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„Breaking Symmetry: Genetic dissection of a lateralized neuronal circuit in *Drosophila melanogaster* "

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Abstract (english)

Anatomical and functional left-right asymmetry of the central nervous system seems to be a general principle in bilateral animals. How and why bilateral symmetry is broken during development is largely unknown. Here I introduce the *Asymmetric Body* (*AB*), the fifth neuropile of the Central Complex in the brain of Drosophila melanogaster, as an experimental model to investigate key questions in brain lateralization. Using a variety of genetic techniques, I could show that the AB circuit consists of two types of neurons: ventral cluster afferent neurons (vANs) and dorsal cluster fan-shaped-body-(FB)-intrinsic neurons (dFBNs). Despite the AB being a bilaterally paired structure, vANs only innervated the right AB. Clonal analyses revealed a different projection pattern of the left versus right vANs while dFBNs develop overall bilaterally symmetric. During Ab development the Fascilcin2-(Fas2)-positive vANs grow in a symmetric fashion and secondarily undergo lateralized remodelling. Comparative studies of Fas2 expression across Drosophila species indicates profound variation in the AB circuitry. These results will support further studies to decipher the genetic programs underlying developmental mechanisms and the functional impact of lateralization on brain function and behaviour.

Zusammenfassung (deutsch)

Die anatomische und funktionale Links-Rechts-Asymmetrie des Zentralnervensystems scheint ein grundlegendes Prinzip innerhalb der bilateralsymmetrischen Tiere darzustellen. Wie sich diese Abweichungen von der bilateralen Köpersymmetrie jedoch während der Entwicklung herausbilden ist weitgehend unbekannt. Der *Asymmetric Body (AB)*, das relativ unerforschte fünfte Neuropil im Zentrum des Gehirns von *Drosophila melanogaster*, bietet wegen seiner namensgebenden Asymmetrie die Möglichkeit grundliegende Mechanismen der Entwicklung und Bedeutung von anatomischer Asymmetrie des Nervensystems und der unterschiedlichen Aufgabenverteilung zwischen linker und rechter Hirnhälfte zu untersuchen. Anhand meiner Daten kann ich zeigen, dass sich der neuronale Schaltkreis des AB aus zwei Klassen von Neuronen zusammensetzt: ventralen afferente Neuronen (vANs) und Fan-shaped Body-(FB)-spezifischen Neuronen (dFBNs), die Information aus dem AB in die dorsalen Schichten des FB weiterleiten. Eine klonale Analyse ergab, dass obwohl der AB ein paariges Neuropil darstellt, die vANs nur den rechte AB ansteuern, wobei sich Morphologie und Verhalten homologer Neurone zwischen rechter und linker Hemisphäre deutlich unterscheidet. Die dFBNs dagegen scheinen bilateral symmetrisch aufgebaut zu sein, obwohl sich auch hier Unterschiede im innervierten AB-Volumen zwischen rechts und links nachweisen lassen. Die fasciclin-2-(Fas2)-positiven vANs scheinen während der Puppenentwicklung ursprünglich symmetrisch ausgebildet zu sein und sich anschließend sekundär asymmetrisch umzuformen. Vergleich der Expression von Fas2 im AB in verschiedenen *Drosophila*-Arten deutet darauf hin, dass die Verschaltung des AB großer interspezifischer Variation unterliegt. Die Variation innerhalb des Wildtyps (*D. melanogaster)* deutet darauf hin, dass sich die asymmetrische Verschaltung der afferenten Neurone außerdem in eine symmetrische überführen lässt. Damit bietet der AB als Modell die Möglichkeit aufgrund der unterschiedlichen Verschaltung homologer Neuronen zwischen den Hemisphären grundliegende Mechanismen der Entwicklung und Bedeutung von anatomischer Asymmetrie des Nervensystems auf Einzelzellniveau und der Lateralisation linker und rechter Hirnhälfte auf Funktionsebene zu untersuchen.

1. Introduction

1.1 Asymmetric Bilateria

In everyday life we tend to think of ourselves as symmetric and view the left body half as the mirror image of the right one. Even the major evolutionary branch housing over 99% of modern-day animal species including humans are named after this left-right symmetrical organization "*Bilateria*"[1]. But this supposedly bilateral symmetry is only true at first glance. Left and right are not the same on many levels from minor imperfections[2] to actual stable large-scale asymmetries like the position of our heart and liver[3,4]. We even exhibit lateralized behaviour (as e.g. we usually prefer to use one specific hand to manipulate objects), and the two hemispheres of our brain seem to process information differently[5,6]. This lateralization appears to be of critical importance. Individuals affected by several different neurological disorders like autism, dyslexia or schizophrenia show a reduced or reversed asymmetry of brain regions[6] while a number of studies showed that animals performed better in cognitive tasks the more pronounced their side preferences were[5,7,8]. Although research investigating the neuronal circuits underlying brain asymmetry is in its beginning[5,6], evidence shows that connectivity of the right and left hemisphere deviates significantly from each other[9–11]. For example the left hemisphere of the human brain possesses relatively more grey matter and a higher density of cells, especially in the frontal and pre-central regions[12]. White matter appears white because of glial sheaths surrounding the axons, which increases action potential propagation velocity and is a characteristic of neurons projecting over long distances. The left hemisphere is thus believed to be involved in functions that require local processing of cells working together in close spatial proximity, while the right hemisphere is assumed to be specialized in functions, which demand integrating information from several different brain areas[12]. Research indicates, that an asymmetric brain is neither a novelty of humans[7] nor limited to vertebrates[13–16], but seems to be a general principle of nature[5]. Since the brain is one of the most energy demanding structures and should thus be subjected to high evolutionary constraints[17,18], the ubiquity of an asymmetric structure of the central nervous system across most animal taxa is remarkable and raises the questions of the developmental mechanisms behind breaking the bilateral symmetry and establishing a left-right axis, of how asymmetry impacts information processing and of what reproductive benefit brain lateralization imposes in the current environment[19].

1.2 Breaking symmetry and left-right differentiation

The central question of brain lateralization is concerning how the consistent left-right patterning develops. On way of telling the story of differential left-right development is that we all start off as ball of cells. The spherical symmetry of this ball is broken first by establishing an anterior-posterior axis and second by development of a dorsal-ventral axis[5]. As a result of these two-steps the ball has transformed to the bilateral symmetry mirroring the urbilaterian organism[20]. The bilateral symmetry then gets broken again and a left-right axis is superimposed on the developing organism. The probably best-established upstream pathway for left-right patterning is the genetic regulatory network called the "nodal cassette"[5,21]. In essence the activation of the *nodal* gene left of the midline causes the downstream activation of Lefty and Pitx2 und thus correct placement of visceral organs[21]. Variations of the nodal cassette were found in different species covering many phyla including snails, fish, frog, chicken, mouse and humans[21,22]. However, cases of normal organ situs despite abnormal nodal expression[23], normal brain lateralization in patients with reversed organ situs[5] and the complete absence of the nodal gene in organisms like Drosophila and C. elegans[22] show that the nodal cassette cannot be the only way to establish left-right asymmetry.

A model that attracted a great deal of attention was the concept of the "F-molecule" proposed by Brown and Wolpert[24]. It theorizes that a chiral molecule with the ability to orientate relative to the anterior-posterior and dorsal-ventral axis (represented by the two arms of the letter F) could cause the break of bilateral symmetry e.g. by the unilateral transport of a diffusible morphogen at the embryo's midline. Although, even Wolpert himself has rejected diffusible morphogens as "too complicated and too messy"[25,26], the model still is intriguing because it takes into account that the basic building blocks of all life forms are handed themselves. Single molecules like amino acids and sugars as well as polymers including DNA, microtubules and F-actin are chiral and typically organisms only use the dextral or the sinistral form exclusively (L-amino acids, D-sugars, etc.)[27]. Chirality is a special case of asymmetry. No matter how hard we might try, our left hand is not superimposable on our right hand. To tighten a screw, we have to turn it in the correct direction depending on whether the thread spirals clockwise or anticlockwise. The idea that the chirality of molecules could be the carrier of left-right information that gives rise to asymmetry on an organism level provides a plausible mechanism.

A truly elegant solution to the problem of breaking bilateral symmetry that is in concordance with the F-molecule model was proposed in the form of the "nodal flow"[28]. A symmetric patch of motile cilia on the organizer tissue (called the "node" in mice) moves

extracellular fluid over the surface of the embryo. Since the molecular motors of motile cilia are assembled in a chiral fashion and the cilia are consistently anchored in a specific orientation relative to the apical-basal polarity of the epithelium, all the cilia rotate in a clockwise direction, which creates a unidirectional leftward flow of the extracellular fluid[21,28,29]. Experimental data supports that this flow both transports vesicular parcels filled with morphogens (sonic hedgehog and retinoic acid) as well as deflects a second type of cilia, which causes Ca^{2+} release and triggers asymmetric ion signaling[29]. However, an increasing number of cases of left-right asymmetry are documented at a time point during development before cilia would be able to create a directed movement of the extracellular fluid (e.g. asymmetric expression of activin receptors (chick embryo), asymmetric gap-junctional communication and H^+/K^+-ATPase activity (*Xenopus* and chick embryos)), while other species lack nodal flow altogether (e.g. chick and pig embryos)[21,23,28,30]. Metabolic differences between left and right blastomeres can be detected with mass spectrometry as early as in the 8-cell frog embryo (*Xenopus*)[31]. Thus, although its importance for left-right determination is supported by research, it seems the nodal flow has to be rejected as the origin of breaking bilateral symmetry[21,23,28,30].

Evidence shows that L/R asymmetry is influenced by a number of factors that might work in parallel and are able to correct each other, namely imprinted chromatin[30], the planar cell polarity (PCP) and the chiral cytoskeleton[21].

When the chromatin is replicated during mitosis, the regions that are activated can differ between the old and the new DNA strand[30]. In embryonic stem cells, in the endoderm and the neuroectoderm the chromatin is not segregated randomly, but is directed[32]. This could be the source of differential left-right gene expression. A protein that was shown to be involved in this selective chromatin segregation is *left-right dynein (lrd)*[32]. *Lrd* is also known to be part of the cilia motor and to be crucial for the correct rotation of nodal cilia in the mouse embryo. Mutations of *lrd* cause randomization of organ placement if mutated[32–34], demonstrating that the same molecule potentially can initiate or amplify left-right asymmetry in more than one specific pathway.

The PCP is an intracellular mechanism, which enables consistent orientation of structures across a tissue e.g. in the wing and eye of Drosophila. Proteins of the PCP pathway interact reciprocally, which leads to the asymmetrical localization of PCP proteins on only one side of the cell relative to the apical-basal axis of the tissue. As demonstrated in the example of the nodal flow, where the PCP pathway coordinates the correct orientation of the cilia, the PCP potentially can work as a large-scale amplifier of left-right asymmetry cues[21,35].

The importance of the chirality of the cytoskeleton for left-right asymmetric development seems to be not only conserved among animals, but also was documented in plants. Even single cells are known to use their cytoskeleton to establish intracellular chirality[36]. The motor protein Myo1D was show to drive visceral left-right asymmetric development in Drosophila by chiral interactions with F-actin even in tissues that are not lateralized in wild types[3,37,38] and the cortex of *Xenopus* eggs shows a pre-existing and consistent chirality of F-actin fibers that, if disturbed, randomizes the visceral left-right orientation[39]. In short, it seems that an embryo is never truly bilateral symmetric and that organisms exploit intrinsic chirality for establishing a stable left-right pattern with downstream amplifying mechanisms like the PCP and gene-regulatory cascades. Left-right pattering is a complex topic that is far from being solved and that is spanning multiple disciplines[21,30,39].

1.3 *Drosophila melanogaster* and the Asymmetric Body

In 2004 a L/R-asymmetric structure was discovered in the central brain of *Drosophila melanogaster*, subsequently named asymmetric body (AB)[40]. The AB is a paired neuropile, densely packed with synapses, located slightly anterior to layer 1 of the fan-shaped body (FB) and thus, part of the Central Complex (CX). Typically, the neuropile appears more prominent on one side in staining, size and structure[41–43]. The asymmetry of the AB circuit was first revealed with a Fas2-antibody staining. In the vast majority of *Drosophila melanogaster* flies Fasciclin2-(Fas2)-antibodies accumulate only in the AB of the right hemisphere. However, in 7.6 % of 2550 tested flies of the wild-type lab stock “Canton Special” both ABs were reported to be Fas2-positive. Testing those flies in associative learning set-ups showed that the long-term memory of flies with bilaterally Fas2-positive ABs is impaired, linking this specific asymmetric region to the formation or retrieval of long-term memory[40]. The asymmetry of the neurons in the AB seems to develop during metamorphosis[37]. The AB was also found in the flesh fly *Neobellieria bullata* utilizing synapsin staining. This indicates, that the AB might be a common feature among the Diptera[42].

Although D. melanogaster has proven to be an invaluable model organism to study body patterning, not much work was invested in the study of left-right asymmetry because of a lack of clear phenotypic L/R markers[3]. In *Drosophila melanogaster* the most used markers for left-right asymmetry are coiled tubular structures: the gut, the genitalia and the testis, which develop independently of the genital disc. The left-right axis in *Drosophila* is of zygotic origin. If the A/P-axis is experimentally reversed, the left-right

axis develops according to the new axis, ruling out determination by maternal factors. Several factors like JNK signalling, Fas2 and Single-minded have been implicated to be involved in left-right determination. However, mutations are of low phenotypical penetrance[3]. The most likely candidates to control visceral left-right asymmetry in *D. melanogaster* are two motor proteins: Myosin 1D (Myo1D) and Myosin 1C (Myo1C). Myo1D drives counterclockwise, circular motility of actin filaments bound to its head domain, which acts as motor region. This chiral interaction seems to mediate left-right asymmetry of visceral organs and is sufficient to drive dextral twisting of cells, organs or the whole body. Myo1D as a dextral determent can induce de-novo asymmetries in organs, which do not exhibit L/R asymmetry in wild-type flies. Absence of Myo1D leads to a situs inversus[38]. In contrast to vertebrates, in Drosophila visceral organs can have their own independent organizers. Myo1D expression is needed in all tissue-specific organizers to develop the wild-type L/R asymmetries[37]. Myo1C acts antagonistic to Myo1D and overexpression leads to a leftward twisting. Ectopic expression of Myo1C causes sinistral looping, but the coiling of Myo1C phenotypes is less pronounced[38]. Myo1D was shown to bind to Dachsous, a protein belonging to the PCP, and thus linking those two mechanisms[44].

However, Myo1D or Myo1C controlling genes do not determine the L/R asymmetry of the brain, suggesting that asymmetry of the central nervous system is under control of an alternative mechanism. Likewise, asymmetry of visceral organs and the lateralization of the brain seem to be controlled by two different mechanisms in vertebrates[37].

1.4 Aims of this thesis

While lateralization research dates back to at least the 19th century with Broca's discovery that damage to a certain area in the left brain hemisphere impairs language, but lesion of the homologue tissue in the right hemisphere does not, progress has been slow and both the mechanisms of how left-right asymmetries develop as well as how exactly asymmetry improves cognition are largely unknown[5]. While visceral and brain asymmetry might be controlled by similar pathways, both seem to develop independently[5,37]. The goal of this thesis is to lay the groundwork for establishing a *Drosophila* model for the development of brain lateralization, with the potential to study the translation of genetic interactions into a lateralized neuronal circuit and function.

At a neuroanatomical level asymmetries have been described in size and shape of comparable brain regions, but little is known about how the asymmetric configuration of individual neurons impacts the functions of the circuit. With the vast array of genetic

tools and three different established binary expression-systems at its disposal[45,46] *D. melanogaster* as a model system is perfectly suited for circuit analyses.

The main focus of this thesis was thus to describe the neurons of the AB circuit. Aim 1 was to describe the cell morphology on a single cell or cell cluster resolution. Aim 2 was to identify axonal and dendritic regions and determine the direction of information flow in the AB circuit. This will also allow determining the level of their asymmetry (aim 3), that is whether the circuit asymmetry lies within a higher prevalence of one component (e.g. synapses or ramifications) on one side or whether some circuit components are exclusive to one specific hemisphere.

At the developmental level it is important to determine how this asymmetric growth in the central nervous system is controlled. Thus, I document here the AB development during the pupa stage (aim 4).

In order to test the functional effects of a “symmetrized” brain as occurring in flies with bilateral Fas2 positive ABs, it would be highly beneficial to find a way to reliable induce this phenotype or even establish a fly strain with symmetric ABs. To get first insights about the control of left-right asymmetric development in the brain of Drosophila (aim 5), I looked at the AB formation in Neuroglian (Nrg) mutants. This L1-CAM homologue[47] is necessary for axons to project over the midline. Mutations of Nrg can lead to a split-brain phenotype lacking horizontal commissures between left and right hemisphere. Studying the ABs in split-brain flies serves multiple purposes: First, if the left-right asymmetry of the AB circuit develops due to a differential expression in the cells of the right hemisphere compared to the right due to different imprinted chromosomes, it would have the possibility to do so without communication with the other hemisphere. Should left-right asymmetry persist in the split brains of Nrg mutants, it would indicate that direct cell-cell communication or a midline organizer are not needed to establish the AB circuit.

Second, if the asymmetric Fas2 expression is due to only the neurons of the right hemisphere expressing Fas2, projections of neurons in the left hemisphere should not be Fas2 positive. In addition, to check if Fas2 is involved in the AB development, I performed a Fas2-kockdown driven in AB neurons.

Third, Nrg mutation disrupts the midline of the brain[48] including glial cells and the transient interhemispheric fibrous ring (TIFR)[49,50]. Because of the close proximity of the AB and the CX to the midline, and because the midline is involved in axon guidance[50] and axis pattering[51] it poses a likely position for a region functioning as organizer of left-right patterning in the brain. If midline structures work as organizers for AB development, the

direction of asymmetry might be randomized or the brain “symmetrized” in split-brain flies, depending on the information relayed by the organizer. Effects on the circuit asymmetry could be an indication of involvement of the midline and cell-cell communication.

At last, I will look at the phylogenetic history of the AB (aim 6). Although the neuroarchitecture of the central complex is conserved throughout the arthropods regions of brain asymmetry seem to be quite dynamic among related species and might reflect rapid cognitive and behavioural adaption to distinct ecological needs and the degree to which the insect central complex is elaborated in a given species is directly correlated with its lifestyle[52,53].

2. Material and Methods

2.1 Fly strains

Flies were kept on standard medium in incubators at 25°C. For a list with all used fly strains and their sources see Table 1. I used five Gal4 lines and their corresponding lexA-lines that show expression in the AB[41]. Morphology of neurons labelled by Gal4-lines were studied by using UAS-mCD8::GFP, 10x UAS-mCD8::GFP and 10xUAS-mTomato, or with 10x LexAop-mCD8::GFP for LexA-lines respectively. To verify overlap between lines, strains carrying different Gal4- and LexA lines combinations (w^-; LexA/cyo; Gal4/TM2) were established and neurons labelled utilizing UAS-RFP and 10x LexAop-mCD8::GFP. For cell cluster and single cell analysis Gal4-lines were crossed to a Flybow strain (w^-; hs-mFlp5/cyo; UAS-FB1.1B/TM6). Clones were induced by heat-shocking late 3rd instar larva for 1.5h in a 37°C water bath.

Fusion proteins of Bruchpilot, synaptotagmin and ICAM5 with fluorescent proteins (GFP or mCherry) under control of an UAS-site were used to tag synaptic regions (w^-; UAS-Brp::GFP;; and w^-; UAS-DenMark, UAS-sytGFP; D/TM6c;). Bruchpilot and synaptotagmin are transported to presynaptic sites while ICAM5 marks dendritic areas. This makes it possible to infer about the direction of information flow between synaptic partners. The *trans*-Tango construct was used to express membrane-tethered red fluorescent protein (mtdTomato) in cells postsynaptic to the cells of a labelled Gal4-line[54]. With this method the morphology of the neurons postsynaptic to the cells of interest can be studied.

Nrg^{849} mutants were tested to study the possible involvement of the TIFR as a central nervous system organizer in AB left-right development. Knockdown of Nrg in AB neurons was also conducted with UAS-Nrg^{RNAi} to control for role of Nrg within AB neurons.

Fas2 expression in the AB was monitored with antibody staining or $Fas2^{GFP397}$ to match cell morphology to the reported phenotypes[40]. $Fas2^{GFP397}$ is an exon trap insertion line tagging three different isoforms of Fas2 [55]. Expression fully overlaps with α-Fas2 antibody staining in the central brain and was hence used instead of antibody staining in experiments not involving Gal4 driven GFP-expression. To study the importance of Fas2 in AB development, Fas2 was knocked down with UAS-$Fas2^{RNAi}$.

To get a rough overview over the interspecies differences in the AB region, I performed a small screen of related *Drosophila* groups. To account for the effect of inbreeding on the asymmetry of the AB and the proportion of the bilateral Fas2 positive phenotype in *D. melanogaster* (the AB was discovered in well-established fly strains held in the lab

for decades), I investigate the prevalence of AB phenotypes in newly caught flies from the wild established as lab fly strain.

Wild type flies were caught in the wild in Vienna and established as fly strains in the lab or were provided by the Miller Group of the Medical University of Vienna.

Table 1. Constructs and Fly-Strains.

Name	Source	Reference
R38D01-Gal4/-lexA R42C09-Gal4/-lexA R52H03-Gal4/-lexA R70H05-Gal4/-lexA R72A10-Gal4/-lexA	Bloomington Drosophila Stock Center	Jenett et al. 2012[41]
UAS-mCD8::GFP UAS-mRFP, lexop-mGFP 10x UAS-mCD8::GFP 10x lexAop-mCD8::GFP UAS-FB1.1B UAS-nlsGFP	Bloomington Drosophila Stock Center	Hadjieconomou et al. 2011[56]
UAS-Brp::GFP	Bloomington Drosophila Stock Center	Fouquet et al. 2009[57]
UAS-sytGFP		Zhang et al. 2002[58]
UAS-DenMark		Nicolaï et al. 2010[59]
trans-Tango		Talay et al. 2017[54]
Fas2^{GFP397}	Bloomington Drosophila Stock Center	Silies and Klämbt 2010[55]
UAS-Fas2^{RNAi}		Perkins et al. 2015[60]
Nrg^{849}	Bloomington Drosophila Stock Center	Dorsch 1985 (diploma thesis) / Strauss and Heisenberg 1993[48]
UAS-Nrg^{RNAi}		Perkins et al.

	2015[60]
D. melanogaster (Vienna J)	Caught in the wild
D. simulans	Caught in the wild
D. malerkotliana	Wolfgang J. Miller
D. prosaltans	Wolfgang J. Miller
D. paulistorum	Wolfgang J. Miller
D. willistoni	Wolfgang J. Miller
D. sturtevanti	Wolfgang J. Miller
D. repleta	Caught in the wild

2.2 Binary Expression Systems

Most experiments in my work use the binary expression systems (Gal4/UAS, LexA/LexAop, QF/QUAS). In binary expression systems a transcription factor (Gal4, lexA or QF) is expressed under the control of DNA sequences in or near a target gene. This creates the possibility to express e.g. Gal4 only in a subset of neurons. Subsequently in cells expressing the transcription factor Gal 4 will bind to its specific activation sequence (in case of Gal4 to UAS) and activate expression of a for example a reporter gene[45,46].

2.3 RNAi Knock Down

RNA interference (RNAi) is a conserved mechanism for controlling gene expression in wich double stranded RNA gets cleaved be the ribonuclease Dicer into pieces of small interfereing RNA (siRNA). This siRNA can be loaded on RNA induced silencing complex (RISC). The double strand siRNA gets unwound by a helicase in RISC and and in turn binds by its antisense strand mRNA with a highly complementary sequence. Bound mRNA is degraded thus inhibiting translation[61]. By introducing a transgene consisting of an UAS-site and inverted repeats of short fragment of the target gene this mechanism can be used to knock down gene expression in specific cells under control of Gal4[62]. In this thesis I used this method to knock down Fas2 and Nrg in AB neurons.

2.4 Immunohistochemistry

Dissection of adult and pupa brains was performed in PBS (phosphate-buffered saline). After the removal of cuticle, tracheae and connective tissues the samples were fixed in a solution of 4% PFA (paraformaldehyde) in PBS for 1 h at room temperature. PFA was

removed and the samples were washed (4×15 min) in 0.3% PBS-T (**PBS containing 0.3% TirtonX-100)** followed by blocking with **goat serum (10%, in PBT)** for 1 h at room temperature. Subsequently, the sampled were incubated with primary antibodies (Table 2) diluted in **goat serum (10%, in PBT)** overnight at 4°C on shaker. After being rinsed (4×15 min) in PBT at room temperature, samples were incubated again overnight at 4°C with the secondary antibodies (Table 2) diluted in **goat serum (10%, in PBT).** After further rinses (4×15 min) in PBT at room temperature, whole brains were mounted in Vectashield (**Vector Laboratories).** Depending on the region of interest the brains were orientated with the anterior or the posterior side facing upwards. Modeling clay was placed between the corners of the cover slips and the microscope slides to protect the brains from pressure.

Table 2. Antibodies used for staining.

Typ	Antibody	Origin	Source
Primary	α-NCadherin (Ncad)	Rat	DSHB
Primary	α-Fasciclin 2 (Fas2)	Mouse	DSHB
Secondary	α-Rat Alexa 647	Goat	Invitrogen
Secondary	α-Mouse Alexa 568	Goat	Invitrogen

2.5 Image acquisition and analysis

Images were collected with a (Leica) Confocal Microscope TCS SP5II using a 20x oil immersion objective. Images were scanned as stacks of optical sections at 1,55 µm spacing with a resolution of 512x512 pixels at a speed of 200 Hz. Acquired 2D images were processed in Fiji[63]. 3D rendering, volume and GFP intensity measurments were performed with Imaris software (Bitplane, Oxford Instruments). Statistical analysis carried out in R (R Foundation for Statistical Computing, https://www.R-project.org/). Laterality index (i) as a measure of side difference compared to the total AB volume or fluorescence intensity side was calculated by the forumula i= (r-l)/(r+l) (r = right side value, l = left side value).

3. Results

3.1 Organization of the asymmetric CX circuit

The AB neurons labelled with the driver lines published in Jenett et al 2012[41] can be roughly divided into two main types, ventral afferent projection neurons and dorsal FB intrinsic neurons (see Fig. 1 B). The cell bodies of the afferent neurons are located ventrolateral to the antennal lobes (Fig. 1). Their neural processes grow upwards and form a bifurcation lateral to the central complex. The medial process projects across the midline and arborizes in the ABs, the dorsal process extends to the SLP to build terminal branches.

The cell bodies of the FB intrinsic neurons form distinct clusters posterior to the fan shaped body around the Protocerebral Bridge. They project bilaterally to the ABs and from there upward to the dorsal FB (Fig. 1 B).

3.1.1 Ventral afferent SLP-AB-neurons

R52H03-Gal4 labels a type of AB neurons, whose cell bodies cluster ventral to the antennal lobe of each hemisphere (Fig. 2 A). The cell processes project upwards and innervate two neuropiles: the AB and the SLP (Fig. 2 A). While the dorsal processes terminate bilaterally in the SLP, typically only the right AB is targeted by the medial processes, thus forming a circuit component, which only exists in one hemisphere, but is absent from the other. Clonal analysis of R52H03-Gal4 revealed a different projection pattern of the left versus right ventral afferent cluster. AB afferents in the right hemisphere innervate only the ipsilateral right AB (Fig. 2 B). In contrast, clones of the left cluster don't arborize in their ipsilateral AB, but project across the midline to the contralateral side, where they innervate the right AB (Fig. 2 C).

The number of R52H03-positive cells varies between brains and hemispheres and ranges from 4 to 8 per hemisphere. However, no side bias was found. On average I found 6,29 cells per cluster with a median of 6 and a maximal difference between left and right of two cells.

In 5 out of 89 adult brains (excluding data from RNAi and Nrg experiments because AB morphology might be effected) and additional 7 brains from the clonal analysis the neurons from both sides were found to innervate both the ispsilateral and the contralateral

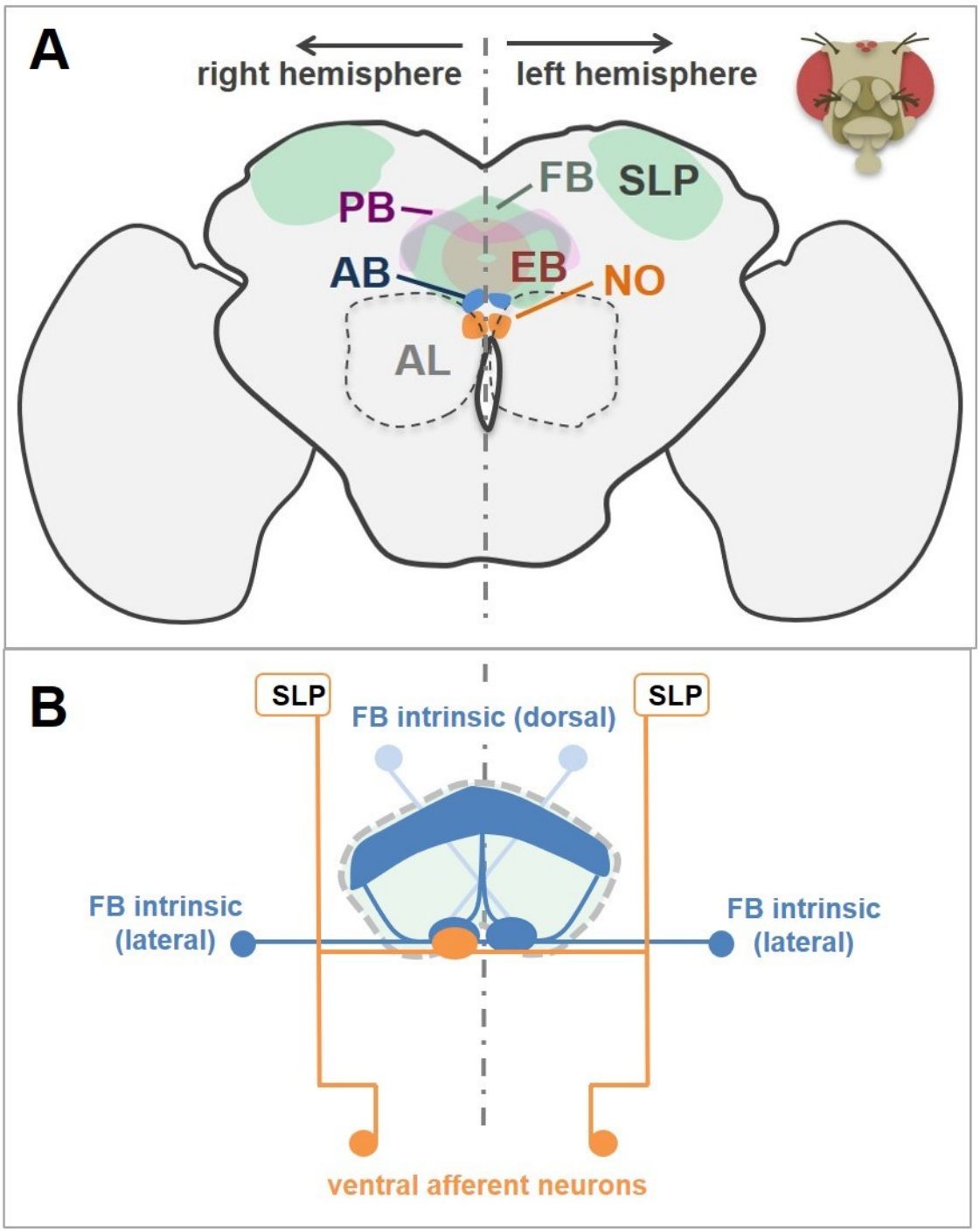

Figure 1. Schematic overview of the brain regions of *Drosophila melanogaster* mentioned in this thesis. A: All images in this study were orientated anterior to posterior. The antennal lobes (AL) are the most anterior region of the brain. The central complex is composed of five neuropiles: the ellipsoid body (EB), the fan-shaped body (FB), the asymmetric body (AB) and the noduli (NO). Posterior to the FB lies the protocerebral bridge (PB). The superior lateral protocerebrum (SLP) is located close to the dorsal surface of the brain. **B:** Main morphological classes of AB neurons: ventral afferent and FB intrinsic.

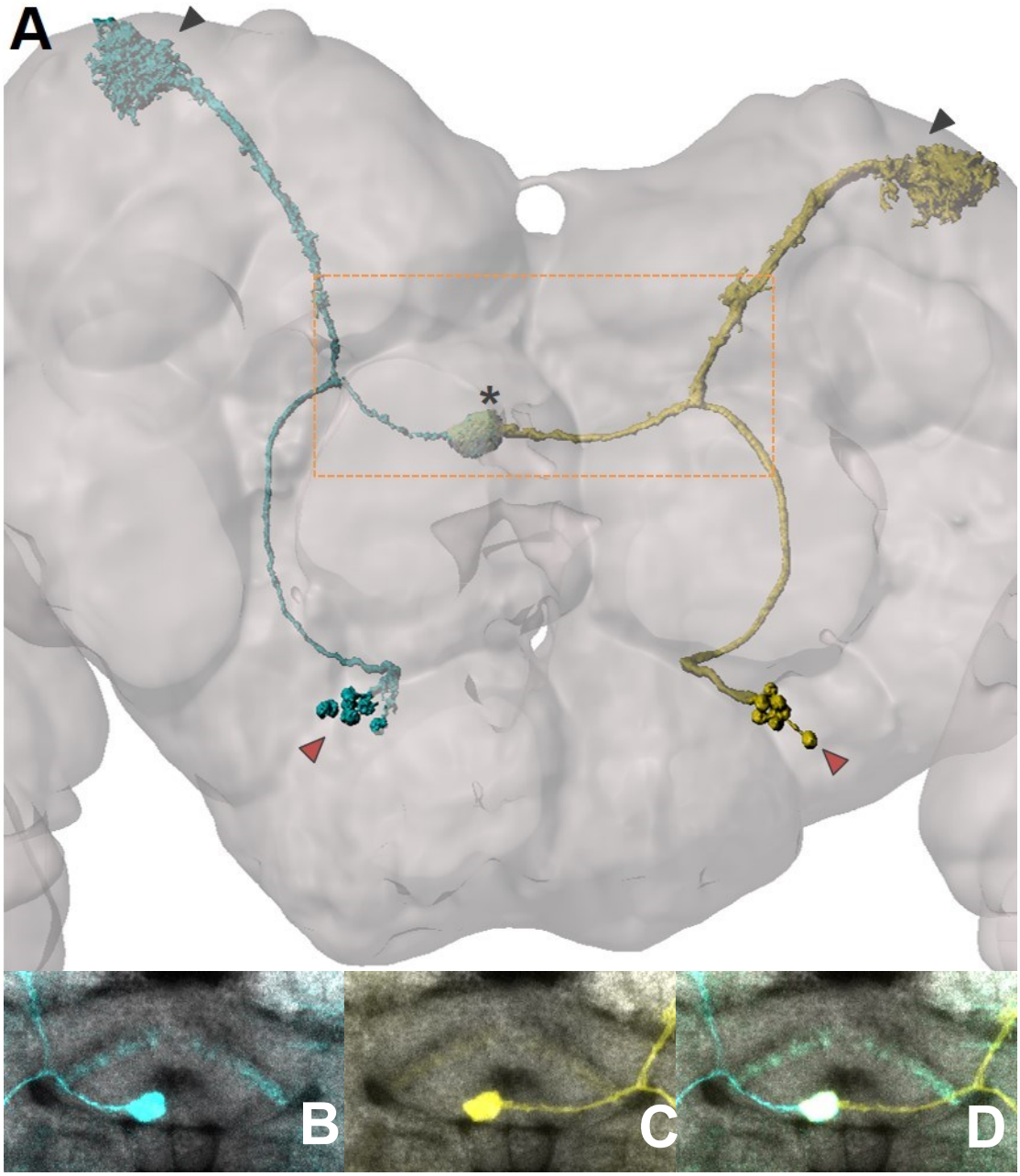

Figure 2. Asymmetric morphology of SLP-AB neurons. A: SLP-AB neurons project from the SLP (grey arrowheads) to the AB (*) in the central complex. The cell bodies are located ventrolateral to the AL (red arrowheads. The orange box indicates the section shown in **B-D**. Neurons in the right hemisphere (cyan) stay ipsilateral **(B)**, while the neurons of the left hemisphere (yellow) and cross over the midline to innervate at the contralateral target site on the right **(C)**. The innervations in the AB from left and right neurons overlap completely **(D)**. α-Ncad-anitbodies were used for reference staining (gray). Neurons are labeled with FlyBow 1.1B (pm-mCitrine and mCD8-Cerulean) driven by R52H03-Gal4.

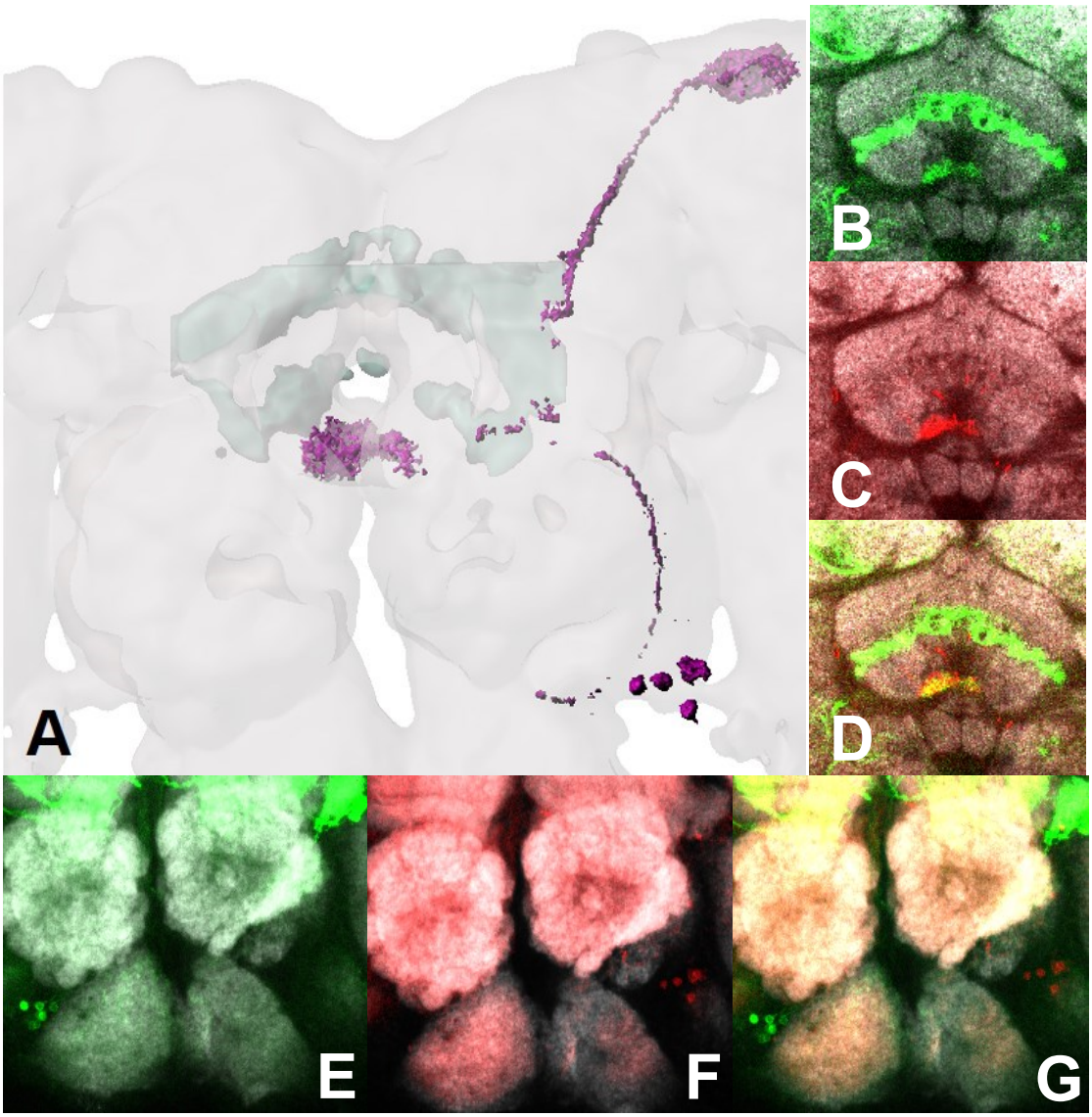

Figure 3. Bilateral AB innervation by SLP-AB neurons. A: Cell clones (magenta) created with FlyBow 1.1B (mCD8-Cherry) innervating left and right AB. Cells from the right hemisphere (green) **(B)** as well as the corresponding neurons from the left hemisphere (red) **(C)** cross the midline and bilaterally innervate left and right AB. The innervations overlap **(D)**. **E-G:** Expression of EGFP is restricted to neurons in the right hemisphere, while mCherry is only expressed by neurons on the left. α-Ncad-anitbodies were used for reference staining (gray). For orientation the FB is highlighted in light green **(A)**.

AB (Fig 3). This coincides with both ABs being Fas2-positive in contrast to a unilateral FasII expression in the right AB neuropile (n=3). Mean Fas2-intensity in flies with bilaterally Fas2-positive ABs does not differ significantly between right and left (*paired samples t-test*, t = -1.8912, df = 4, p-value = 0.1316). However, the mean Fas2-intensity seems to have a tendency to be higher in the right AB even if bilaterally Fas2 positive.

The cellular polarity of R52H03-Gal4 neurons was tested with the presynaptic markers Bruchpilot and synaptotagmin, which were tagged with GFP and with the dendritic marker DenMark, a hybrid protein of ICAM5/Telencephalin and the red fluorescent protein mCherry[59] (Fig. 4). The innervating processes in the AB contain high concentrations of Brp and sytGFP, while almost no presynaptic markers can be seen in the SLP. The SLP is almost completely void of any signal from presynaptic or postsynaptic markers. Sparse DenMark-signal could indicate a dendritic region. Similar results were described by a recent study from the Janelia labs by Wolff and Rubin[43]. Thus, these cell type seems to mainly project information from the dendritic areas in the SLP to the central complex via the AB or process information over short distances within the AB and were therefore named SLP-AB-neurons following the nomenclature suggested by Wolff et al.[43,64].

Fas2-expression in the adult brain completely overlaps with the innervation of the AB by the SLP-AB-neurons. RNAi-knockdown driven by R52H03-Gal4 leads to a complete loss of α-Fas2-antibody staining in the AB (Fig. 5). Thus, SLP-AB neurons seem to be the source of Fas2 expression in the AB at least in the adult flies.
Co-expression of UAS-RNAi and UAS-GFP did not reveal any changes in overall SLP-AB cell morphology nor did it affect the ratio of unilateral to bilateral AB innervation.

3.1.2 FB intrinsic AB-FB neurons

The second main morphological class of neurons in the AB circuit consists of FB intrinsic neurons interconnecting AB and FB. AB-FB-neurons labelled by R70H05-Gal4 innervate both ABs (Fig. 6). These neurons seem to from presynaptic sites in the two ABs and in the FB layers l6, l8 and l9. Dendritic regions are mostly located in the ABs (Fig. 7). The morphology appears overall bilateral symmetric. However, in the majority of brains which show a unilateral, right Fas2-positive AB, the volume of AB innervation

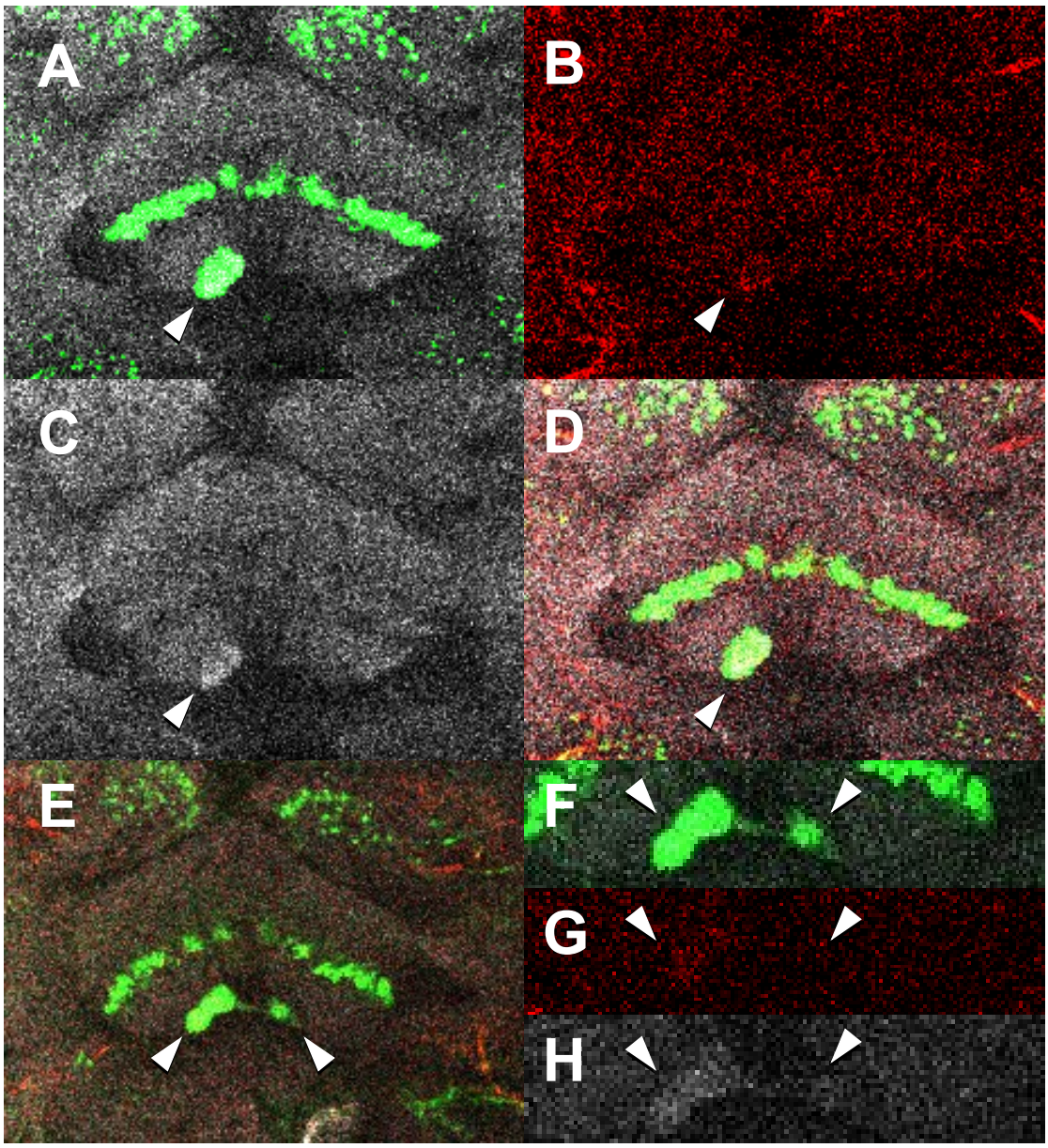

Figure 4. Dendritic and presynaptic regions of SLP-AB neurons (R52H03-Gal4). A: The unilateral AB innervation (arrowhead) is strongly labeled by the presynaptic marker sytGFP (green). The signal in the FB does not originate from SLP-AB neurons. **B:** DenMark (red) also shows weak expression in the right AB (arrowhead). **C:** The combination of α-Fas2- and α-Ncad-antibodies label the fas2-positive AB-innervations of SLP-AB neurons in addition to the overall brain structures (gray). **D:** Composite of all channels showing the overlap of presynaptic and dendritic regions of SLP-AB-neurons and the Fas2-signal. **E:** Composite showing dendritic and presynaptic regions of SLP-AB neurons innervating both ABs. Innervations on both sides are strongly labeled by sytGFP **(F)** while DenMark-signal **(G)** is sparse. AB innervations are Fas2-positive on both sides (**H).**

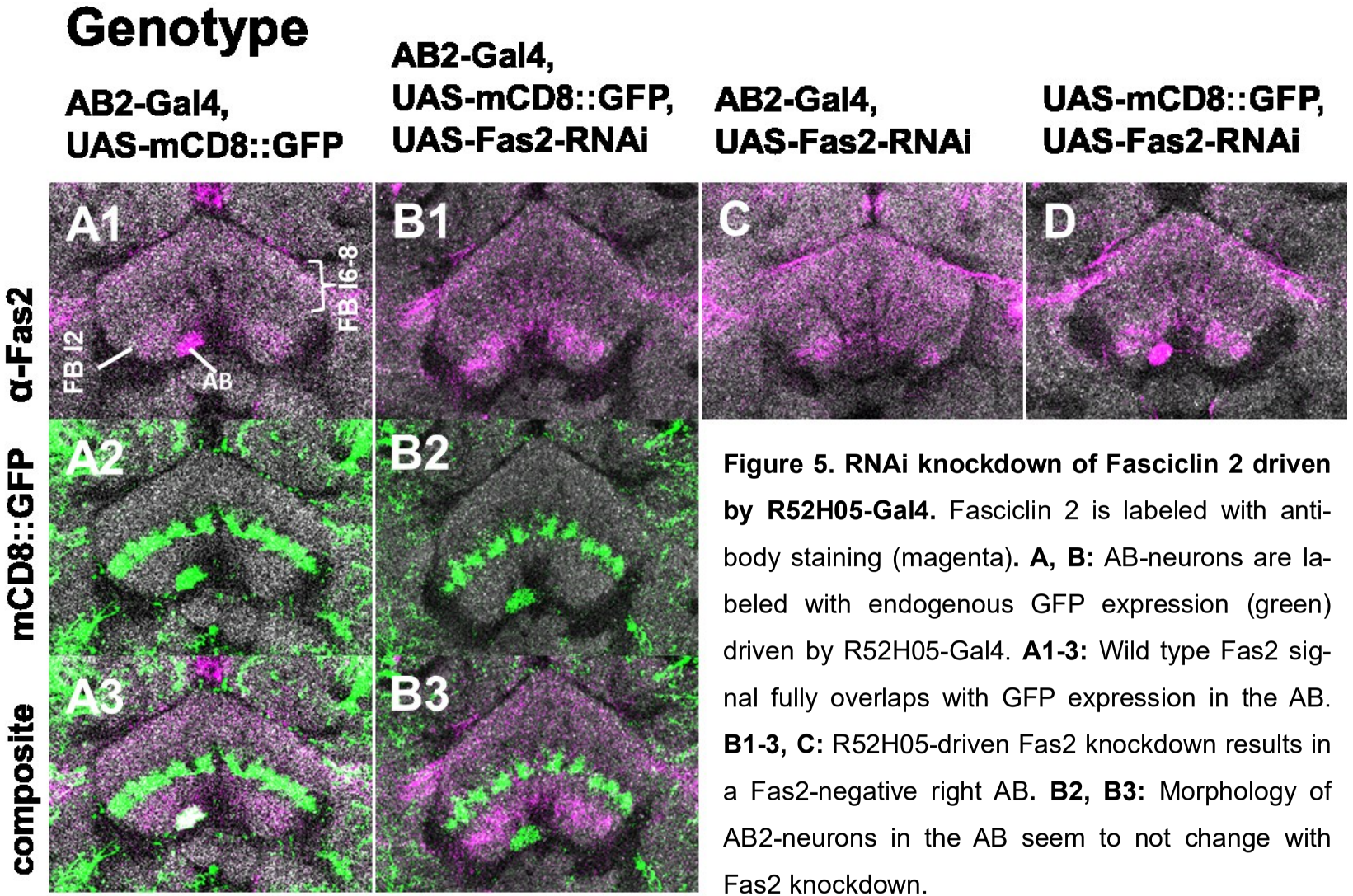

Figure 5. RNAi knockdown of Fasciclin 2 driven by R52H05-Gal4. Fasciclin 2 is labeled with antibody staining (magenta). **A, B:** AB-neurons are labeled with endogenous GFP expression (green) driven by R52H05-Gal4. **A1-3:** Wild type Fas2 signal fully overlaps with GFP expression in the AB. **B1-3, C:** R52H05-driven Fas2 knockdown results in a Fas2-negative right AB. **B2, B3:** Morphology of AB2-neurons in the AB seem to not change with Fas2 knockdown.

measured by GFP expression is significantly bigger on the right compared to the left (*paired samples Wilcoxon test,* V = 9, n = 15, p-value = 0.002014). The relative difference between left and right is 15 % of the total innervated AB volume on average. I found one exception in a single brain with a 7% bigger volume on the left but a unilaterally Fas2-positive right AB.
Innervation density measured as mean GFP-intensity can vary up to 14% between a right Fas2-positive and a left Fas2-negative AB. However, this variation is not side-biased *(paired samples t-test*, *t = 0.8153, df = 14, p-value = 0.4286)* and GFP-signal can be more intense on either side. Fas2 knockdown driven by R70H05-Gal4 did not affect the Fas2 expression in the ABs.

In the small portion of flies with bilateral Fas2-positive AB innervation (n=5), the intrinsic volume difference of R70H05-Gal4 driven GFP expression between left and right AB was no longer recognized (*paired samples Wilcoxon test*, V = 0, p-value = 0.0625). Nonetheless, the AB-innervation volume of all analysed bilateral Fas2-positive brains was at least marginally bigger on the right, ranging from 0,04% to 43,61% volume difference (Fig. 8).

Clonal analysis revealed that the R70H05-positive neurons can be classified in four different clusters per hemisphere according to the position of their cell bodies relative to the fan-shaped body and to their overall morphology (Fig. 7 C, Fig. 9): dorsal-medial (documented by 9 clones), dorsal-central (6 clones), dorsal-lateral (8 clones) lateral (2 clones). Co-labelling shows that these neurons can be labelled with R38D01-Gal4, R38D01-lexA, R70H05-Gal4 and R70H05-lexA.

The dorsal-medial (DM) cell cluster is the most anterior and the closest to the midline. Neurons of this cluster project contralaterally to the AB and from there upwards to the l8 layer of the fan shaped body (Fig. 9 A).
The dorsal-central (DC) cluster lies between the to other dorsal cell clusters and like the DM cluster, its neurons project to the contralateral AB and dorsal FB (Fig. 9 B).
The dorsal-lateral (DL) cluster is the only cluster with cells projecting both to the ipsi- and the contralateral AB. The cells of each hemisphere project from the AB further to the topmost FB-layer l9, where they arborize (Fig. 9 C). The dorsal projections are divided into to separate bundles. One is positioned directly on the midline, the second one takes a curved projection path through the ipsilateral hemisphere. Cell processes con-

dense in FB-l2 and form a glomeruli-like structure. Surprisingly, this structure is also not labelled by Brp::GFP, syt::GFP or DenMark driven by R70H05-Gal4 (Fig. 7).
The lateral (L) cell cluster is positioned lateral to the FB neuropile and is the most ventral cluster. L neurons project to both ABs and extend from there to the ipsi- and contralateral dorsal fan shaped body layers. The arborization is much smaller on the contralateral AB and in the FB is restricted to a small area on the lateral end of l6-l8 (Fig. 9 D).

Although, the four mentioned AB-FB neurons clusters form areas densely packed with cell processes in the ventral FB (l1 and l2), neither presynaptic markers nor DenMark do not do not enrich in these regions following R70H05-Gal4 transgene expression (Fig. 7). Innervations in FB-l6 are clearly visible in 10x-UAS-GFP images and are also labelled by presynaptic markers, but labelling is absent in l7. In contrast, clones created with FlyBow 1.1B show innervation of l6-l8 by three of the neuron clusters.

To further determine the neuronal connectivity of the asymmetric afferent ventral neurons and the FB-intrinsic neurons a recently developed trans-synaptic labelling technique was applied, which allows expressing the fluorescent protein tomato in neurons postsynaptic to Gal4-positive neurons. *Trans*-Tango driven in SLP-AB neurons (R72A10-Gal4) shows their asymmetric output to the FB (Fig. 6 E). The construct labels ispsilateral FB-l1-4 and l8 and l9 as well as the contralateral l6. Most postsynaptic partners labelled with *trans*-Tango seem to be PB-FB-NO neurons with tmt-expression in left and right Noduli as well as the contralateral PB_{G2-4} and the ipsilateral $PB_{G7-9.}$ The potential postsynaptic partners to SLP-AB neurons within the class of AB-FB neurons fitting the data from *trans*-Tango would be the dorsal-medial and dorsal-central clusters in the contralateral hemisphere.

3.1.3 Ventral SLP-FB-AB and FB-NO neurons

R42C09-Gal4 shows broad but weak Gal4-expression in the FB that is not specific to the AB. However, the corresponding lexA-line shows a very different expression pattern (Fig. 10). It labels a ventral cluster of neurons with a similar morphology to the SLP-AB neurons labelled with R52H03-Gal4. Cell bodies are located ventrolateral to the antennal lobes (Fig. 10 F). Dorsal projections connect to the SLP and the CX (Fig. 10 D,E). In contrast to SLP-AB neurons, the medial neuronal processes branch out in ventral FB and show condensed innervation at both ABs (Fig. 10 A). The volume of AB-innervation

is significantly higher on the right than on the left (*paired samples t-test*, t = -6.7328, df = 10, p-value = 5.151e-05). The right side is also more densely innervated (*paired samples t-test*, t = -2.3968, df = 10, p-value = 0.03752). For a comparison of relative differences of left and right AB see Figure 8. Polarity of the R42C09-lexA positive SLP-FB-AB neurons was not tested. Morphological similarity with R52H03-Gal4 positive neurons might indicate that they also are afferent to the AB-circuit. Since all analysed brains were of the unilaterally Fas2-positive AB-phenotype, I was not able to collect data if and how the morphology of these neurons is changed in the bilaterally Fas2-positive phenotype.

Interestingly, the same regulatory element which controls R52H03-Gal4 expression leads to a related but distinct expression pattern as the corresponding lexA line (R52H03-lexA) (Fig. 11). These neurons have a similar dorsal projection to the CX, but instead of targeting the AB exhibit a broad innervation of the ventral FB (Fig. 11 B). In addition, they seem to project from there to the Noduli (NO). Although GFP-signal slightly overlaps with the Fas2-positive AB, the neurons show no form of condensed or specific AB innervation as neurons labelled by R52H03-Gal4 do. These FB-NO neurons also completely lack the projection and innervation of the SLP (Fig.11 C).

3.2 Fas2 expression across *Drosophila* species

Fas2-expression in fan-shaped body was found to be conserved across *Drosophila* species and even other Diptera (Fig. 13). In all species the ventral and dorsal regions of the FB are strongly labelled by α-Fas2 antibody staining, especially the layers l2 and l6-9 corresponding to the classification in *D. melanogaster*. L5 seems to be Fas2 negative in *D. melanogaster*, *D. simulans*, *D. willistoni*, *D. paulsitorum* and *D. prosaltans* while weak Fas2 expression can be observed in l4. *D. malerkotliana* and *Zaprionus sp.* seem to express Fas2 expression in l5 but not l4. Layers l1 and l3 seem in general to be Fas2-negative. Fas2 expression in the ABs is subjected to variation (Fig. 12). All analysed ABs in adult wild type *D. melanogaster* (n=38) as well as in *Drosophila simulans* (n=12) caught in natural environment were unilaterally Fas2-positive. *D. malerkotliana* seems to have bilaterally Fas2-positive ABs (n=6).

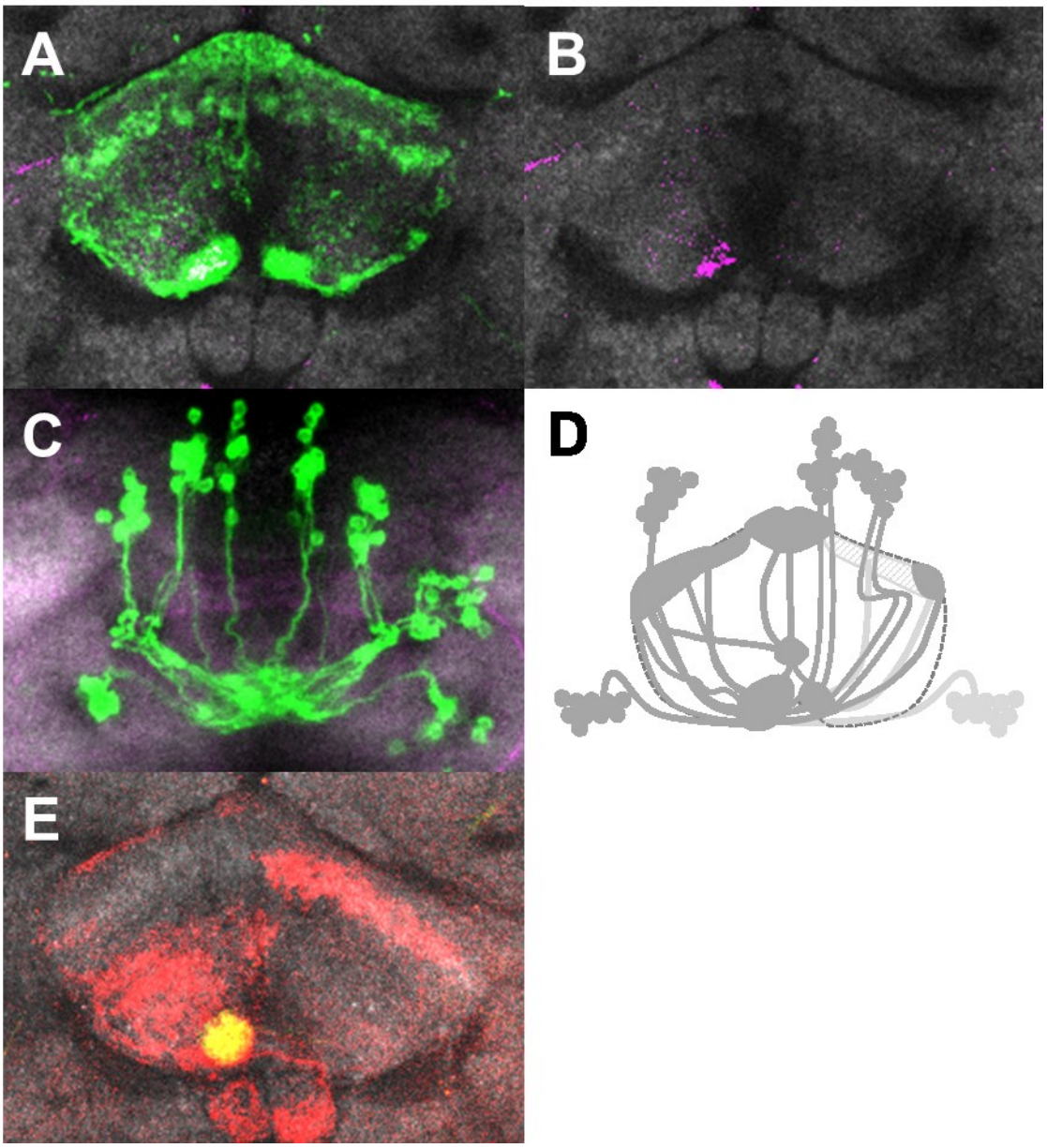

Figure 6. Cell morphology of AB-FB neurons labeled with mCD8::GFP (green) driven by R70H05-Gal4 and the corresponding lexA-driver. A: Section through the AB. AB-FB neurons connect to both ABs and several FB-layers (l1, l2, l6, l8 and l9). **B:** The same section as in A) without GFP-signal shows that only the right AB is Fas2-positive (α-Fas2 staining, magenta), to check that SLP-AB neurons innervate only the right AB. **C:** Merged images from the posterior FB to the posterior end of the brain. AB-FB neurons are grouped in four clusters per hemisphere around the posterior FB and the PB. **D:** The schematic shows the clusters to potentially receive asymmetric input from the SLP-AB neurons based on FlyBow clone analysis (Figure 13). **E:** Transtango driven in SLP-AB neruons. Postsynaptic neurons are labeled red, the yellow region is where SLP-AB neurons and postsynaptic partners overlap. α-Ncad-antibody staining (gray) was used as reference.

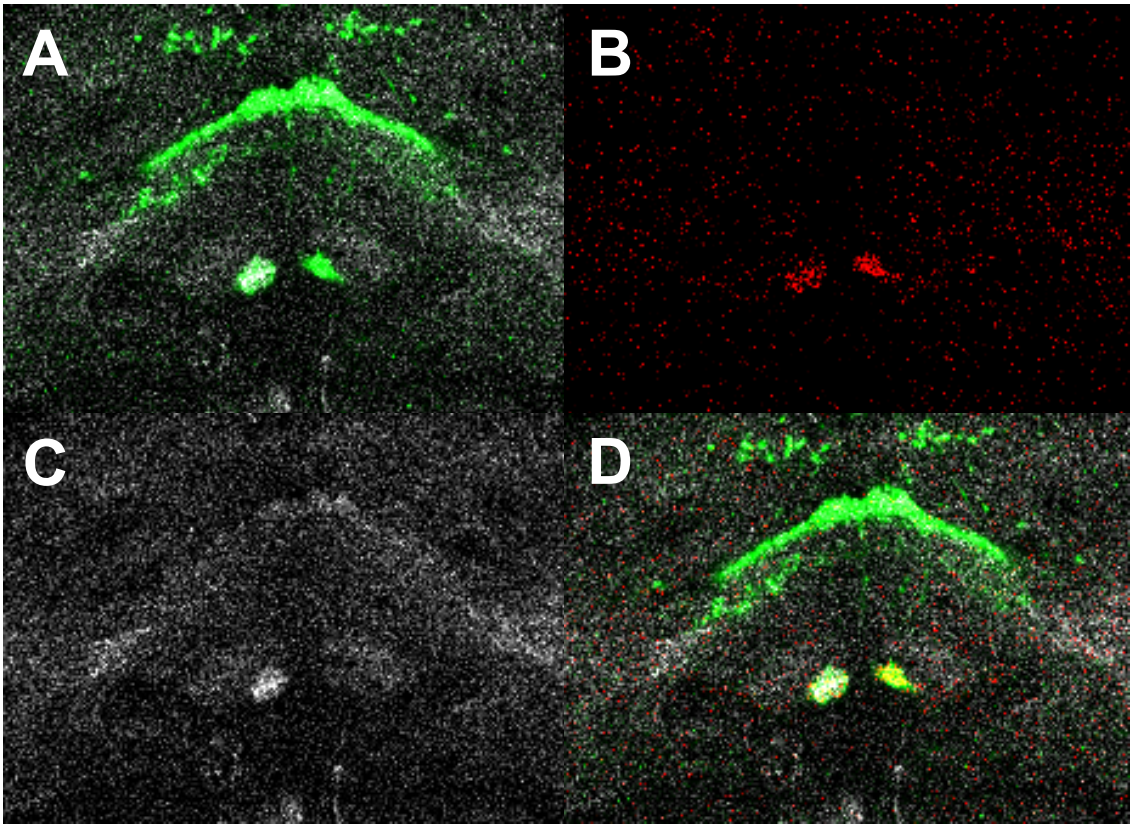

Figure 7. Dendritic and presynaptic regions of AB-FB neurons (R70H05-Gal4). A: Presynaptic regions labeled with sytGFP (green). Presynaptic regions of AB-FB neurons are located in the left and right AB as well as in the dorsal FB (l6, l8 and l9). **B:** Dendritic regions labeled with DenMark (red) are located in the ABs. **C:** The combination of α-Fas2- and α-Ncad-antibodies label the AB-innervations of SLP-AB neurons in addition to the overall brain structures (gray). **D:** Composite of all channels showing the overlap of pre-synaptic and dendritic regions of AB-FB-neurons and the right-side innervations by SLP-AB neurons. Note that the cell processes in FB-l1 (Figure XX) are not labeled with sytGFP nor DenMark. α-Ncad-antibody staining (gray) was used as reference.

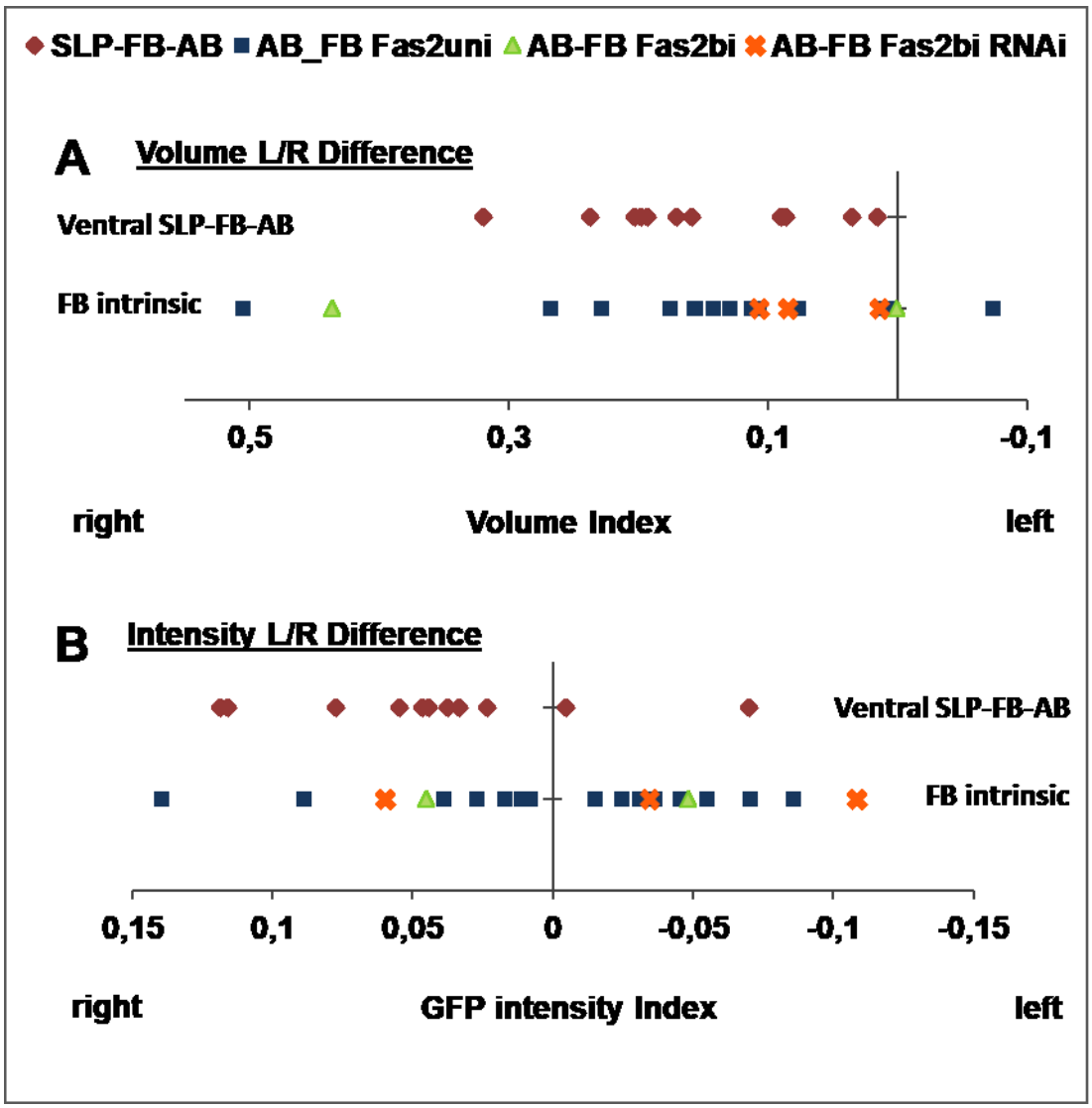

Figure 8. Differences between left and right AB in volume and GFP-intensity in SLP-FB-AB and AB-FB neurons. A positive index value on the x-axis indicates that the value measured for the right AB is relatively larger than the corresponding one of the left AB, and vice versa while a 0 value stands for no difference. Volume and meanGFP intensity were measured by the mCD8::GFP signal driven by R42C09-lexA or R70H05-Gal4 respectively. **A:** Difference in volume relative to the total combined AB-volume. **B:** Difference in meanGFP intensity relative to total meanGFP intensity of both ABs. Fas2uni: only right AB was Fas2-positive. Fas2bi: left and right AB were Fas2-positive. RNAi: To get a better understanding of changes in bilateral Fas2-positive ABs three brains from the Fas2-RNAi knockdown experiment were included in the figure. Keep in mind that these data points were not collected in wild-type flies.

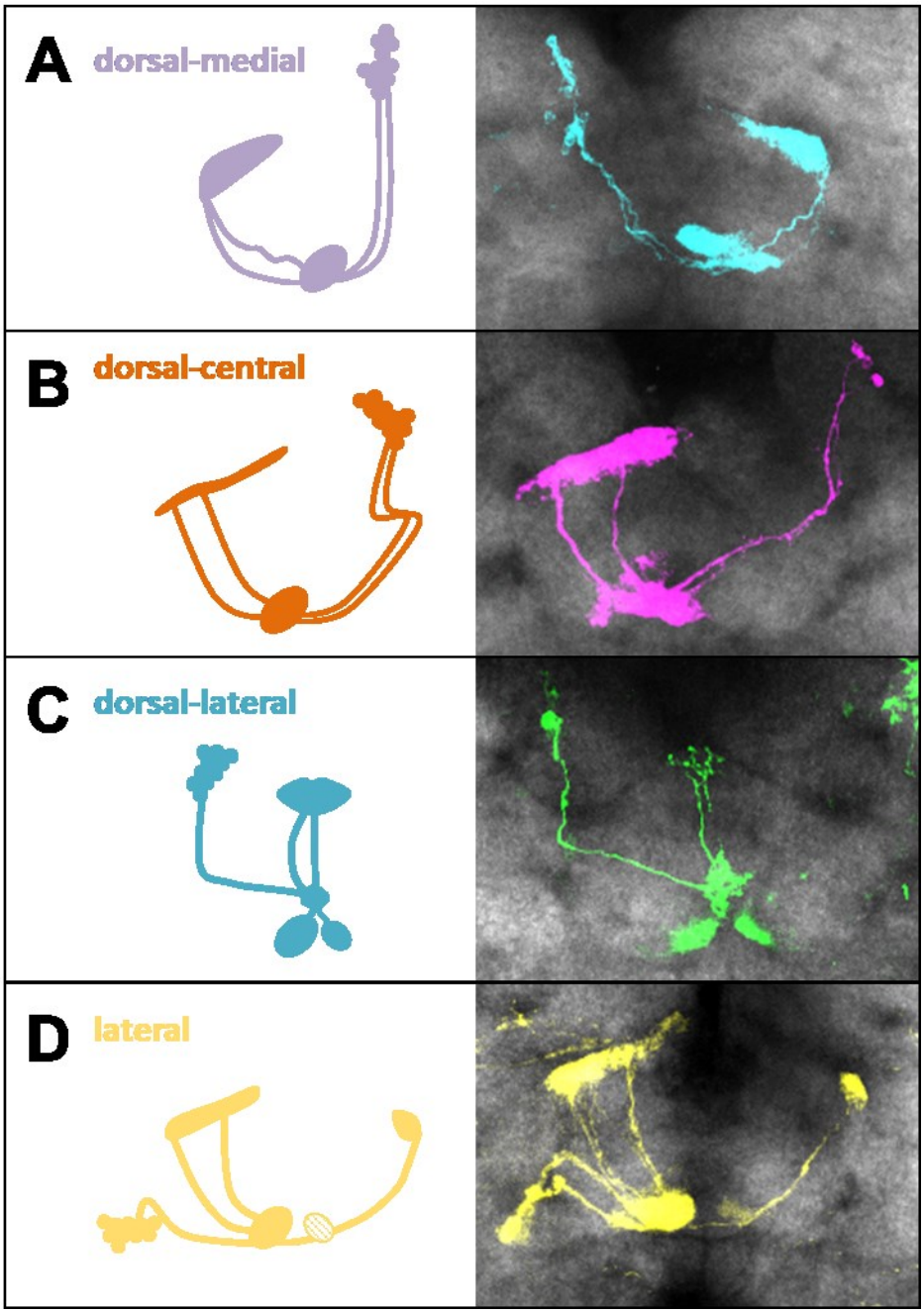

Figure 9. Cell morphology of different AB-FB neuron clusters (R70H05-Gal4). The clusters are named after their position relative to the FB. The dorsal-medial **(A)** and the dorsal-central **(B)** clusters exclusively project to the contralateral hemisphere, where they innervate the AB and the FB-layers l1, l7 and l8. The dorsal-lateral **(C)** and lateral **(D)** clusters innervate bilaterally and connect to both ABs. The dorsal-lateral neurons project to l1, l7 and l8. The lateral cluster meanders at the midline in l2 and projects to l9. All Images were produced by merging along the anterior-posterior axis. Brain structures are stained with α-Ncad (gray). Other colours are signal from the membrane-tethered fluorescent proteins of FlyBow 1.1B driven by R70H05-Gal4.

In *Drosophila willistoni* (n=7) the ABs are not clearly recognizable; however six flies showed a small Fas2 positive area in the left hemisphere, which could potentially be homologue to AB-Fas2-expression in *D. melanogaster*. In the species *D. paulistorum* (n=14), *D. prosaltans* (n=12), *D. sturtevanti* (n=8) and *D. repleta* (n=7) the AB was Fas2-negative. The same was true for the phylogenetic more distant species *Zaprionus sp.* (n=13) and *Rhaegoletis cerasi* (n=1).

3.3 Development

The expression onset of all tested driver-lines seems to start after AB development during pupa formation or Gal4 expression was too weak to trace the neurons the used reporters (single copy UAS-GFP and 10x UAS-tmt). Therefor I used the Fas2-signal as a marker to monitor AB formation in the early pupa stages (Fig. 14). As described in 3.1.1 the AB Fas2-signal originates from the ventral afferent neurons (SLP-AB neurons) at least in the adult fly. In the first 20h after pupa formation (apf) a large Fas2-positive layer forms in the developing central complex (Fig. 14 A), which mainly contains contralateral projections of prospective EB and FB neurons. The layer undergoes substantial remodelling from 20 to 30 apf and gives rise to three Fas2-positive subunits anterior to the ventral FB: one medial located on the midline and two lateral ones (Fig. 14 B). The Fas2 gradually weakens first in the medial structure around 30h apf and from 30 to 40h apf in the left lateral one (Fig. 14 C). At 45h apf only the right structure remains Fas2-positive and develops to the adult Fas2-expression pattern in the AB. Frequently, I could observe a subdivision of the Fas2-positive AB area of mid-pupa brains into two smaller compartments, which seems to be finally fused to a single neuropile by 80h apf (Fig. 14 D).

3.4. Nrg^{849} disrupts AB development

Because the formation of Nrg^{849} disrupts the formation of horizontal commissures, it can be used to separate left and right afferent ventral neurons to check for evidence different transcriptional programming. In general, Nrg^{849} mutant flies exhibit a broader Fas2 expression in adults that not fully overlaps with neither SLP-AB nor AB-FB neurons (Fig 15 A,B). The pupal EB and FB are split in right and left halves as is the Fas2-positive medial structure (Fig. 15 C,D).

SLP-AB neurons always innervate only their respective ispsilateral AB and neurons from neither side cross the midline. The ventral afferents thus appear overall symmetric

in Nrg^{849} mutants. Some AB-FB neurons, probably projections from dorsal-lateral cluster, clump at the midline.

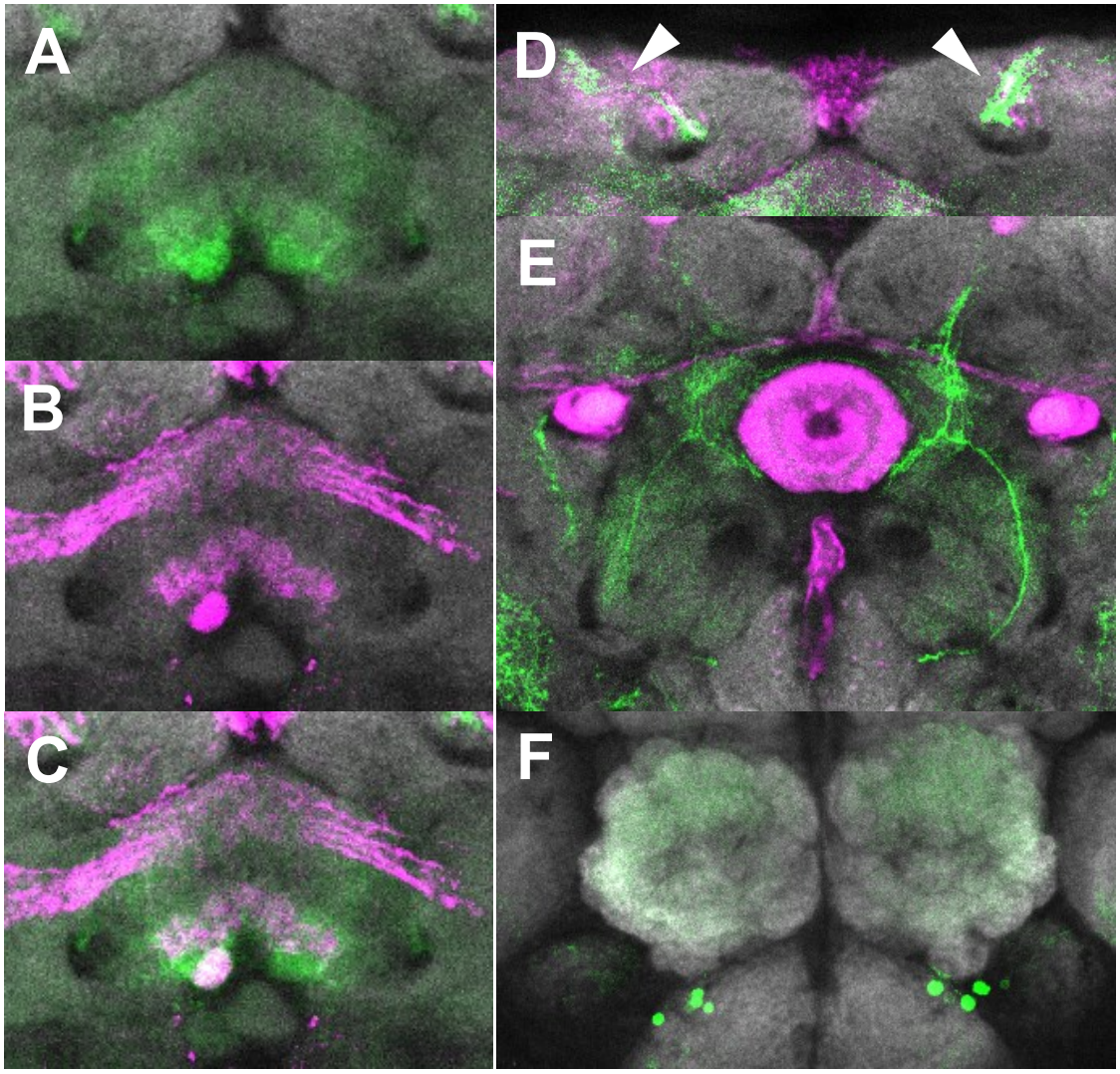

Figure 10. Morphology of SLP-FB-AB neurons labeled by mCD8::GFP (green) driven by R42C09-lexA. A: Innervation of the ventral FB and left and right ABs. **B:** α-Fas2-anitbody staining shows the innervation of the right AB by SLP-AB neurons. **C:** Composite showing the overlap of SLP-FB-AB neurons with the Fas2-signal in the right AB. **D:** Innervations in the SLP (white arrowheads). **E:** Upward projections from the cell bodies ventrolateral to the AL **(F)** to the FB/AB and to the SLP. α-Ncad-anitbody staining was used as reference (gray).

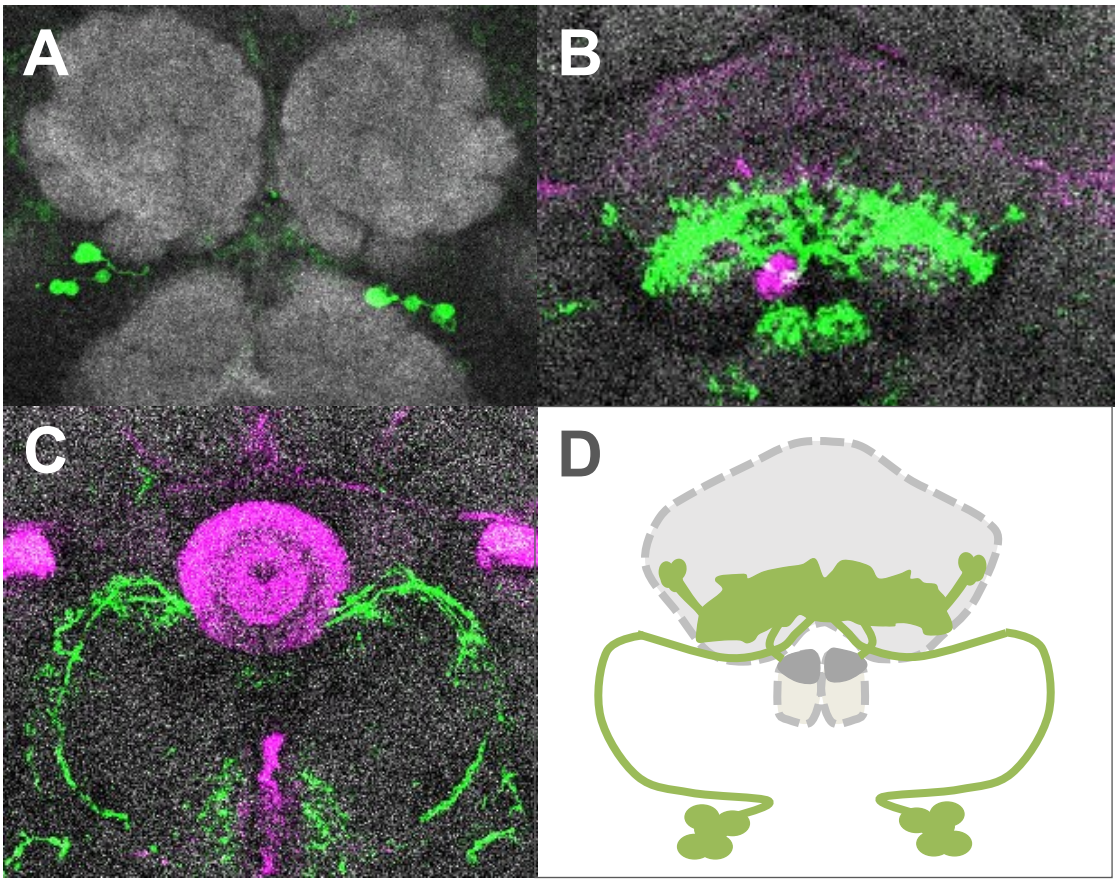

Figure 11. Morphology of FB-NO neurons labeled with mCD8::GFP (green) driven by R52H03-lexA. The neurons project dorsally **(C)** from the cell bodies ventrolateral to the AL **(A)** to the FB. **B:** Innervation of the ventral FB and the NO. α-Fas2-anitbody staining (magenta) shows that the innervations for the most part do not overlap with the AB-innervation of the SLP-AB neurons. Images are not to scale. α-Ncad-anitbodies were used for reference staining (gray). **D:** Schematic of the morphology of R52H03-positive FB-NO neurons.

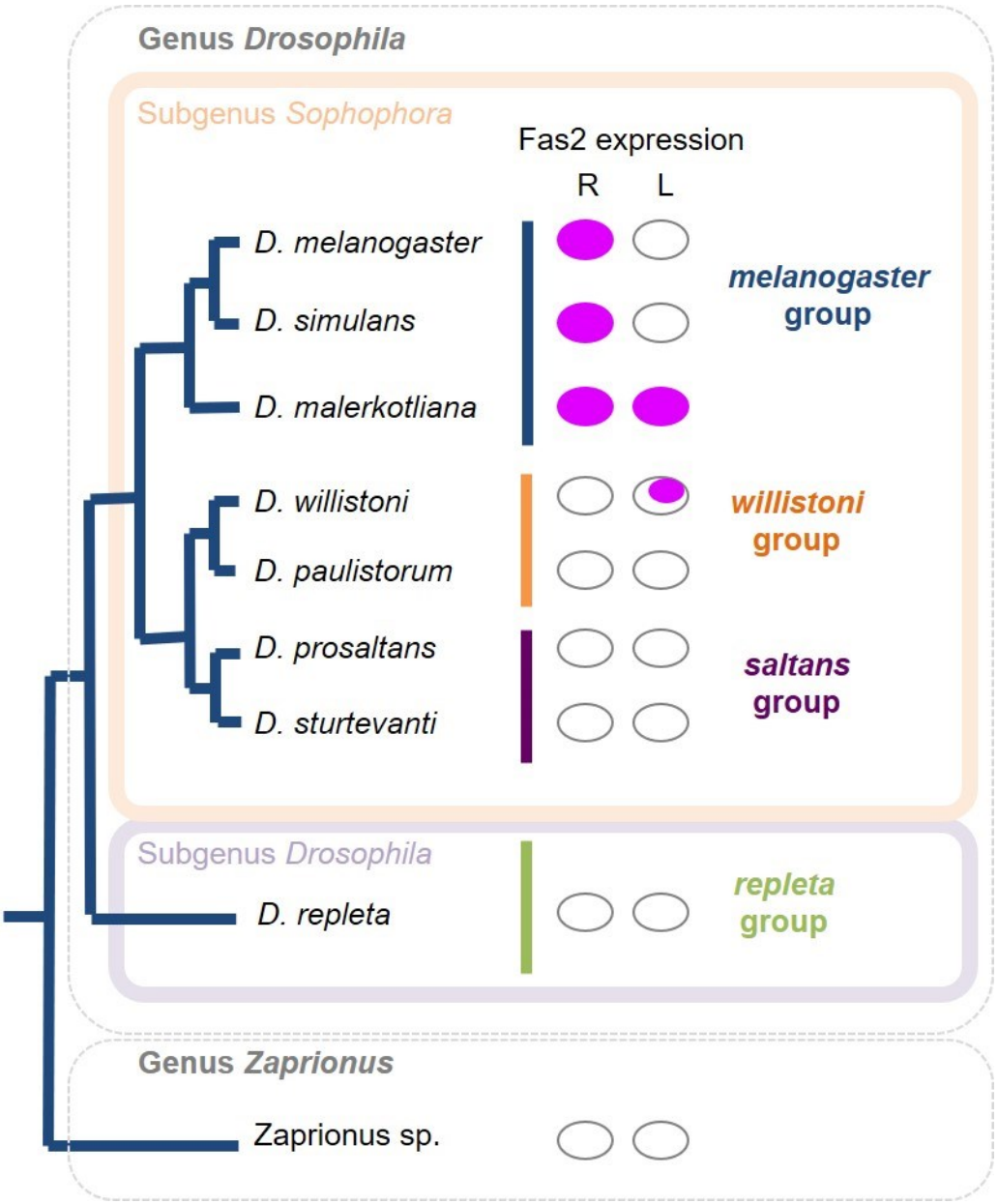

Figure 12. Cladogram of tested *Drosophila* species. Most prevalent Fas2 expression pattern in the AB is shown in Magenta for each species (R=Right, L=Left). Consistent Fas2 expression was only found in the melanogaster subgroup. Asymmetry and direction of lateralization seem to be subjected to variation even across species exhibiting Fas2 expression in the ABs.

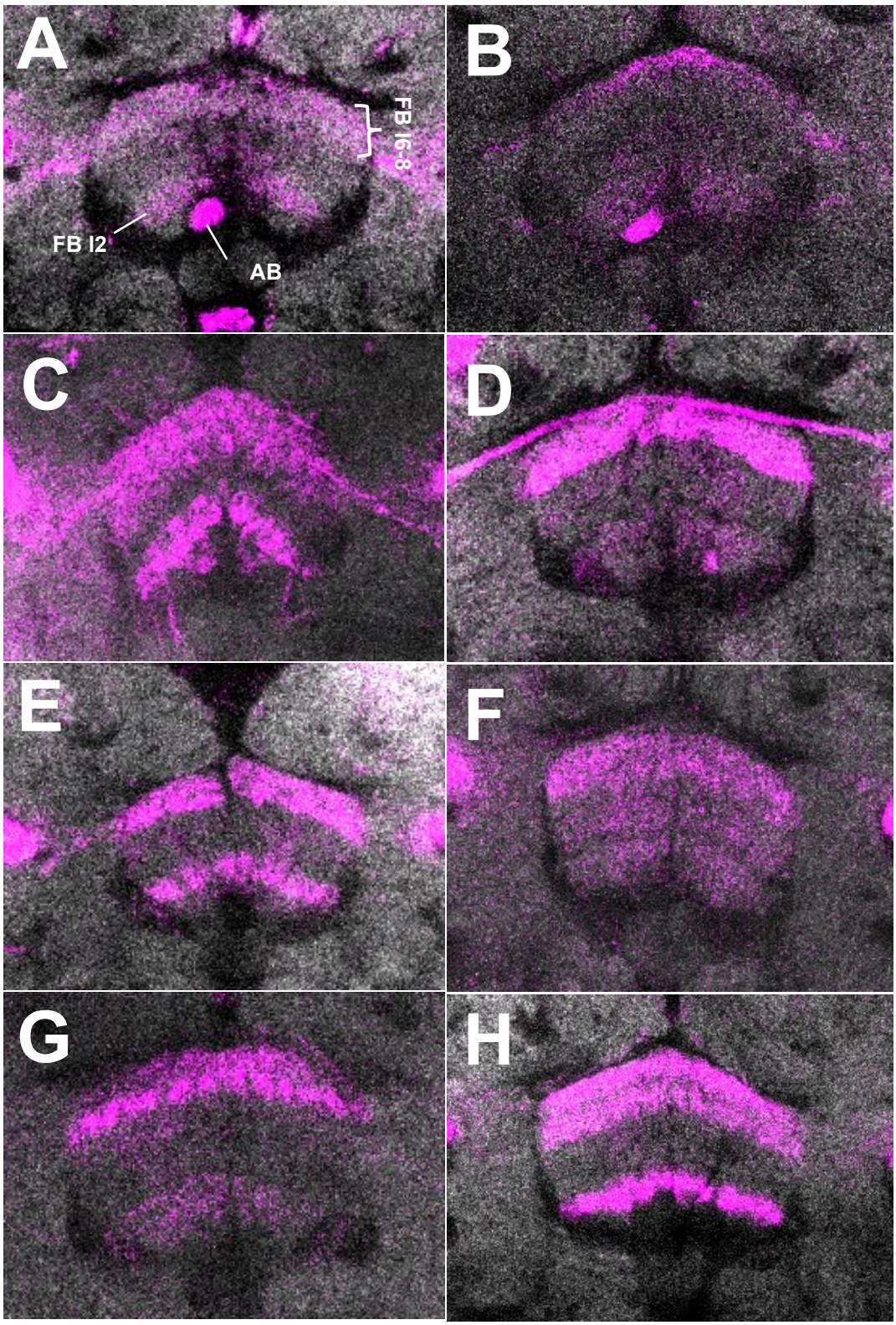

Figure 13: Fas2 expression in the FB and AB across *Drosophila* species. A: *Drosophila melanogaster* **B:** *D. simulans.* **C:** *D. malerkotliana.* **D:** *D. willistoni.* **E:** *D. paulistorum.* **F:** *D. prosaltans.* **G:** *D. sturtevanti*. **H:** *Zaprionus sp.* Magenta: α-Fas2-antibody, gray: α-Ncad-antibody. Pictures are not to scale.

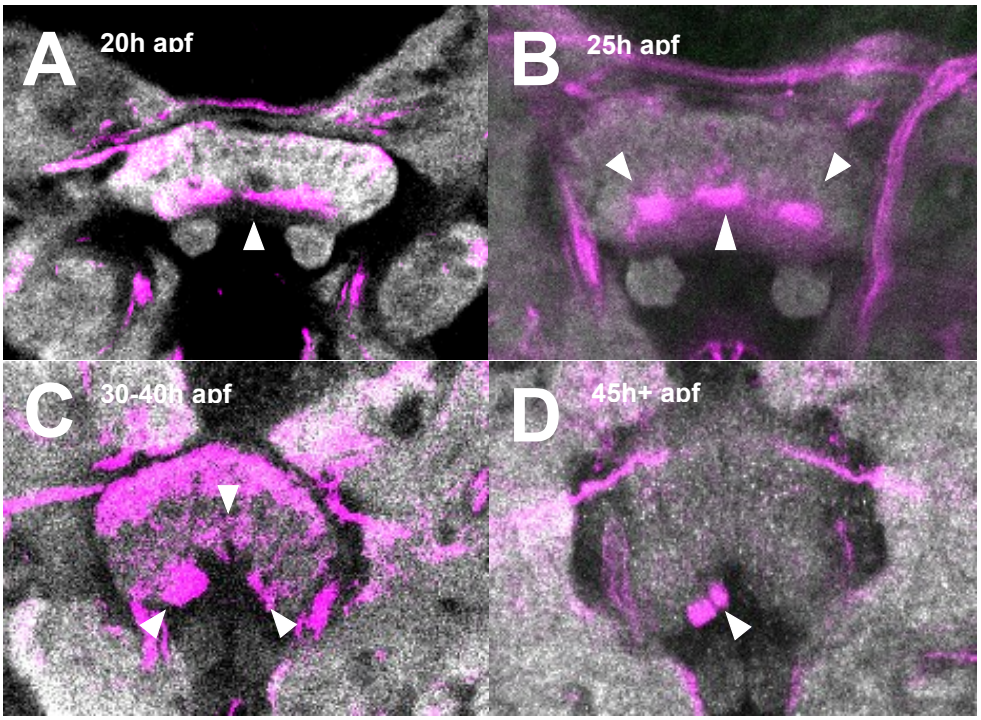

Figure 14: AB-formation and Fas2-expression during pupa development. A: Fas2-positive layer in the CX 20h apf. **B:** The ABs and a third Fas2-positive midline structure become recognizable around 25h apf. **C:** Fas2-expression in the left AB and the midline node get weaker between 30-and 40h. **D:** Fas2-expression in left and middle structure ceases around 45h. Subdivision of the AB can remain visible up to 80h.

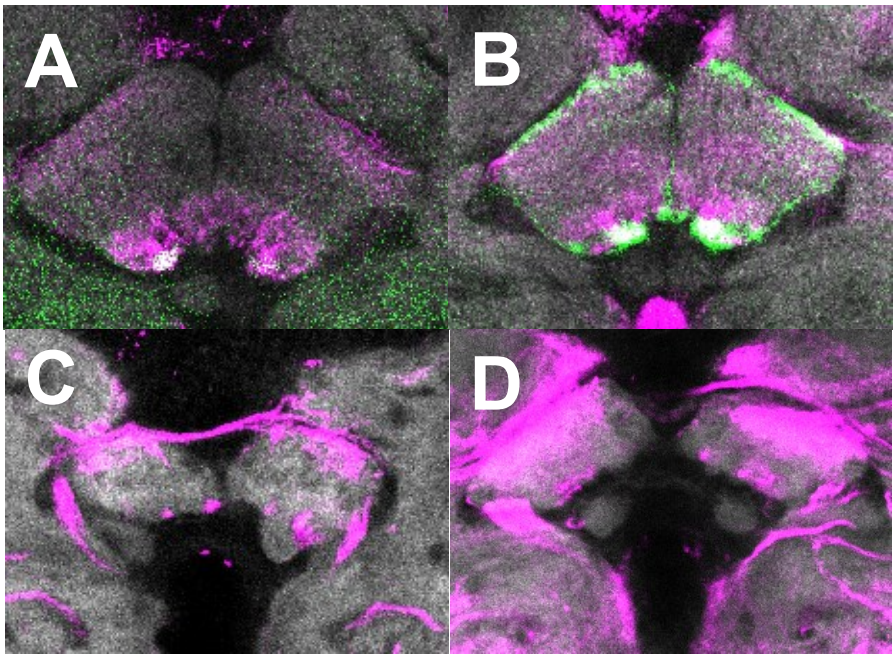

Figure 15: Effects of nrg^{849} on AB-formation. Brains were stained with α-Fas2-antibody (magenta) to track AB-development independent of Gal4-drivers. **A:** Expression of mCD8::GFP (green) driven by R52H03-Gal4 in SLP-AB-neurons. The neurons innervate left and right AB. **B:** Some Projections of AB-FB neuron (R70H05-Gal4) seem to clump at the midline. **C,D:** In pupa development (around 25h apf) the fan-shaped is not fused at the midline. The Fas2-positive structure at the midline is split in a right and a left half.

4. Discussion

As shown in the results the AB circuit comprises of at least two principal types of neurons, afferent projecting neurons from a ventrolateral cluster next to the antennal lobes and a group of related bilateral neuron clusters, located dorso-laterally to the central complex and closely associated with the Fan-shaped body neuropile. According to pre- and postsynaptic labelling the ventral SLP-AB neurons relay information from the SLP to the right AB, while AB-FB neurons further project from both ABs to the dorsal regions of the FB.
Lateralization of the AB circuit seems mainly, or exclusively, determined by the unilateral ventral SLP-AB innervation, as in all cases of bilateral SLP-AB projections the size difference is at least reduced.

Regarding the developmental analysis, it seems that the formation of a bilateral-symmetric circuit organization is the default program, with a slow degradation of the SLP-AB innervations of the left AB during pupa development. Considering the data from the pupal development, both ABs are Fas2-positive from around 20h-40h apf with the signal from the left getting gradually weaker. Since I was not able to follow SLP-AB development with Gal4-lines due to late expression onset or maybe too weak expression for the reporter-construct chosen and used the Fas2 expression to monitor AB development. Unfortunately, the origin of Fas2 expression is ambiguous. Fas2 seems to be expressed broadly in the developing CX and the Fas2 signal cannot be conclusively linked to SLP-AB neurons. However, because SLP-AB neurons seem to be the origin of adult Fas2 expression in the AB, I further discuss the developmental data under the assumption that the Fas2-expression matches the axonal processes of SLP-AB neurons.

The directional right-side bias of the AB circuit is extremely robust. In all analysed wild type lines, I did not find a single adult fly with a unilaterally Fas2-positive left AB.
The same was only observed in *D. simulans*. The variation of Fas2 expression in the AB region even within the closely related *melanogaster* subgroup indicates that the *D. melanogaster* expression pattern is probably a more recent evolutionary innovation. It would be interesting to investigate if this variation also matches the innervation pattern of the ventral afferent neurons in the tested species. The unilateral AB innervation might derive from an originally bilateral innervation state, as it seems to be present in *D. malerkotliana* (based on Fas2 expression) and seems to still exist in pupal development stages of *D. melanogaster*.

Experimentally inducing a *D. melanogaster* brain to retain the original innervation probably might not return the circuit fully back to its hypothetical ancestral functional state, as indicated by the impaired long-term memory in flies with bilaterally Fas2 positive ABs[40]. However, if this bilateral state can still be found in other extant *Drosophila* species, it would allow a direct comparison of innovated and ancestral state.

Should a bilateral innervation correlate with absence of Fas2 in the ABs, which was observed in the majority of tested Drosophila species, it also raises the question if Fas2 might play a critical role in establishing the unilateral innervation pattern of the *melanogaster* AB circuit.

4.1 Asymmetric Circuitry

The AB circuit combines two types of asymmetry[10]: On one hand, it possesses a strictly unilateral component (Fig. 16 B) that only exists in the right hemisphere in form of the exclusive right side AB innervation by the SLP-AB neurons (Typ 1). Each SLP-AB neuron from the left hemisphere projects contralaterally, while the homologue neurons from the right hemisphere target the ipsilateral AB neuropile, so that left and right neurons of this type have a distinctly different morphology.

On the other hand, AB-FB neurons form circuit components that are mirrored by homologue components in the opposite hemisphere, meaning that when a certain neuron projects ipsilaterally, its homologue in the opposite hemisphere will also project ispsilaterally and when a it projects contralaterally, its homologue will do the same. All components of AB-FB neurons are present on the left and right. So, regardless of ipsi- or contralateral trajectories, neurons of this type follow an overall bilaterally symmetric circuit morphology. FB-intrinsic neurons nonetheless exhibit asymmetry regarding the more voluminous innervation of the right AB (Typ 2). Right and left sides may also differ in the prominence of structural components e.g. in number of synapses as well as in identity of synaptic partners. Although my data indicates that the asymmetry of the AB circuit might mainly be due to the unilateral input from the afferent neurons, it could be that the Typ 2 asymmetry is the evolutionary older feature, since AB size asymmetry seems to a be conserved[42] while the unilateral innervation by the afferent neurons might be a more recent innovation based on the Fas2 expression pattern.

Functionally, the first type has the potential to affect the circuit function more heavily, because information from both hemispheres, left and right SLP is relayed asymmetrically and converges in the right hemisphere. This hardwired left-right asymmetry allows us

to infer straight up that the right hemisphere processes information differently than the left hemisphere.

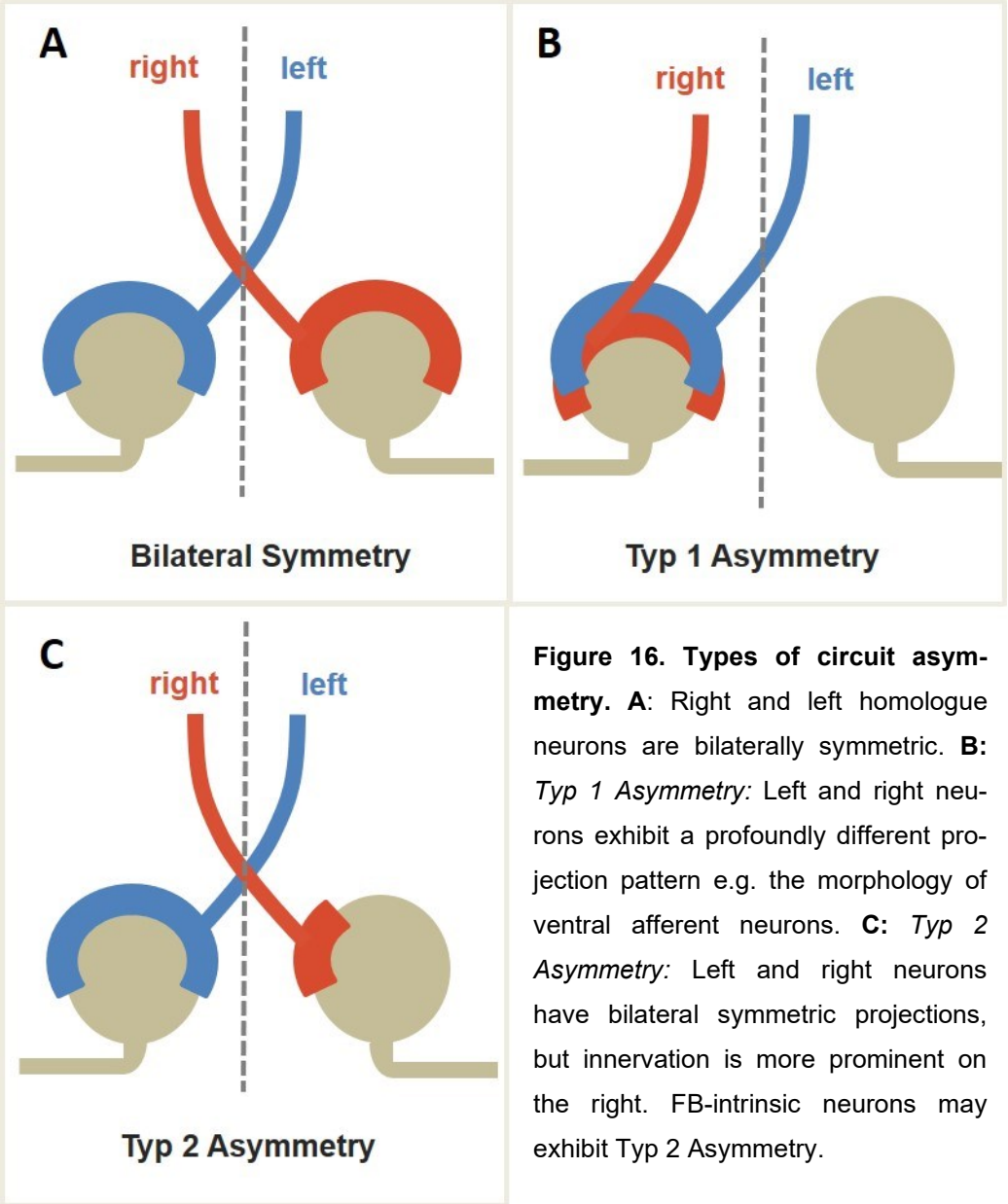

Figure 16. Types of circuit asymmetry. A: Right and left homologue neurons are bilaterally symmetric. **B:** *Typ 1 Asymmetry:* Left and right neurons exhibit a profoundly different projection pattern e.g. the morphology of ventral afferent neurons. **C:** *Typ 2 Asymmetry:* Left and right neurons have bilateral symmetric projections, but innervation is more prominent on the right. FB-intrinsic neurons may exhibit Typ 2 Asymmetry.

4.2 Development from DNA to current form

The find an explanation for the asymmetric neuronal growth from genetic regulators to the adult circuit organization is very central to this project. Most likely, molecular chirality constitutes a basic left-right asymmetry from the earliest embryonic stages. The developmental mechanism is thus probably acting upon the left-right information provided by the cytoskeleton or by the DNA itself amplifying the molecular properties over several scales of magnitude.

The known pathways that could achieve such amplification can be classified into two types[65].

The first is based on early transcriptional programming. Adult lateralization can be predetermined by an ontogenetically asymmetric state. An early symmetry break occurs during the first cell divisions and “marks“ descendent cells as left and right. From this event onwards a transcriptional cascade leads to a side specific gene expression and the divergence in a left and right cell lineage. In this way, asymmetry can develop controlled by intrinsic regulation without the need for further external signals.

An example how such a mechanism could work is the development of directed asymmetry in the ASE gustatory neurons in C. elegans[66]. In this case, the left (ASEL) and right (ASER) variants are bilaterally symmetric regarding their morphology, but express different chemoreceptors. ASEL and ASER descend from different, but equipotent blastomeres. Their cell fate depends on Notch signalling at the four-cell stage. Notch receptors are expressed in the precursors of ASER, but not in those of ASEL. Activation of these receptors causes the repression of T box transcription factors and through a feedback loop the differentiation into an adult ASER neuron. Without the activation, T box transcription factors unpack the chromatin at the lsy-6 locus, “priming” it for transcriptional activation. This chromatin status persists over several cell divisions and functions as a cell fate memory. Transcribed lsy-6 miRNA causes through a regulatory cascade ASEL cell fate determination[65,67]. The findings of selective chromatin segregation of differently imprinted sister chromatids in mice is a clear indication that such a transcriptional left-right control is at least thinkable also in other organisms[30].

The second type, or signalling model, is based on the idea that asymmetry is superimposed on the bilateral symmetric state of a tissue in reaction to an external signal without relying on *a priori* left-right asymmetries. This model seems convincing with regard to the fact that many molecules are handed themselves and the source for breaking bilateral symmetry could be the “conversion” from chirality on a molecular level to multicellular laterality. A chiral molecule aligned to the anterior-posterior and the dorsal-ventral axes could due to its intrinsic handedness drive the asymmetric transport of a morphogen as proposed by Brown and Wolpert in their “F-molecule“ model. The best described example for a model probably is the unconventional myosin Myo1D in *Drosophila melanogaster*. Although not strictly speaking a signal, the fact that it can elicit asymmetric growth *de novo*, that is, even in otherwise bilateral symmetric tissue and that it interacts with the global module of the PCP makes it the most complete model to

date. Although Myo1D seems to be not involved in the AB development, it allows us to infer about the potential properties of an organizer of neuronal asymmetric growth in *Drosophila*. As shown by Myo1D the potential for left-right asymmetric growth is inherent to more tissues than actually show bilateral asymmetry in the adult fly[38]. Myo1D further demonstrates that one mechanism can be sufficient to both break symmetry and to determine the direction of asymmetry. Expression of Myo1D seems to act as a binary switch from symmetric to asymmetric visceral development requiring only upstream alignment of polar F-actin filaments and the presence of downstream components interacting with Myo1D. The potential for breaking symmetry *de novo* also shows that the downstream pathways are not special to left-right asymmetric tissues but seem to be expressed globally. The extremely stable direction of asymmetry of the AB lets it seem likely, that a similar switch e.g. a chiral molecule, already carrying the side-bias information in its structure, changes the AB circuit from a bilateral symmetric default to asymmetric development. Thus, if the upstream components of this pathway (e.g. the cytoskeleton) are intact, expression of such a switch molecule would cause an automatic asymmetric growth to the right, because the direction of the asymmetry is predetermined by the chirality of the switch molecule. A reversal of the asymmetry would in such a scenario only occur if the upstream components were disturbed (Fig. 18).

Applied to the AB in *D. melanogaster*, I can think of three alternative mechanisms controlling L/R-asymmetry development (Fig. 17).
First, there is the possibility that the asymmetry of the AB circuit is caused by different “priming” of left and right SLP-AB neurons. However, in an otherwise symmetric brain, this model would demand an enormously complex signalling if the assumption of bilateral AB innervation as default state were correct. The mechanism would have to be able to distinguish left from right AB, as well as to instruct the right and left neurons to retract from the left AB. This would require differentiation between left and right AB innervation within the same neuron. That such a complex mechanism, and probably error prone mechanism would evolve to control the stable and directed asymmetry of a single neuropile seems unlikely to me, especially since alternative models would in principle only require a single signalling molecule.

An alternative mechanism to explain SLP-AB development would be the expression of a signal molecule in the left AB, relaying the instruction to retract cell processes when interacting with SLP-AB neurons (Fig 17 C). This signal would act as a switch, transform-

ing the symmetric default state into an asymmetric state. This external signal could act upon a global intrinsic cell-mechanism and would not require an *a priori* difference between left and right SLP-AB neurons. There are two sets of data supporting that the bilateral SLP-AB innervation is the default state: the bilateral innervation phenotype of SLP-AB neurons in adults and the Fas2-expression during the pupa stage. In bilaterally Fas2 positive ABs left and right neuropiles are innervated by contra- and ipsilateral SLP-AB projections. This is in concordance with the developmental data, where it appears that at first left and right are innervated both and the ramifications in the left AB are degraded secondarily. If the switch-signal is deactivated or otherwise impaired, the neurons would always innervate both ABs as we can observe in the bilateral Fas2-positive phenotype.
Reduced signal expression or effectiveness could explain why the volume of the right AB innervation is most of the times still larger than the left (Fig 18 C).
In a model combining priming and signalling any neuron type could drive the asymmetry, if its neurons in one hemisphere only innervate the AB of one side (e.g. the dorsal-medial and dorsal-medial AB-FB neurons clusters). If the neurons in the hemisphere innervating the left AB would be primed to express a “switch” signal (e.g. a cell adhesive molecule), while neurons of the opposite hemisphere do not, it would result in the wild type phenotype. If it turns out that SLP-AB neurons reach the ABs before the AB-FB neurons it would further support this hypothesis.

Information about the identity of potential organizer neurons can be concluded from AB phenotype in nrg^{849} mutant flies. Although the effects on the AB-FB neurons were not studied in depth, the decussating projections across the midline of the dorsal clusters seem not to be disrupted. According to this model left AB innervation should at least be reduced, if AB-FB neurons are the organizers. Since this was not observed in the nrg^{849} flies, at least the dorsal-medial and dorsal-medial AB-FB neurons clusters seem to be unlikely candidates.

Because of the proximity of the ABs to the midline a third alternative presents itself in form of a midline L/R organizer (Fig. 17 B). In such a model the asymmetric transport of a signalling molecule or asymmetric positioning of a transmembrane protein could initiate the retraction of the axonal ramifications. Since the signal has only to be presented during approximately 20-40h after pupa formation, this organizer could be transient structure e.g. the TIFR. The midline plays an important role in organizing and patterning

during development and might also be important for establishing the left-right asymmetry of the AB. The disruption of the midline in nrg^{849} mutants results in SLP-AB neurons to only innervate their respective ispsilateral AB. While we can only speculate whether there are transcriptional differences between left and right, it demonstrates that at least neurons in both hemispheres meet the necessary requirements to recognize their respectively ipsilateral AB and are able to form condensed ramifications there. With regard of the role of glial cells in remodelling neuron ramifications and in axon guidance[68,69] it would be possible that the asymmetric projections are directed by interactions of the neurons with midline glia cells, maybe even the TIFR, which also is disrupted in nrg849 mutants. As a next step I would thus recommend a RNAi screen driven in AB neurons and in midline glia cells knocking down proteins of the PCP pathway, known transmembrane proteins involved in axon guidance and of unconventional myosins. Ablations could be used to screen for potential organizer cells.

4.3 Translation of brain asymmetry to lateralization: Function and possible adaptive value

The arthropod central complex exhibits extensive homology with the vertebrate basal ganglia in its circuitry and in its mediation of sensory and motor integration, motivational and affective behaviour, and cognition[52]. Impairment of its substructures can result in a variety of behavioural defects ranging from motor phenotypes to attention and sleep disorders.

While AB seems to be connected to long-term memory formation or retrieval, it was shown in a number of vertebrate studies that a pronounced lateralization improves performance in discrimination tasks[5]. Boosting cognitive abilities should definitely be beneficial to survival. That all dissected flies caught in the wild as well as their offspring exhibited only the unilateral Fas2-positive wild type phenotype, while about 10% of flies from traditional lab strains, which were less exposed to natural selection for many generations, exhibit the bilateral phenotype, indicates a strong selection against aberrations from the wild-type asymmetry in nature and underlines the potential importance of the AB for survival of the adult fly. Although the AB region shows morphological variance between different Drosophila species, evidence indicates that the AB lateralization is conserved across *Diptera* taxa[42]. These seem strong arguments that the asymmetry of the AB circuit imposes an adaptive value rather than being a by-product of genetic drift, giving it credence as a model to study lateralization.

When looking for possible functions of the AB, a reasonable approach is to search for lateralized behaviour. Handedness and side biases have been reported in flies. Individual flies show persistent preferences for one direction when circling in an arena or choosing an arm of a Y-maze as well as for folding their left wing on top of the right or the right on top of the left[70]. These locomotion biases are independent from leg length and other morphological features like the twisting direction of the gut. Since the central complex is also involved in locomotion control, a large-scale asymmetric circuit like the AB could constitute the neuronal basis for locomotion side biases or handedness.
However, the volume difference and the Fas2-expression show extremely pronounced side biases on a population level. If the AB would be processing motor or sensory information, I would expect that the direction of behavioural lateralization is mirroring the direction of asymmetry of the AB. Thus, in the case of the AB one would expect a strong population wide lateralization to the right or left, with a minority of flies with reduced lateralization. None of the described lateralized locomotive behaviour of Drosophila meets this assumption[70,71]. On the contrary, handedness and lateralization of locomotion in Drosophila is randomly distributed among individuals with one half of the flies showing a preference for left and the other half for the right. There is no evidence of a population-level asymmetry. Involvement of the AB in locomotion seems thus unlikely.

The only documented lateralization in *Drosophila* on a population level that I am aware of is the side bias in the olfactory system. The left antenna was shown to contribute relatively more than the right when tracking an olfactory cue[72]. In fact, the olfactory system is already lateralized in larva[73]. If the AB indeed develops during pupa stage, as indicated by my results, the AB is thus probably not the cause for lateralization of sensory information and even its involvement is questionable since the results from Pascual and colleagues tells us, that all flies were able to establish associative (shot-term) memory[40].

This means, that flies with symmetrized ABs are able to correctly process the features of the conditioned stimulus (odour) und the unconditioned stimulus (electric shock) and successfully form an association. Therefore, the AB probably does not participate in sensory perception either. Considering that the only known effect of AB anomaly is an impaired long-term memory, the AB might not be linked to lateralized behavioural output at all.

An often mentioned feasible benefit of brain asymmetry is reduction of energetic cost by avoiding duplication and by decreasing circuit size[5,7,19]. While AB asymmetry probably reduces overall cost, the difference should be very small, since the left AB, although smaller still seems to be well connected in the central complex and serving its own function with bilateral SLP-AB innervation resulting in severe implications.

Another interesting idea is that by two homologue structures processing different processing, it would allow to talk on two otherwise incompatible tasks in parallel[7,19]. Experimental support for this hypothesis was found in the domestic chick. Chicks use their right eye for searching food with the connected side of the optic tectum more sensitive to fine details while the other one is looking out for predators with the respective tectum more sensitive to moving objects. This way the lateralization enables efficient and simultaneous processing of different kinds of visual information and thus increases the chance of survival. Since the SLP-AB neurons converge input of supposedly bilaterally symmetric presynaptic circuits to the right hemisphere, it could serve to enable parallel processing of higher cognitive functions. However, documented examples for parallel processing usually are accompanied by lateralized sensory systems and behaviour (e.g. the right ear advantage in especially verbal auditory processing and the speech-relevant centers located in the left hemisphere of the human brain[74]. As discussed above, such a lateralization seems to be absent in case of the AB.
Instead of parallel processing asymmetric circuits could also be involved in deciding between two incompatible binary options[19] such as adaptive behaviours that cannot be executed simultaneously. For example, the asymmetry of the habenular circuit seems to modulate fear responses. The fish has to decide whether to freeze and stop moving or to escape. The inactivation of the left-right asymmetric dorsal habenula in Zebrafish inhibits fear induced escape responses resulting in the fish freezing in reaction to a stimulus. This type of lateralization does not have to be associated with motor or sensory handedness and seems thus as a likely candidate to explain the function of asymmetry in the AB circuit. But what could be a binary option modulated by the AB, that would result in loss of long-term memory if the SLP-AB connections are symmetrized? Since it is unclear how information is processed in the central complex, it is impossible to deduce AB function from connected brain regions conclusively. Direct affiliated neuropiles are the fan-shaped body and the SLP and probably also to the noduli and the protocerebral bride. So-called tangential neurons, which connect the superior protocerebrum with specific layers of the FB and the NO are crucial for visual short-term memory. Dis-

tinct neurons projecting from the superior medial protocerebrum to the FB are relevant for memorizing different pattern parameters like elevation and contour orientation and seem to involved in visual associative memory or relay information about learned associations from the superior protocerebrum to the central complex[42,75,76]. The morphology of these neurons differs from SLP-AB neurons and they arborize in the superior medial protocerebrum (SMP) instead of the SLP. However, injected dye in the SMP reveals neurons connecting to the SMP and the ABs in very similar fashion to SLP-AB neurons (compare Philips-Portillo and Strausfeld 2012, Fig. 7[42]). It might be a circuit feature common to the neuropiles of superior protocerebrum to project some information to the FB while taking a detour through the ABs. Should the SLP have a similar function for olfactory memory as the SMP for visual memory, projections from the SLP might relay information about learned associations to the FB. How asymmetry would contribute in this scenario, remains unexplained.

An option in concordance with the findings from Pascual and colleagues would be that the AB processes the behavioural reaction to aversive stimuli e.g. regulating the motivational state towards it. SLP-AB neurons could converge relevant information about learned aversion from the SLP to the right hemisphere allowing modification of a specific response according to past experience. If activation of neurons postsynaptic to the left AB would result in one behaviour and activation of the right in an alternative response by a winner-takes-it-all principle, bilateral activation might randomize the reaction to the stimulus. Therefore, even if the fly had formed long-term memory of the condition, the response would appear as if it had no memory.

Both the parallel processing and the binary option model would require the postsynaptic circuits to work separately and independently from each other, meaning that the *Drosophila* brain would have to be more lateralized than originally expected. It also means that effects of lateralization may be subtle and hard to test in behavioural setups without prior knowledge of its function. Thus, it is important to first determine in which brain functions the AB is involved e.g. by silencing or ablation.

4.4 Next Steps

To learn more about the developmental mechanism a RNAi knockdown screen of candidate genes probably would be crucial to identify potential signal molecules and involved pathways (e.g. the PCP). Should the signalling model be correct, disruption of directional cues (e.g. cytoskeleton or chiral motors) is expected to randomize the direc-

tion of asymmetry, while interfering with the signal's function should increase the number of symmetrized brains.

In preparation to behavioural testing of lateralization effects, first it is necessary to determine the function of AB e.g. is silencing or ablation of neuron clusters in total or in a single hemisphere. It would for example be interesting if the bilateral innervation phenotype applies specifically to olfactory associations or also to visual ones.

To verify that bilateral SLP-AB innervation is causal to long-term memory defects, I would also recommend bilateral activation or repression of the neurons postsynaptic to the SLP-AB neurons. Inhibition or excitation of AB-FB neurons should in principle have the same effect as bilateral SLP-AB innervation and should impair olfactory long-term memory.

Knowledge of AB function in addition to a method to reliably induce symmetrized brain development would allow find suitable behavioural set-ups to compare correctly lateralized flies with symmetrized ones to study the effects of lateralization. This might be expanded even to other *Drosophila* or *Diptera* species to explore translation of different morphologies and the direction of asymmetry into behavioural output.

4.5 Conclusion

Far from solved, the topic of bilateral asymmetry of the central nervous system and lateralization remains an exciting and complex topic. The asymmetry of the AB circuit in the *Drosophila* is an example for a stable and directed asymmetry, which most likely provides adaptive value by improving the processing capability of higher cognitive functions. Because of the vast array of genetic methods combined with the high translational value to other species, *Drosophila* seems to be an ideal model organism to contribute to lateralization research both in development, in asymmetric information encoding and its implications for cognitive processing. Variations of the AB circuit across *Diptera* taxa in the otherwise very conserved central complex makes the AB also a suitable model to study the evolution of lateralization.

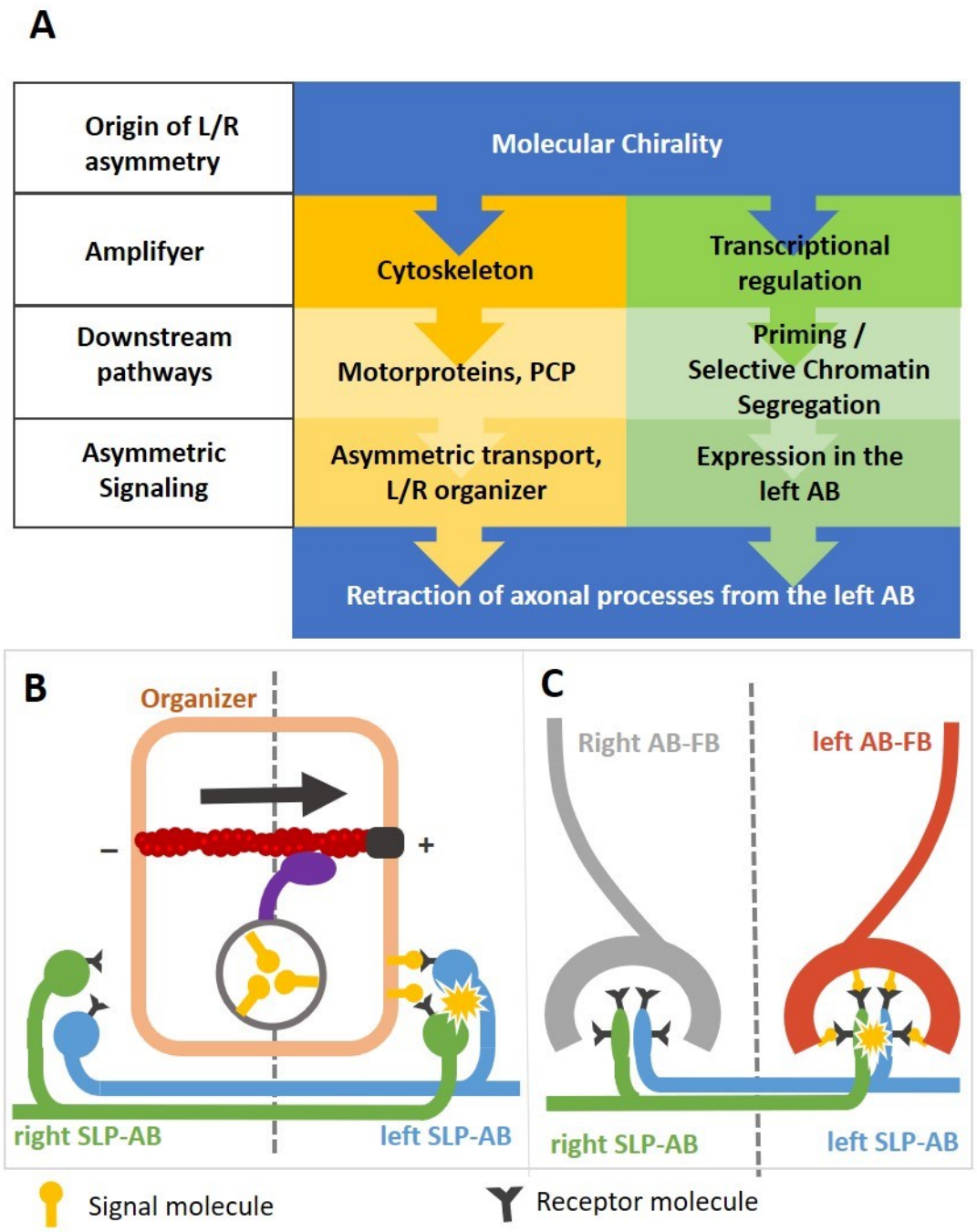

Figure 17: Models for asymmetric development of the AB circuit. **A**: Comparison of the mechanisms which could translate molecular chirality into circuit asymmetry. **B:** Schematic of a midline organizer model. Proteins with chiral motors (purple) move in a fixed direction along an anchored actin filament (red) and transport the signal molecule unilaterally to the left. **C:** Schematic of a transcriptional regulation model. Only the left individual neurons of a cluster type express a signal molecule.

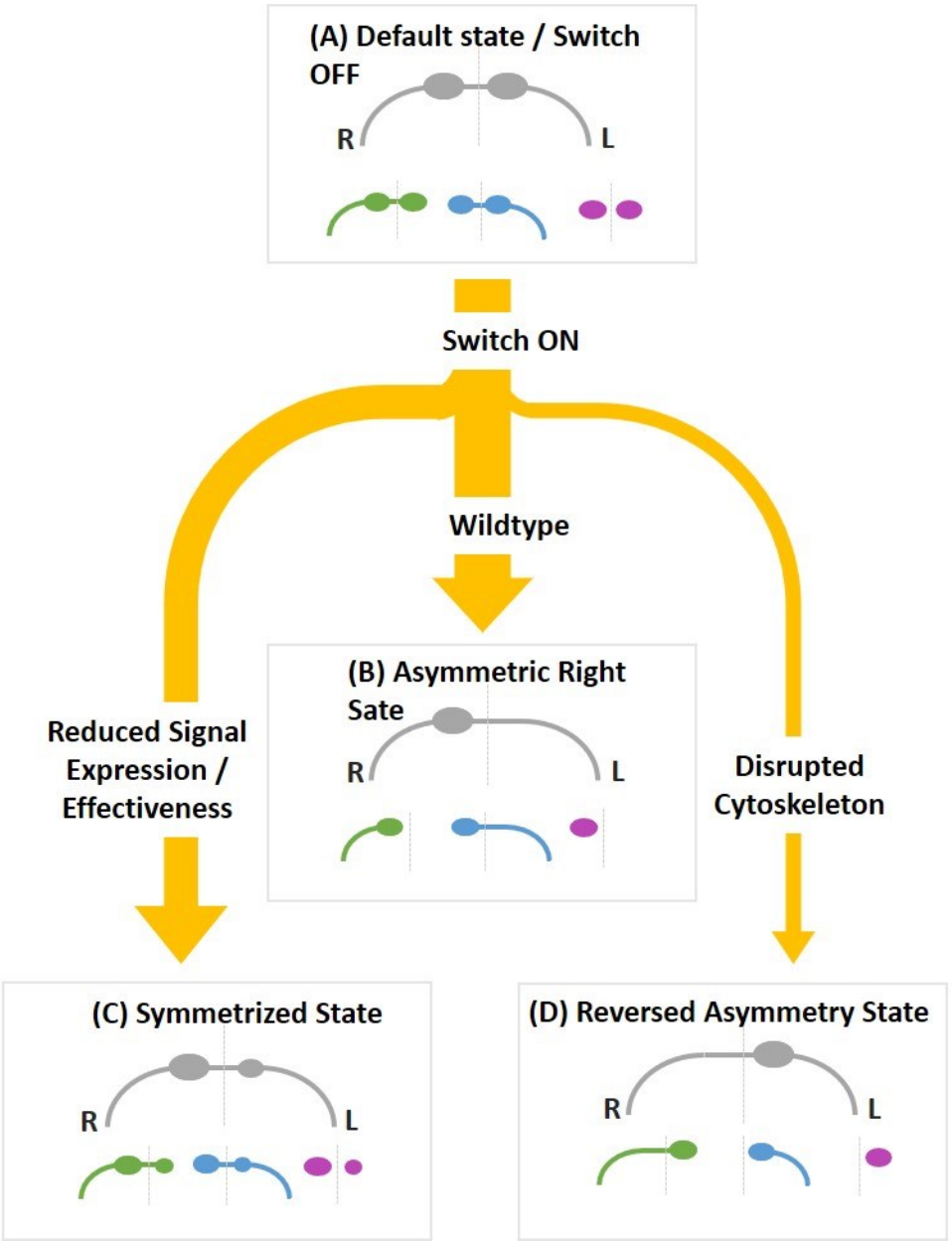

Figure 18. **Explanation for SLP-AB neuron phenotypes based on the "switch-signaling" model. (A):** Default state modeled after the Fas2-staining during pupa development and after left-right innervation pattern in adult bilateral Fas2-positive brains. Left and right neurons innervate both their respective ipsi- and contralateral AB. **(B):** Wildtype AB innervation is caused by a switch signal, that instructs the neurons to retract their axonal processes from the left AB. **(C):** Impaired signaling results in an incomplete retraction. **(D): T**he direction of asymmetry might be randomized by disrupting the cytoskeleton or by mutating necessary motor protein domains, resulting in flies with a left-side only AB innervation. **Green**: Axonal processes of right SLP-AB neurons. **Blue**: Axonal processes of left SLP-AB neurons. **Magenta:** Fas2-expression.

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