing in buffer containing 0.3% Triton-X-100 (Sigma- Aldrich), primary antibody treatment was performed overnight at 10 °C. After at least 30 minutes, buffer exchange every ten minutes, of washing specimen were incubated with secondary antibodies for 2 hours at RT. Subsequently, specimen were washed for one hour, exchanging the buffer every 15 minutes The following antibodies were used:
Antibodies and their dilutions as used in this study are listed in table 4.2,

4.2.5 Expansion Microscopy

According to the method described in Gärtig et al. (2019). For expansion microscopy samples were immunostained as described above, followed by incubation in 1 mM Methacrylic acid N-hydroxysuccinimide ester for 1 hour at room temperature (Chozinski et al., 2016). To minimize tissue warping in high saline buffer, samples were incubated in 30% and 60% monomer solution (MS) (1xPBS, 2 M NaCl, 2.5% (wt/wt) acrylamide, 0.15% (wt/wt) N,N'-methylenebisacrylamide, 8.625% (wt/wt) sodium acrylate) for 15 minutes each and 100% MS for 45 minutes at 4°C. Gelling was performed at 37 °C for 1 hour after adding ammonium persulfate (Sigma-Aldrich), TEMED (Roth) and TEMPO (Sigma-Aldrich) to MS. After gelling, excess gel was removed and embedded specimen were placed in digestion buffer (1X TAE, 0.5% Triton X-100, 0.8 M guanide HCL) with 8 units/ml Proteinase K (NEB) for 2 hours at 37 °C. The gel was expanded in deionized water for a total of 1 hour. Water was exchanged every 15 min. Gels were mounted on PLL coated cover slips for imaging.

4.2.6 Image Acquisition

4.2.6.1 Confocal Imaging

Image acqusition for confocal images (including expansion microscopy) was performed using a custom built spinning disk confocal field scanner. The scanner was mounted on a Nikon Ti-E microscope stand. A spinning disc from CREST Optics (`https://crestopt.com/`) was used and all images were acquired with a pinhole size of 70 μm . Images were acquired using a 60x/1.2 N.A. Olympus water immersion objective. A Photometrics Evolve Delta camera was used with an effective voxel size of 0.267 x 0.267 x 0.300 μm .
Laser from the company Omicron (`https://www.omicron-laser.de/`) were used with emission wavelengths of 450 nm, 488 nm, 515 nm, 561 nm and 638 nm.
The following other parts were used.
Z-Focus: ASI piezo-controlled z-stepping - ASI MS-2000
Motorized filter wheel: CAIRN Optospin IV (Cairn Research, United Kingdom)

Emission filters:

- Semrock, bandpass 630nm/92)
- Chroma, bandpass 480nm/40)
- Semrock, bandpass 525nm/45)
- Semrock, bandpass 542nm/27)

Dichroic mirrors:

- Chroma, triple band 440/514/561
- Chroma, quad band 405/488/561/640

Either NIS Elements (Nikon) or MicroManager (NIH, Edelstein et al. (2014)) software was used for image acquisition.

4.2.6.2 Image acquisition at stereoscope

Images on stereoscope used for larval surface area measurements (see section 4.2.7.3, figure 2.12) or jeb^{BOnSTOP} phenotype (figure 2.2) were acquired using the Nikon SMZ1270 Binocular and either a CMOS Hamamatsu C11440-22CU or CCD Hamamatsu C4742-80-12AG camera with a pixel size of 6.5 μm x 6.5 μm and 6.45 μm x 6.45 μm , respectively. Image acquisition was controlled by MicroManager (NIH, Edelstein et al. (2014)).

4.2.7 Image Analysis

Basic image processing was performed with the image processing package Fiji (`http://fiji.sc/`).

4.2.7.1 Analysis of Neuronal Morphology

Dendritic trees and axonal branches were digitally reconstructed using Amira 6.0 (FEI), supplemented with statistical algorithms developed by Jan Felix Evers (hxskeletonizations, (Evers et al., 2005; Schmitt et al., 2004)). Dendritic and filopodial dynamics were scored manually by comparing 3D reconstructions at different time points. The more complex, dendritic reconstructions were performed as follows: In a first step, third instar and second instar were compared, as dissected VNC at 48 hrs yielded the best image quality. Next, second to first and, lastly, also first to third were compared. Comparison of dendritic arbors was started from the primary neurite following the largest branches until finally comparing thin, terminal structures. For each branch the following criteria were taken into consideration:
(1) position of the branch origin (=branchpoint) along upstream branch
(2) position of the origin relative to other branches along the upstream branch (if applicable)
(3) direction in which the branch grows relative to the upstream branch, primary neurite, and cell body
(4) shape of the branch (curvature/bend)
(5) length of the branch
Regarding the direction of branches (3) a change below 90 degrees for a branch was considered as a modification of the branch not as a new branch. We assumed an error due to the orientation of the neuron in X, Y and Z during the different imaging sessions and the lower Z-resolution than X,Y-resolution. Additionally, we have observed slow rotations of branches in 10 min interval live imaging in embryonic CNS (data not shown).
From data showing stretching of stable structures, we expect that any increase in length of an existing branch from 0 to 24 h up to a factor 1.3 is not tip growth and from 24 to 48 hrs up to a factor of 1.4. Greater length was considered new length.
Csv files were exported from Amira and processed using the programming language R for statistical computing. Customized scripts were used written by Jan Felix Evers, Aaron Ostrovsky and Phil-Alan Gärtig.

See also Gärtig et al. (2019) method section.

4.2.7.2 Quantification of Synaptic Contacts

Manual quantification of synaptic contacts was performed according to the method described in Gärtig et al. (2019), which is shortly paraphrased here. Images of RP2 motoneurons after expansion microscopy were processed in FIJI by applying a Gaussian 3D filter and subtracting background noise using the rolling ball algorithm. Drep2 puncta were counted manually with the FIJI Cell Counter plugin `http://rsbweb.nih.gov/ij/plugins/cell-counter.html`). Markers were cross

checked with membrane staining, if available, and lastly only puncta juxtaposed by nc82 signal were quantified. Intensity of fluorescence was measured for Figure 2.9 D along a line drawn across the synaptic contact. Distance was measured and divided by the previously experimentally determined expansion factor 3.7.

4.2.7.3 Calculation of Larval Surface Area

In order to measure larval surface area animals were placed in water and imaged on a stereoscope. Subsequently, both the length L from anterior to posterior end as well as the diameter D of the animal at the widest region were measure in FIJI. As in Zwart et al. (2013), animals were approximated as ellipsoid and the surface area (SA) was calculated according to the following function with P=1.6075:

$$SA = 4\pi\left(\frac{2\left(\frac{L}{2}\right)^P\left(\frac{D}{2}\right)^P+\left(\frac{D}{2}\right)^{2P}}{3}\right)^{\frac{1}{P}}$$

4.2.8 Statistical Analysis

Statistical comparisons were made using pairwise Student's t test for multiple comparison of parametric data. All statistical tests were performed using the statistical programming language R (R-project). Graphs show the mean of the samples and single data points unless otherwise noted. Furthermore, standard error of the mean (SEM) is indicated. All plots were generated with in R using the package ggplot2 (`https://cran.r-project.org/web/packages/ggplot2/citation.html`). From Gärtig et al. (2019)

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Over last four years, while working on this project, and the years before during my studies I had immense support from many people. I want to thank all the people who made the time I worked on this thesis at COS a memorable and positive experience. The hours spent in the lab are only as enjoyable if they are spent with the right people. I had the great luck of sharing the ups and downs of working as a scientist with many wonderful colleagues.

I thank Jan Felix Evers who provided me with the opportunity to work in his lab on a fascinating topic and on an excellent microscope that allowed me to visualize biology no one has seen before. Thank you for advice, feedback and support over the last years.

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scripts in R and all the other little tips and tricks of scientific work.

Always just a bay away were the fellow PhDs from the Grossmann lab Rik, Philipp, Vanessa and Anna. Thanks for making long days in the lab more enjoyable, thank you for the coffee breaks, beer hours and all the yummy birthday cakes. Thank you Rik, for being there when I needed you the most. Thank you Anna, for listening to my problems and complains but also for sharing yours with me. I was happy to have you as a colleague and I don't know how I would have made it through some of the days without your awesome, energetic High Fives.

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Glossary

A27h A27h interneuron. 26, 63, 71–73, 77

aCC aCC motoneuron. 25, 26, 34, 48, 63, 66, 68, 69, 71, 73, 77, 87, 89–91

ALK Anaplastic lymphoma kinase. 11–17, 82, 85

Alk *Drosophila* anaplastic lymphoma kinase. 11–17, 29–31, 33, 35, 36, 53, 54, 57, 59, 61, 77–79, 82–85

ALKALs ALK and LTK ligands. 12

APS ammonium persulfate. 103

BDNF brain-derived neurotrophic factor. 9

BMP bone morphogenetic protein. 10, 11, 13

Brp Bruchpilot. 7, 36, 43, 46, 48, 50, 71, 79, 81

CAM cell adhesion molecules. 8, 9, 11

CNS central nervous system. ix, 3–18, 21, 24, 25, 27, 29, 30, 33, 35, 43, 44, 48, 52, 53, 61–63, 76, 78, 81, 86, 88–94, 105, 106, 108

Drep2 DNA fragmentation factor related protein 2. 7, 43, 44, 46, 48, 49, 52, 60, 66, 68, 79, 80, 90, 92

EDTA Ethylenediaminetetraacetic acid. 103

GABA gamma-aminobutric acid. 23, 63, 69, 87–90

IN$_{lat}$ lateral interneuron. 25, 26, 34–36, 39–41, 43, 49, 53, 59, 77, 81, 92

Jeb Jelly belly. 11, 13, 14, 16, 30, 33, 53

LTK Leukocyte receptor tyrosine kinase. 12

MA-NHS methacrylic acid N-hydroxysuccinimide ester. 103

MOPS 4-Morpholinepropanesulfonic acid. 103

NF1 Neurofibromatosis type 1. 15–17, 82, 84

Nf1 Neurofibromin 1. 15, 83–85

NMJ neuromuscular junction. 7–11, 14, 17, 20, 43, 89

PFA paraformaldehyde. 103

PLL poly-L-lysine. 103, 106

PTX picrotoxin. 23, 27, 62, 63, 66, 68, 69, 71, 73, 77, 86–90

RP2 RP2 motoneuron. 10, 16, 24–26, 30, 34, 35, 46, 48, 49, 52, 53, 57, 61, 63, 66, 68, 69, 73, 76, 77, 80–83, 85, 89–92, 105

RTK receptor tyrosine kinase. 11, 12

SDS sodium dodecyl sulfate. 103

TEMED N,N,N',N'-Tetramethylethylenediamine. 104, 106

TEMPO 2,2,6,6-Tetramethylpiperidinyloxin. 104, 106

TGF-β transforming growth factor beta. 9, 10, 13

VNC ventral nerve chord. 5, 6, 10, 25, 26, 31, 34, 36, 39, 40, 43, 47, 58, 63, 71, 78, 84, 92, 105, 106, 108

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