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Brain lateralisation in *Drosophila*: developmental mechanisms of neuronal remodelling underlying structural asymmetry

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Abstract

Although the overall architecture of most animals seems to be bilaterally symmetric at first glance, brain lateralisation is a common feature in nearly all organisms. The underlying developmental mechanisms that control the formation of different neural structures between the left and the right hemisphere are largely unknown. A bilateral neuropil in the central brain of *Drosophila*, the Asymmetric Body (AB), displays a striking left-right asymmetry in terms of volume and synaptic connections. This structural asymmetry is induced by the innervation of a distinct class of bilateral projection neurons (SA1/2 neurons), which first build transient symmetric AB connections followed by a neuronal remodelling process to retract from the left AB and maintain the right AB contacts. In this thesis, I am studying two candidate mechanisms of AB circuit remodelling, differential cell-cell adhesion and a signalling pathway regulating cytoskeleton dynamics within growing neurons. The cell adhesion molecule Connectin is expressed in various neuron types during AB development and Connectin null mutants show defects in AB circuit remodelling. In addition, I could observe synergistic interactions between Connectin and the SA1/2-specific adhesion molecule Fasciclin2, indindicating adhesion as a critical player in asymmetric connectivity formation. In a second project, I analysed the evolutionary conserved Fog signalling pathway, which regulates cell shape organization in neurons and non-neuronal tissues via actin/myosin activation. A receptor of the secreted Fog ligand, Smog, is expressed in the AB circuit during neuronal remodelling and targeted receptor/ligand knock-down interferes with various steps of AB asymmetry development. Based on these results I am proposing cell-autonomous and non-autonomous mechanims of circuit lateralisation in *Drosophila*.

Keywords: *Drosophila melanogaster*, Asymmetric Body, Fas2, Connectin, LRR, Fog, Smog, neuronal remodelling, non-muscle Myosin II

Zusammenfassung

Obwohl der Bauplan der meisten Tiere auf den ersten Blick symmetrisch erscheint, ist Lateralisation im Gehirn ein weit verbreitetes Merkmal. Die entwicklungsbiologischen Mechanismen, welche der Formation von verschiedenen neuronalen Strukturen zwischen der rechten und der linken Gehirnhälfte zugrunde liegen, sind trotzdem zumeist unbekannt. Ein bilaterales Neuropil im zentral-Hirn von *Drosophila*, der Asymmetrische Körper (AB), weist eine faszinierende Rechts-links Asymmetrie, bezüglich Volumen und synaptischen Verbindungen, auf. Diese strukturelle Asymmetrie wird durch die Innervierung einer neuronalen Untergruppe von bilateralen Projektionsneuronen (SA1/2 Neuronen) induziert. Diese Neuronen bilden erst symmetrische Verbindungen zwischen den ABs und bilden sich dann aus dem linken AB zurück, während sie im rechten AB erhalten bleiben. In dieser Arbeit fokussiere ich mich auf zwei Kandidaten-Mechanismen des AB remodelling, die differentielle Zell-Zell Adhäsion und den Zell Signalweg der Plastizität des Zytoskeletts in wachsenden Neuronen reguliert. Das Zelladhäsionsmolekül Connectin wird in zahlreichen Neuronentypen während der Entwicklung des ABs expremiert, Connectin Null Mutanten zeigen Defekte im AB remodelling. Zusätzliche zeigten sich synergistische Interaktionen zwischen Connectin und einem AB-spezifischen Adhäsionsmolekül Fasciclin2, was vermuten lässt, dass Adhäsion einen wichtigen Faktor in der Entwicklung der asymmetrischen Konnektivität darstellt. In einem zweiten Projekt habe ich den konservierten Fog Signalweg untersucht, der Zellform und Organisation in Neuronen und nicht neuronalen Strukturen via Actin/Myosin Aktivierung reguliert. Ein Rezeptor des sekretierten Liganden Fog, Smog, wird im AB Netzwerk während des neuronalen remodelling exprimiert und gezielte Rezeptor/Ligand knock-down Experimente stören die Entwicklung der AB Asymmetrie in verschiedensten Stadien. Diese Ergebnisse lassen vermuten, dass ein Zusammenspiel von Zell-autonomen und nicht-autonomen Mechnanismen, der Netzwerklateralisation in *Drosophila* zu Grunde liegen.

Schlüsselwörter: *Drosophila melanogaster*, Asymmetrischer Körper, Fas2, Connectin, LRR, Fog, Smog, neuronales remodelling, nicht-Muskel Myosin II

Contents

Acronyms

AB Asymmetric Body

APF after puparium formation

Caps Capricious

CX Central Complex

EB Ellipsoid Body

Fas2 Fasciclin2

FB Fanshaped Body

Fog folded gastrulation

GFP green fluorescent protein

GPCR G-protein coupled receptor

LRR leucin rich repeat

MB Mushroom Bodies

MCFO multi colour flip out

NO Noduli

PB Protocerebral Bridge

PBS phosphate-buffered saline

PCP planar cell polarity

PFA paraformaldehyde

PMG posterior midgut

RISC RNA-induced silencing complex

RNAi RNA interference

RTP receptor tyrosine phosphatase

siRNA short interfering RNA

SLP superior lateral protocerebrum

SMP superior medial protocerebrum

UAS upstream activating sequence

VF ventral furrow

1 Introduction

1.1 Symmetry and asymmetry in bilaterian organisms

Everywhere we look we are tuned to recognize symmetric patterns. From plants or animals to architecture or in music, symmetry is surrounding us. Humans and even animals tend to find symmetric ornaments more appealing than asymmetric ones (Huang et al., 2018; Rensch, 1958). Symmetry is a very principal concept which can be observed to some degree in almost every organism. While some sessile animals like jellyfish and sea anemones exhibit radial symmetry, the majority of animals, including humans, have a bilaterally symmetric body plan. This means we have a left and a right body half that are symmetric to each other. The bilateral symmetric architecture is the defining feature for the big taxa of the *Bilateria* in the animal kingdom (Moubayidin, 2015).

If we take a closer look, bilaterian organisms have several asymmetric properties, visceral asymmetry for instance is one of the more obvious ones. Our heart is situated in our left body half contrary to the liver, which is located mostly in the right body half, but there are far more delicate examples. Not only do most humans use preferably the right hand when they write or complete smaller tasks, we also have a favourite ear (left or right ear advantage) and it could be observed that there is even a directional bias when we lean in to kiss one another (Güntürkün and Ocklenburg, 2017a).

Asymmetries do exist on several levels, which are (1) behavioural asymmetries like handedness; (2) asymmetries of information processing like in our language system in the brain, and lastly (3) structural asymmetries like the visceral one (Güntürkün and Ocklenburg, 2017b). Of course, it is impossible to separate these levels from one another, rather they could be seen in a bottom-up fashion. For example, researchers could show that the green toad (*Bufo viridis*) is more prone to show an escape response if a simulated predator was presented in the left visual field compared to when it was presented in the right visual field (Lippolis et al., 2002). This represents the behavioural level of asymmetries. But these results also indicate that there is lateralisation in the toad's brain regarding neuronal structures that are involved in escape responses. It seems that

neuronal tissue in the right hemisphere is more specialised for those tasks than in the left hemisphere (Lippolis et al., 2002). Such asymmetries can be different: (1) fluctuating asymmetries, which means the given asymmetry is seen as developmental noise; (2) directional asymmetries meaning they are consistent asymmetries in all organisms and (3) anti-symmetries, which are consistent asymmetries among all organisms but the direction in which they occur are random and not fixed (Grimes, 2019).

But if asymmetries evolved so widespread across animal taxa, then what is the function or the benefit? At the first glance, it does not seem to be so beneficial if a toad shows higher escape reactivity if the predator comes from the left side. However, if the escape response shows lateralisation in the brain, maybe the attacking behaviours of the predators do so as well. Indeed, in some fish species a certain degree of oscillating asymmetry in the attacking behaviour was observed (Hori, 1993). In these terms, oscillating means that a population of predator fish attacking mostly from the behind-right side lead to prey fish expecting the predator from this side. This, in turn, leads to a predation advantage of fish attacking from behind-left, resulting in an oscillating directional bias of attacking behaviour (Hori, 1993). In other words, lateralisation of brain tissue increases the efficiency, because certain areas of the brain are more specialised and processing two different stimuli in parallel becomes possible. Furthermore, to establish the dominance of one hemisphere also means that it is not possible to elicit two incompatible responses (Vallortigara, 2006). Overall, it can be beneficial for an animal to have a certain degree of asymmetry, but how does it even come so far? What are the underlying mechanisms of lateralisation in organisms?

1.2 Developmental mechanisms of inducing asymmetry

For the establishment of left/right symmetry and consecutively for visceral asymmetry, in some vertebrates, a process called "nodal flow" has been reported to be crucial (Hirokawa et al., 2006; Shiratori and Hamada, 2014). It is involved in breaking the symmetry which happens in an early developmental stage and leads to left-right patterning. In mice during gastrulation, a midline structure called the node is formed. On this specific set of cells, leftwards rotating cilia are located, generating a consistent leftward flow. Consequently, gene products from the nodal signalling cascade are flushed through this flow to the left body half (Grimes, 2019; Hirokawa et al., 2006; Shiratori and Hamada, 2014). This represents a very elegant solution for this issue, but it

does not account for the asymmetries we can observe in the human brain (Güntürkün and Ocklenburg, 2017b; Kennedy et al., 1999). Another group of signalling pathways through which cell and tissue polarity can be provided are the conserved Wnt signalling pathways. The Wnt signalling transduction pathways are a big group, harbouring many subgroups of pathways that are critical for diverse developmental processes, such as cell fate determination, polarity, primary axis formation, and organogenesis. They can be broadly categorized into canonical Wnt signalling (which means β-catenin-dependent) and β-catenin-independent pathways. The group of β-catenin-independent pathways includes, among others, the planar cell polarity (PCP) pathway (Komiya and Habas, 2008; Seifert and Mlodzik, 2007). The numerous different proteins that are part of the PCP pathways interact convoluted and become asymmetrically localized in to form a polarized cell (Seifert and Mlodzik, 2007). In *Drosophila* the PCP pathway plays a role in orientation of bristles at the apical surface of wing cells, whereas in mammals it is involved the precise orientation of stereociliary bundles at the surface of sensory hair cells in the organ of corti (Verghese, 2011).

While the nodal flow gives us at least partly an explanation of how the visceral asymmetry is formed in some species, it is neither involved in human brain lateralisation nor does it play a role in lateralisation in *Drosophila*. Nevertheless, humans and the fruit fly exhibit lateralisation in the CNS which means there are other programmes inducing this asymmetry. However, the underlying mechanisms of human brain lateralisation are largely unknown making this field of research so attractive. This thesis aims to investigate two potential mechanisms underlying brain lateralisation in *Drosophila melanogaster*.

1.3 Inside the brain of Drosophila melanogaster

1.3.1 Axon growth and neuronal reorganisation

To establish symmetry or induce asymmetry in neuronal circuits, precise axon guidance, growth and target finding are required. But how does a neuron even find its way in the dense network of a nervous system? Axons grow via a growth cone that is sensitive to environmental cues like chemoattractants or repellents. In many organisms the midline is one of the most important sources of such cues (Evans and Bashaw, 2010). One of the best studied examples for this are probably the proteins Slits and their receptors Robo. They belong to the family of the leucin rich repeat (LRR) proteins and are

perhaps best associated with their function in axonal midline crossing in the ventral nerve cord of *Drosophila melanogaster*. In the first step the axons are attracted towards the midline by the chemoattractant Netrin that binds to DCC receptors on the axons. The second step, for commissural axons, is to cross the midline. Ipsilateral axons must stay ipsilateral and grow alongside of the midline. On the one hand, ipsilateral neurons in the ventral nerve cord of *Drosophila* express the Robo 1 receptor which senses its ligand Slit. In these neurons Slit functions as an environmental repellent cue which means the ipsilateral neurons stay on their side and do not cross the midline. On the other hand, in commissural neurons the Robo1 receptor is downregulated, hence insensitive for Slit. This makes it possible for them to cross the midline. Robo 1 is then upregulated in these neurons to prevent them from crossing the midline again (De Wit et al., 2011; Kidd et al., 1999). Neuronal growth processes exhibit a certain dynamic, which means that connectivity patterns that have been established in the course of development may be "outdated" in the adult brain. Some of those connections may need to be eliminated to make place for new ones or more refined ones. Neuronal remodelling displays an inevitable process for circuit development. This involves elimination of neurites without cell death, which is also referred to as pruning (Luo and O'Leary, 2005; Yaniv and Schuldiner, 2016; Yu and Schuldiner, 2014). Pruning covers the elimination from single synapses to whole dendritic trees and with this, it paves the way for neuronal plasticity. *Drosophila melanogaster* provides a fitting model organism to study pruning, since some neuronal areas undergo stereotypical remodelling during development. The Mushroom Bodies (MB), a structure in the central brain of *Drosophila* that is especially important for learning, memory and olfactory processing, is one of them. The MB consists of five lobes per hemisphere, the α, $\alpha\prime$, β, $\beta\prime$ and γ lobes. Three types of MB neurons project into these five lobes, for instance the γ lobe consists of the axons of one MB neuron type. So do the α and β lobe, they are made of the axons of the second MB neuron type, and the third MB neurons type forms the $\alpha\prime$ and $\beta\prime$ lobe (Crittenden et al., 1998). In a first step during development, the γ neurons extend their dendrites and axons to medial and dorsal lobes. In a second step, during metamorphosis, the neurites are pruned in a stereotypic fashion. after puparium formation (APF) the axons of the γ neurons regrow, until it resembles the connectivity pattern of the mature brain (Yu and Schuldiner, 2014).

1.3.2 The central complex of Drosophila melanogaster

Once the neurons have found their targets, they branch out and form different neuropils that can be grouped into functional and structural units. One of those areas is the Central Complex (CX), which is a midline structure that lies in the centre of the brain (Figure 1). It consists of four core structures which are sometimes also referred to as the central body. The biggest one is the Fanshaped Body (FB), above lies a structure called the Protocerebral Bridge (PB) which exhibits a unique shape that reminds one of a handlebar. Directly in front of the FB the Ellipsoid Body (EB) is localized, which has a doughnut-like appearance. The Noduli (NO), a paired structure, are located right beneath the FB-EB complex (Honkanen et al., 2019; Turner-Evans and Jayaraman, 2016; Young and Armstrong, 2010). Although minor differences in the CX of different species have been reported, it is considered to be a highly conserved structure that originated more than 400 million years ago (Honkanen et al., 2019).

The CX is involved in a lot of different behaviours such as courtship behaviour (Popov et al., 2003), navigational strategies (Honkanen et al., 2019) and memory (Liu et al., 2006). It is seen as integration centre processing sensory information. This requires a dense wiring within the substructures of the CX and as well to the brain regions surrounding the CX. Each of the neuropils in the CX are organised in columns and layers (Wolff et al., 2015; Wolff and Rubin, 2018). To give a quick example, the FB of *Drosophila melanogaster* is comprised of 9 horizontal layers, vertical columns and layers from anterior to posterior called shells (Wolff et al., 2015). The neurons innervating the CX are categorized into small field neurons and large field neurons. Small field neurons are arborizing in a single structure of the CX and therefore either connecting subunits of that neuropil, or they are connecting different neuropils within the CX. For instance, the vDeltaA neuron (Figure 1) arborizes into both ABs and connects them to upper layers of the FB. These neurons show an asymmetric degree of innervation within the ABs which possibly already lateralizes the output they give to the FB. Large field neurons on the other hand build the bridge to brain regions located next to the CX, or to those in close connection. They only innervate single structures of the CX connecting them with associated brain areas. The SA1 neuron serves as an example for a small field neuron here (Figure 1), it innervates the right AB forms mostly axons there and projects to the SLP where it arborizes and forms dendrites, which means that information flow is possibly from outside of the CX into the CX. As always, there are exceptions to that categorization (**Hanesch1989a**). In the following text the neuron-nomenclature used on *neuPrint* (Clements et al., 2020), which is an interactive website (hemibrain.org) to

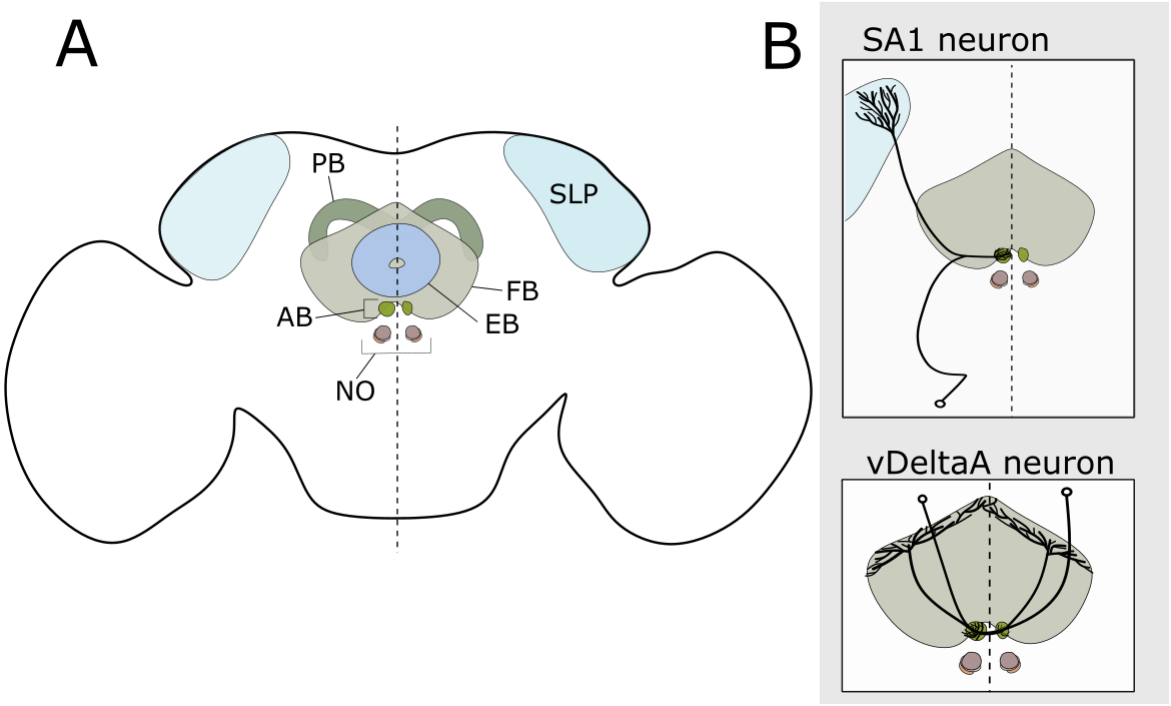

Figure 1. **Central Complex of *Drosophila melanogaster*:** **A** shows a scheme of the neuropils and their relative position of the CX in the brain of the fruit fly. The CX consists of the PB (Protocerebral Bridge), the FB (Fanshaped Body), EB (Ellipsoid Body), AB (Asymmetric Body) and the paired NO (Noduli). The SLP (Superior Lateral Protocerebrum), here depicted in light blue, is an associated brain region but not directly part of the CX. **B** shows example neurons inervating the CX. The SA1 neuron was chosen as an example for large field neurons. It connects a neuropil of the CX (the AB) with an area that is not part of the CX but in close connection (the SLP). Right beneath an example for small field neurons is depicted, the vDeltaA neurons. These neurons innervate the AB as well and provide an information flow into the upper layers of the FB. The vDeltaA neurons connect therefore two neuropils within the CX.

explore neurons, was adopted.

1.3.3 The asymmetric AB

In 2004 Pascual et al. reported the finding of a previously unknown neuropil in the CX of *Drosophila melanogaster*. It is round, about 10 µm in diameter and seemed to be located in 90 % of the flies in the right hemisphere between the EB and the FB, exhibiting therefore a striking structural asymmetry. The researchers gave the neuropil the suitable name Asymmetric Body (AB) and reported a correlation of asymmetry and performance in conditioning assays, linking the degree of asymmetry of the AB to performance of memory (Pascual et al., 2004b).

In the course of more extensive studies of the CX it was shown that the AB is indeed a bilateral neuropil existing in both hemispheres. Nevertheless, it is bigger on the right side and the cell adhesion molecule called Fasciclin2 (Fas2) is exclusively expressed in a subgroup of neurons that only innervate the right AB (Wolff and Rubin, 2018). Even though the AB is a bilateral neuropil, the structural asymmetry is still given. The AB is innervated by many neurons connecting it to other regions of the CX. A subset of neurons, the SA1 and SA2 neurons are the main players that constitute to this striking asymmetry (Wolff and Rubin, 2018) (Figure 2, A). This neuron type has a cluster of cellbodies in each hemisphere beneath the olfactory lobes. The neurons originating in the right hemisphere innervate ipsilateral into the right AB and then project to the superior lateral protocerebrum (SLP) of the same hemisphere. The neurons originating in the left hemisphere innervate contralaterally into the right AB and then project ipsilaterally into the left SLP (Wolff and Rubin, 2018). It has been shown that in the development of *Drosophila* the AB is first symmetrically innervated by the SA1/2 neurons, giving rise to a Fas2 positive layer (Figure 2, B). Between 20 hours APF and 30 hours APF the layer clusters to three distinct dots of Fas2 signal. About 10 hours further in development the middle and the left dot are showing less and less signal until only the right AB is Fas2 positive. This indicates that the processes in the left AB are remodelled and remain in the mature brain in the AB of the right hemisphere (Markovitsch, 2019). In the adult fly brain, the SA1 and SA2 neurons represent a source of input for other neurons innervating the right AB (Wolff and Rubin, 2018). Although not yet proven, it is believed that there are two distinct mechanisms which together create the asymmetry of the AB. The first one leads to the overall asymmetric innervation of neurons in the AB, this includes the remodelling process described before. The second one leads to the directional asymmetry of the AB.

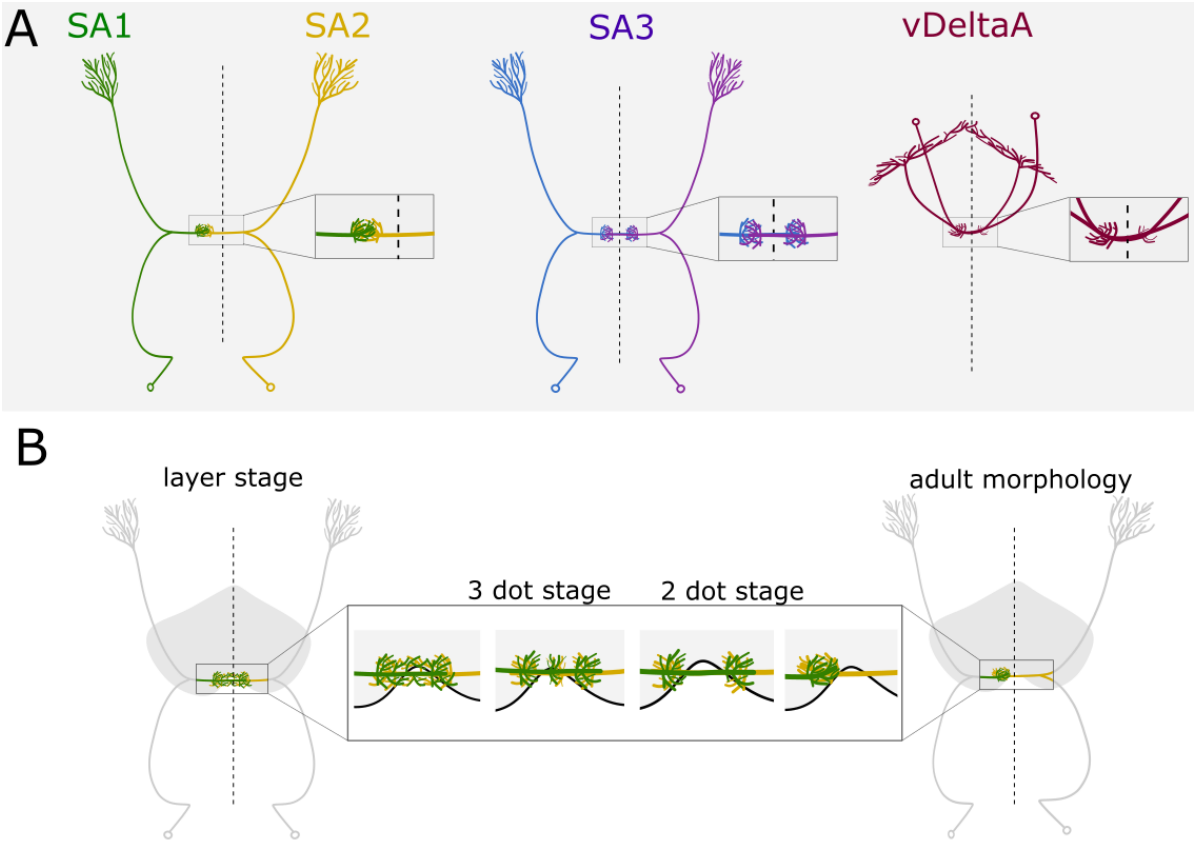

Figure 2. **AB neurons and AB formation: Scheme A**: The SA1/2 neurons (green and yellow ones) innervate the right AB and therefore are the main neurons that constitutes to the asymmetry displayed in the ABs. They mainly give information to other neurons innervating the AB therefore facilitating information flow from outside the CX into the right AB. The SA3 neurons (blue and purple) innervate the ABs bilaterally. They show postsynaptic sites as well as presynaptic sites in the area of the AB, but about twice as much postsynaptic sites in the right AB then in the left AB, providing perhaps an information flow pattern contrary to the one from the SA1/2 neurons. The vDeltaA neurons (red ones) also innervate both ABs but more densely in the right hemisphere, and project then into the upper layers of the FB. These neurons have mostly postsynaptic sites in the AB area which means they receive input from other AB neurons and probably convey it further to the FB. **Scheme B**: the AB is first organised as a Fas2 positive layer and aggregates further into three dots. Later, the dot on the midline vanishes and only two Fas2 positive dots remain. In this stage the AB is symmetrically innervated by the SA1 and SA2 neurons. After the remodelling process only the right AB remains innervated by those neurons that express Fas2.

The bilateral asymmetry of the AB in *Drosophila melanogaster* that is first symmetrically innervated and experiences a break in symmetry during development, displays an extraordinary chance to investigate the underlying mechanisms. The SA1 and SA2 neurons are a rare case of neurons exhibiting morphological asymmetry to a strong degree, which makes them such an interesting study object concerning cerebral lateralisation. The remodelling process in the AB could represent another pruning event in the development of *Drosophila*, similar to the one in the MBs. But are the underlying mechanisms comparable? Which neurons are involved and which signalling pathways induce and maintain this asymmetry? These are just some of the complex questions regarding the asymmetry of the AB, but on these questions will be focussed on in this thesis.

1.3.4 Fasciclin 2 dynamics and AB development

Although not focused on in this thesis the dynamics of Fas2 in the AB will be mentioned briefly in this chapter. In this thesis Fas2 was primarily used as tool to analyse the phenotypes but one can ask the question what the function of Fas2 in the AB is and does it maybe on its own has an important role in the remodelling process. It could be shown recently that both, overexpression and knockdown of Fas2 (with Fas2Gal4) led to significantly more bilaterally innervating SA1/2 neurons. Since Fas2Gal4 is not active in those neurons during the remodelling phase this indicates that Fas2 expression in neurons different to the SA1/2 neurons play a crucial role in the remodelling process. The proposed model how this could work includes Fas2 positive neurons that are located between the SA1/2 neurons and the vDelta neurons during development. These neurons are thought to downregulate Fas2 in the left hemisphere during remodelling phase, which opens a critical window where there is opportunity of synaptic plasticity to take place and therefore, the possibility for retraction of the SA1/2 neurons (Mitic, 2021). This means Fas2 dynamics provide a frame for the retraction of the SA1 and SA2 neurons out of the left AB but the exact mechanisms remain unsolved.

1.4 Candidate mechanisms potentially underlying brain lateralisation in the fruit fly

During a recently performed RNAi screen a special protein called Connectin, a cell adhesion molecule, belonging to the LRR protein family, attracted attention. A knockdown of Connectin produced variable AB phenotypes because of which it was selected as a

candidate protein to study its dynamics in AB development. It has been shown that Connectin is expressed in very distinct patterns in the CX during development. It is first expressed by columnar neurons forming 4 columns in the FB but then undergoes a change from columns to layers. Further there seems to be a very close interplay of Connectin and Fas2 expressing neurons (Vokac, 2021). When the layer of Fas2 expressing neurons fragmentises into three dots, then two dots and eventually one adult AB on the right side the Connectin expressing neurons seem to grow into the space which was formerly occupied by Fas2 expressing neurons. This "taking over the place" suggests an interaction of Connectin expressing neurons and Fas2 expressing neurons in respect of AB formation. On the one hand Connectin has a very distinct expression pattern in the course of FB development, and on the other hand the AB is a neuropil which is structurally very densely wired with the FB during development. Connectin seems to play a role in the separation and the detachment of the AB. For this reason, it was chosen as potential candidate protein being involved in the formation of the AB.

As the asymmetry of the AB is mostly constituted by the SA1 and SA2 neurons, this subtype of neurons is also studied more closely. Both neurons retract their processes out of the left AB which means that the SA1 neurons retracts contralaterally and the SA2 neurons retracts ipsilaterally. A signalling pathway that was first identified in morphological tissue changes following the event of gastrulation caught interest here. In case of *Drosophilas* gastrulation the folded gastrulation (Fog) signalling pathway was reported to stepwise activate Myosin II which leads to constriction of cells that results in the end in irreversible cell shape changes required for invagination (Costa et al., 1994). The signalling pathway is activated by Fog which is a protein that binds to different G-protein coupled receptor (GPCR) that are called Mist and Smog. The presence of the ligand Fog induces homoclustering of the receptor Smog on the apical surface of cells. The GPCRs activate the G protein Concertina (Figure 3) which in turn activates RhoGEF2-Rho-RhoKinase leading in the end to Myosin II activation and accumulation (Dawes-Hoang et al., 2005; Jha et al., 2018; Kerridge et al., 2016a). It is thought that Fog acts as an autocrine signal (Ratnaparkhi and Zinn, 2007) which would mean that the SA1 and SA2 neurons themselves express Fog leading to non-muscle MyosinII activation which results in the end in their own remodelling.

The first project examines Connectin that has a substantial interaction with Fas2 and acts more from the surroundings and the neighbouring neuropil (FB) which has a very close connection to the AB when seen from a developmental perspective. The second candidate mechanism, the Fog signalling pathway, deals with the more direct signal from

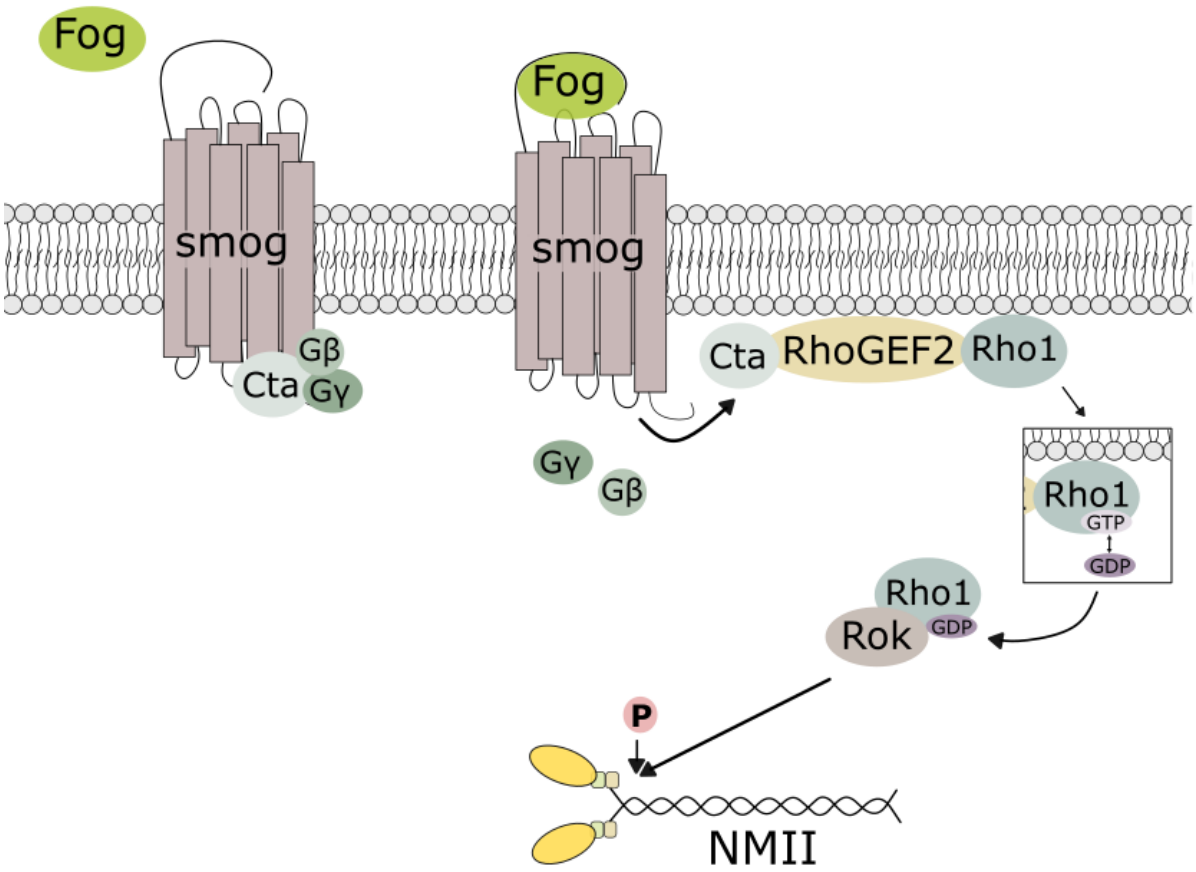

Figure 3. **Fog pathway:** Upon binding of the ligand Fog on the receptor Smog, Concertina (Cta) dissociates from the Gprotein subunits Gγ and Gβ and recruits RhoGEF2 to the membrane. This facilitates an exchange from GDP to GTP in the GTPase Rho1 which activates this protein that can now in turn activate the Rho Kinase (Rok). Rok phosphorylates now the regulatory light chain of non-muscle Myosin II which activates it and leads to subsequent contractility.

the SA1 and SA2 neurons to initiate and regulate their own remodelling process. The candidate mechanisms should not be seen as two completely separate topics as they are very likely to have interactions and influence each other, in order to make a proper remodelling possible.

1.5 Aims of this thesis

In this thesis the focus will lie on the LRR protein Connectin and the Fog signalling pathway to address the big questions of how this asymmetry is generated in the brain of *Drosophila melanogaster* and its underlying mechanisms.

Connectin

Given the remodelling process of the SA1 and SA2 neurons in the course of development and the patterning of the LRR protein Connectin in the CX, one of the aims of this thesis is to clarify the role of Connectin in the development of the AB. The working hypothesis concerning the Connectin approach is: The Connectin positive neurons that form first columns and then segregate in layers, could have a critical impact on the neurons that are first symmetrically innervating both ABs and then remodel out of the left AB. To investigate the putative role of Connectin in this process it was tried to identify the neurons expressing Connectin. Further, knock-down experiments were performed, to see, if a loss of Connectin induces again a symmetric innervation of the Fas2 positive SA1 and SA2 neurons.

Fog

With this approach specifically the neurons innervating the AB and their impact in the remodelling process were targeted. Therefore, it was hypothesised that neurons innervating the AB express the GPCR Smog and are therefore directly a part of the folded gastrulation signalling pathway. This pathway may play a role in transducing the signal for AB remodelling. To investigate the putative role of the GPCR Smog in the process of AB remodelling, again knockdown experiments were performed targeting one of the receptors Smog, as well as the ligand Fog.

2 Material and Methods

2.1 Fly husbandry and fly strains

Flies were reared on standard food at 25 °C in small (⌀ 25 mm) or big (⌀ 100 mm) *Drosophila* breeding vials. The stocks were flipped every second week into new vials containing new food. Crosses were usually set up with 1 to 5 female virgin flies and 1 to 4 male flies. After the females laid eggs for a few days, the parental flies were flipped out into another vial. If not stated otherwise, all RNAi crosses were raised in incubators at 29 °C. For a complete list of all used fly stocks see table 1.

2.1.1 Reporter-lines

In all experiments two reporter-lines were used. First, and most often the AB1lexAlexAop ::GFP reporter-line, which labels the SA1/2 neurons was used. The constructs AB1lexA and lexAop::GFP were recombined for this reporter-line. To drive the green fluorescent protein (GFP) in the SA1/2 neurons, the enhancer for the Smog gene was used in the driver line construct AB1lexA. Second, the AB4lexAlexAop::GFP reporter-line was used, which labels the vDeltaA neurons that are innervating into both ABs. In this reporter-line, the enhancer of a dopamine receptor was used to drive the lexA construct. These neurons project into the upper layers of the FB and do not express Fas2.

2.2 Genetic techniques

2.2.1 Binary expression systems

One of the most used binary expression systems in *Drosophila* is the Gal4/UAS system. The protein Gal4 derives from the yeast *Saccharomyces cerevisiae* and functions as a gene regulator. Gal4 binds on the so-called upstream activating sequence (UAS) which functions as an enhancer and promotes expression of certain genes. The Gal4/UAS system is not only in *Drosophila* a very widely used method to drive the expression of

Table 1

Fly lines used:

Line	Source	reporter line used
RNAi lines		
UAS-43135 (smogRNAi)	Bloomington	
UAS-51705 (smogRNAi)	Bloomington	
UAS-36790 (fogRNAi)	Bloomington	
UAS-110641 (smogRNAi)	VDRC	
UAS-28967 (ConRNAi)	Bloomington	
Driver lines		
R11F10-Gal4	Bloomington	AB1lexA lexAop::GFP
R14B01-Gal4	Bloomington	
Fas2-Gal4	Bloomington	
elav-Gal4	Bloomington	AB1lexA lexAop::GFP
R72A10-Gal4 (AB1-Gal4)	Bloomington	
442-Gal4	Bloomington	
AB4-Gal4	Bloomington	AB1-and 4 - lexAlexAop::GFP
Con-Gal4	Bloomington	
elav-Gal4,hsfFlp,UAS mcd8::GFP/Frt42,Gal80	Bloomington	
Other lines		
CantonS	Bloomington	
Fas2eb112	Bloomington	
Condef	Bloomington	
DF9062	Bloomington	
FogDP(y)	Bloomington	
36284 ($Fas2^{EB112}$)	Bloomington	
Frt42,Gal80	Bloomington	

reporter proteins in a specific time window in tissue specific manner. To do so, flies carrying the UAS site upstream of the gene of interest (e.g., marker protein) have to be crossed with flies carrying the Gal4 construct fused to a wanted driver. In the offspring, the UAS site is present in every cell, but the Gal4 construct is only present in the cells where and when the driver is expressed. Consequently, in neurons where the driver is expressed also Gal4 will be expressed, which binds to the UAS site which leads to expression of the gene of interest (Duffy, 2002; Jenett et al., 2012). The LexA/LexAop system is a second binary expression system that is widely used. Here, LexA acts similar to Gal4 and binds on LexAop, a promotor, which leads to the expression of the target gene. Both binary expression systems can be combined to drive the expression of different target genes in a tissue specific manner (Pfeiffer et al., 2010).

2.2.2 Knockdown via RNA interference

RNA interference (RNAi) is an efficient tool to silence the expression of specific proteins. Therefore, a DNA construct of the target gene which produces a double stranded RNA must be in the cell. This double stranded RNA is then cleaved into smaller pieces of short interfering RNA (siRNA) by a protein called Dicer. These siRNA pieces associate with another protein complex called RNA-induced silencing complex (RISC). RISC unwinds these pieces and subsequently binds mRNA with a high degree of complementary sequence. The target mRNA is then cleaved which leads to a knockdown of the protein of interest (Ambesajir et al., 2012). In the following experiments a lot of fly strains carrying RNAi constructs (e.g., Connectin, Smog or Fog) were used. These RNAi constructs were expressed under the Gal4/UAS system to ensure the knockdown of the proteins in tissue specific manner.

2.2.3 Multi colour flip out technique

The multi colour flip out (MCFO) technique is a very useful and flexible method to label individual neurons. It marks specific neurons stochastically in distinct colours. The basic components are a UAS-heatshock-flippase construct and the so-called MCFO construct, that both have to be present in a parental fly. This fly is then crossed to flies carrying the Gal4-driver construct. The MCFO construct contains epitopes like HA and Flag that are inserted in a nonfluorescent GFP. Stop-cassettes flanked by FRT sites are inserted upstream of those epitopes. If the flies that carry all components (the UAS-heatshock flippase, an MCFO construct and a Gal4-driver) are heatshocked, the flippase

binds to the FRT sites and cuts out the stop cassettes randomly. Consequently, the epitopes are expressed stochastically in the neurons where the driver is active. Finally, the epitopes can be labelled with antibodies to visualize the neurons with fluorescent colours (Guo et al., 2019; Nern et al., 2015).

2.3 Immunohistochemistry and mounting

For preparation of the dissected brains the protocol that is used in our laboratory was followed. This includes anesthetising the flies prior to dissection in CO_2 and killing them in 96% ethanol. The adult flies were dissected in a droplet of phosphate-buffered saline (PBS) whereas pupae were first opened in dry and then dissected in PBS. Afterwards, the brains were fixated 1 hour in 4% paraformaldehyde (PFA) and PBS solution. The fixated brains were then 4 times washed with 15 min. in between in a 0.3% PBT solution (PBS containing 0.3% TritonX-100). For blocking, the fly brains were next incubated for 1 hour in goat serum (10% goat serum in 0.3 % PBT), and subsequently kept overnight on 4 °C in a solution containing goat serum and the primary antibodies. For a list with all used antibodies and their dilutions see table 2. The samples were incubated in a solution with secondary antibodies after an intermediate step of washing again for 1 hour in PBT solution.

Before mounting the brains in Vectashield (Vector Laboratories) the brains were washed again 4 times á 15 min. The brains were orientated with the anterior side facing upwards. After orientation the coverslips were provided with small amounts of modelling clay in the four corners and then carefully placed on the droplet containing the brains. If not immediately imaged, the slides were stored in a slide folder that was kept permanently at 4°C.

2.4 Image analysis and acquisition

A Leica Confocal Microscope TCS SP5II with a 20x oil immersion objective was used to collect images. Frame size was usually 512x512 pixels at a scanning speed of 400 Hz. However, to improve the resolution for some MCFO images I used a frame size of 1024x1024 pixels. All images were scanned as stacks with optical sections at 1.55 µm spacing. Laser power was adjusted throughout the scanning process to maximize image quality. After imaging, the confocal stacks were analysed and edited using the opensource software ImageJ. All statistical analysis were performed with the opensource

Table 2

Antibodies used to stain the samples

Type	Origin	Dilution	Antibody	Source
Primary	Mouse	1:50	anti-Connectin (Con)	DSHB
Primary	Mouse	1:5	anti-Fasciclin2 (Fas2)	DSHB
Primary	Rabbit	1:1000	anti-GFP	Invitrogen
Primary	Mouse	1:2000	anti-Flag	NovuBio
Primary	Rabbit	1:2000	anti-HA	NovusBio
Primary	Rat	1:10	anti-Ncadherin (Ncad)	DSHB
Secondary	Goat	1:500	anti-Rabbit 488	Invitrogen
Secondary	Goat	1:500	anti-Mouse 488 (highly cross absorbed)	Invitrogen
Secondary	Goat	1:300	anti-Mouse 568 (highly cross absorbed)	Invitrogen
Secondary	Goat	1:500	anti-Rat 647	Invitrogen

software R and the environment RStudio. Grouping of graphs and confocal pictures, as well as all schemes were created with Inkscape.

3 Results

3.1 Connectin expression pattern: characterization of Con positive neurons via MCFO

The first goal was to find out where Connectin is expressed in detail in the CX. For this purpose, brains of *Drosophila melanogaster* where dissected and stained with antibodies against Connectin. The results showed that the LRR protein is expressed strongly in three distinct domains of the FB (Figure 4). The three domains consist of the ventral FB layers, the medial layers and the very top layers of the FB which are all Con positive. It could be shown that neurons innervating the NO also express Connectin. The PB shows Connectin staining as well, but to a lesser degree than the domains in the FB. Similar in the EB, the signal of Connectin staining was weaker.

To pin the expression pattern down to distinct neurons, the genetic technique MCFO was used to generate single-cell clones. Given that the observed signal was very broad in all of the 62 brains, single neurons could not be fully traced. Nevertheless, distinct signals per neuropil could be observed and analysed regarding potential neuron candidates (Figure 5). In the FB in 82 % of the brains, clones of neurons innervating in the upper layer of the FB and then projecting to the SLP (Figure 5, e.g., neurons FB6A) could be observed. In some cases neurites coming through the channel of the EB could be traced, leading to the assumption that this neuron-type could be the ExFl2 (FB6A on hemibrain) and FM3 (Young and Armstrong, 2010) neurons, which are showing remarkably similar morphology (Figure 5, FB6A neurons). Another candidate that could be responsible for this signalling pattern is the FB7D neuron or the FS4A neuron which innervates in the AB and projects then to an area lying on the border of superior medial protocerebrum (SMP) to SLP. In 80 % of the brains a signal could be observed in the middle layers of the FB. The FS4A neurons also innervate the bottom layers of the FB and the AB which could be responsible for the signal in lower layers in about half of the brains. In 37 % of the brains also the area of the AB was labelled which means that neurons innervating the AB are possibly also expressing Connectin. However, the

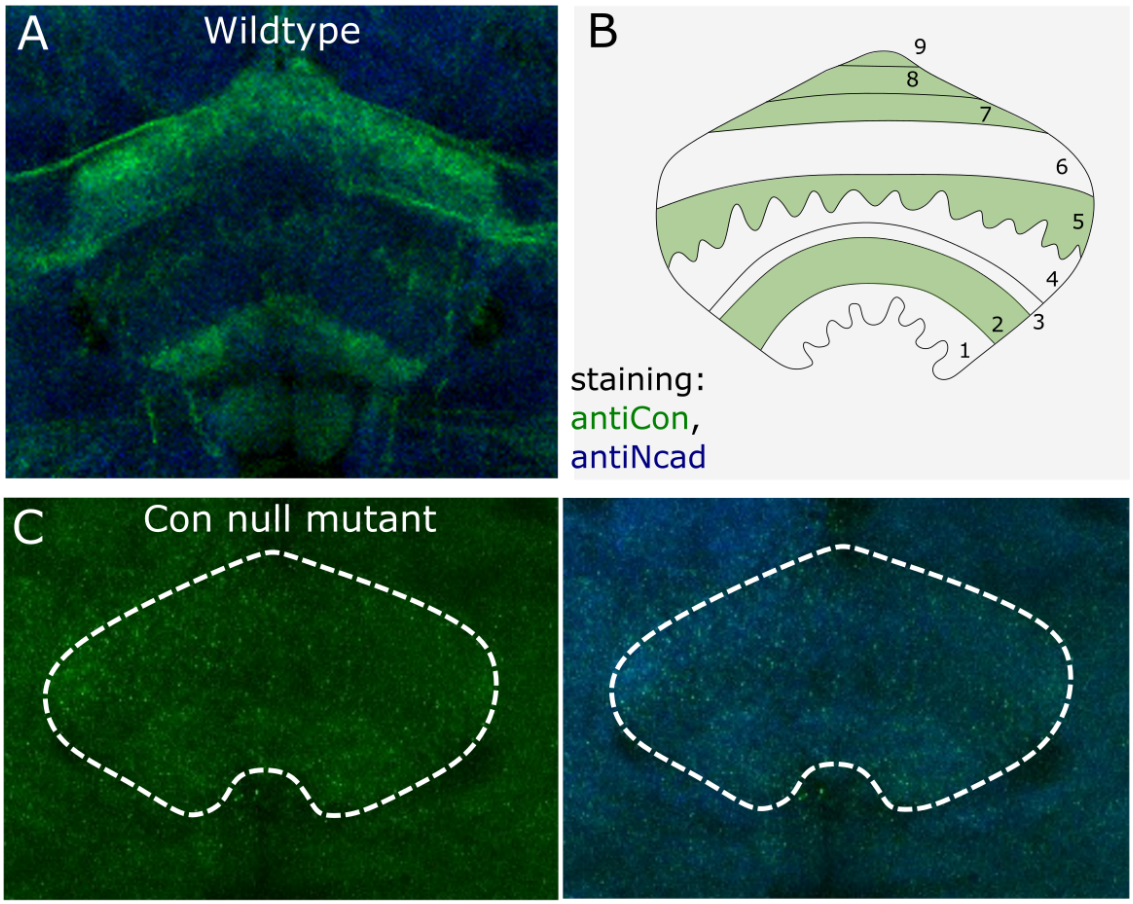

Figure 4. **Expression pattern of Connectin:** **A** displays the wildtype expression pattern of Connectin. The green signal implies an expression of Connectin in 3 main layers in the FB. **B** shows a schematic representation of the FB layers and which layers (for instance layers 2, 5 and from 7 to the very top) the con-antibodies could have stained (light green). The two pictures in **C** show the Con null mutants stained against Connectin. It is visible that the brains have lost almost all of their green signal. All brains were stained against Connectin (green) and Ncad as reference staining (blue).

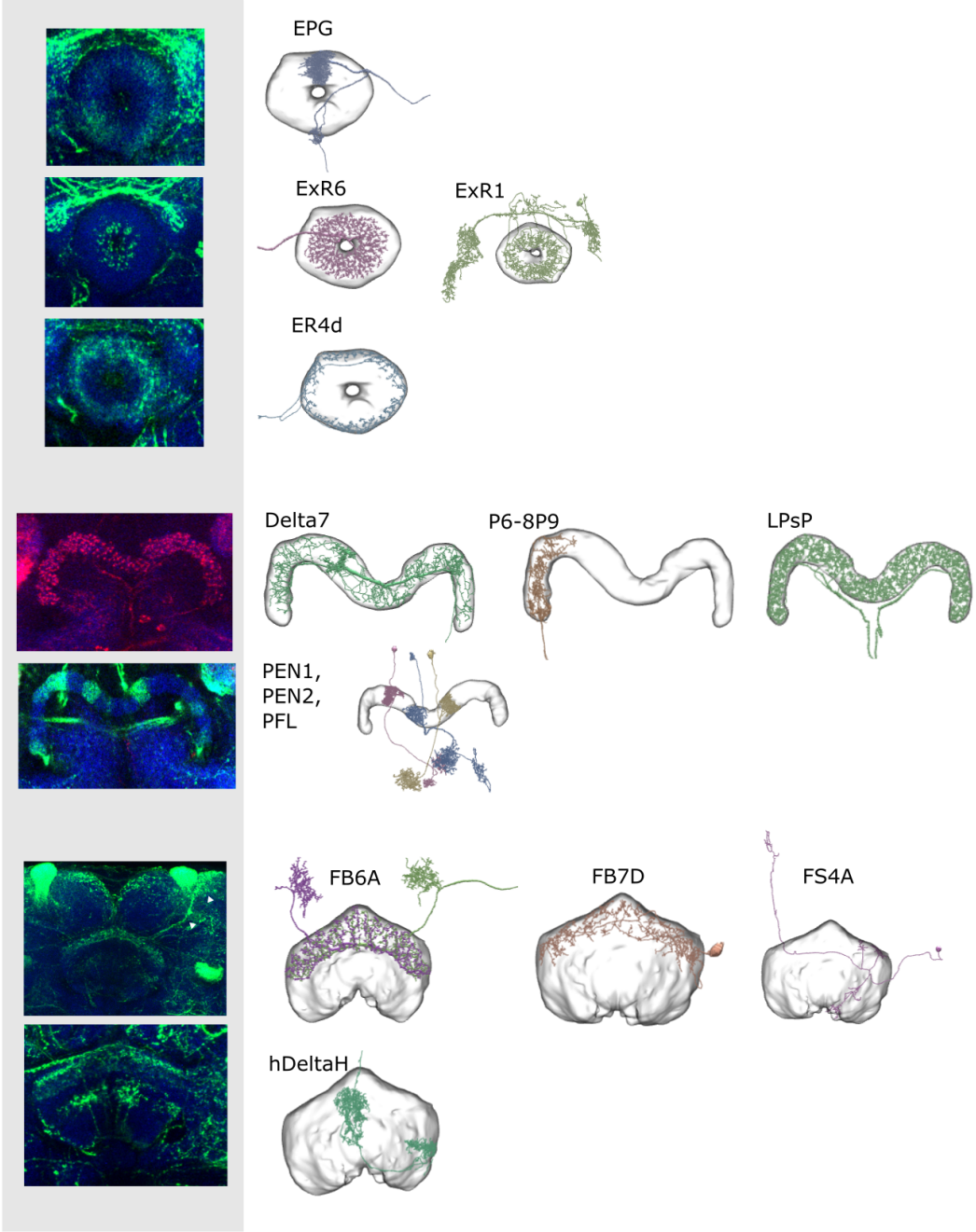

Figure 5. **MCFO clones of Con positive neurons:** Different representative pictures were chosen from the website hemibrain.org to display the MCFO clones of Con positive neurons. As the signal was very broad, the neuropils of the CX were separately analysed. Besides the confocal pictures are neurons displayed from the website hemibrain that could serve as candidates for the signals that can be seen in the respective neuropils. The neurons from hemibrain only serve as examples, numerous other neurons could be possible candidates for expressing Connectin and being responsible for the seen signal. The staining was with antibodies against the epitopes HA (red), Flag (green) and the membrane protein Ncad (blue) as reference staining.

signal in the AB was always sparse and the typical innervation pattern of the SA1 and SA2 neurons could not be detected, which indicates that other neurons innervating the AB could be the ones expressing Connectin. In about 30% of the brains MCFO clones in the EB could be observed as well. Mostly concentric rings of the EB were marked, which means that potentially large field neurons of the EB (the ring neurons) express Connectin (Figure 5, e.g., ExR6, ExR1 and ER4d). Furthermore, at least in one brain, a wedge of the EB was labelled (e.g., Figure 5) potentially from small field neurons, for instance the EPG neuron. In 32% of the brains numerous distinct glomeruli of the PB showed signal (e.g., the PEN1, PEN2 and PFL neurons) and in about 20 % the whole PB showed signal, which could derive from large field neuron, for instance the PB18.s-GxΔ7Gy.b (Delta7 on hemibrain) or the PB.b-LAL.s-PS.s (LPsP on hemibrain) neuron (Wolff et al., 2015). Another candidate, the PBG6–8.sG9.b neuron (P6-8P9 on hemibrain) could also contribute to this kind of signal. The schematic representations of CX neurons listed in Figure 5 are only examples of candidate neurons that could be a part of the Connectin expressing network contributing to the Connectin expression pattern (Figure 4). In the majority of brains clones of FB neurons could be detected (Figure 6) and in a third of the brains clones in the EB and the PB. The overall collection of randomly generated MCFO clones under the driver-line ConGal4 validates the expression pattern of Connectin seen in figure 4 but to confirm the exact types of neurons further experiments with more specific driver-lines are necessary.

To conclude, through this MCFO technique it could be shown that firstly Connectin is expressed by a high number of different neuron types and secondly broadly distributed across the CX. Interestingly, in some cases also the area of the AB was labelled, indicating that those neurons innervating the AB are also Con positive. To get more exact results on which neuron types are the main source of Connectin, experiments with more specific driver lines must be conducted.

3.2 Connectin knockdown via RNAi

To investigate the role of Connectin in the AB remodelling process, it was knocked down in two different driver lines. As described above the SA1 and the SA2 neurons exhibit a striking asymmetry. In the wildtype morphology these neurons innervate solely the right AB and express Fas2. However, in the course of development, the AB undergoes a drastic change from a layer-like organisation to three dots and then to two dots both innervated by the SA1/2 neurons. In the adult brain, as mentioned above, the right AB

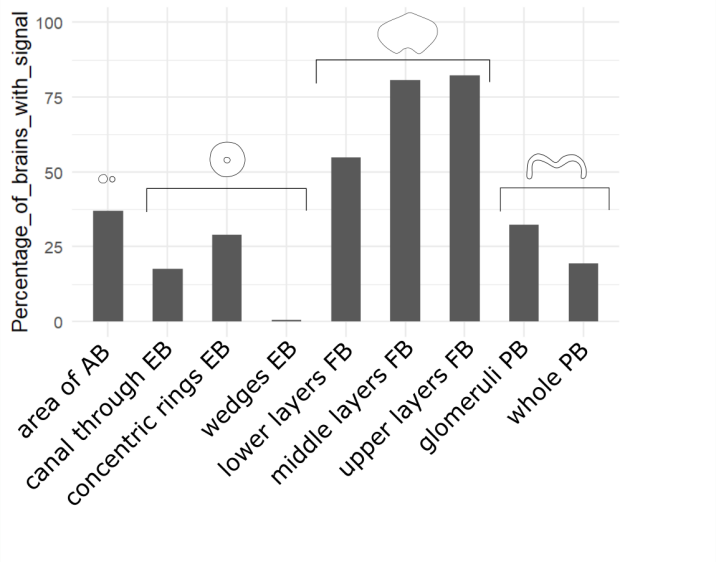

Figure 6. **Distribution of MCFO clones in the CX:** The different areas in the CX are shown on the x-axis and the number of brains (in %) in which signal could be seen on the y-axis. In most of the brains neuronal clones of the FB could be detected. In about a third of the brains were visible clones of the other neuropils.

remains innervated, meaning that a remodelling process needs to take place beforehand (Figure 2).

When knocking down Connectin with two different driver lines, 3 different phenotypes deviating from the wildtype morphology could be observed (Figure 7). The first abnormal phenotype showed a directional disturbance, as the Fas2 positive AB was located in some brains on the left side. The other two abnormal phenotypes showed a structural bias, as the innervations of the two ABs could not be separated and remained as one merged AB on the midline (hereafter referred to as ”merged” or ”midline” phenotype) (Figure 7, C). Another possibility is that the merged AB phenotype is the result of the AB being ”stuck” in the developmental layer stage and could not be fully separated from the FB. The third abnormal phenotype was Fas2 positive innervations in both ABs (hereafter referred to as ”bilateral” phenotype) (Figure 7, D).

The Connectin knockdown was performed in two different driver-lines, R14B01Gal4 and ConGal4, and it produced all three classes of phenotypes in both driver-lines (Figure 8, A). None of the experimental groups showed significantly more abnormal phenotypes than the control group. When Connectin was knocked down with the driver ConGal4, the frequency of different abnormal phenotype classes was almost one-third for each class. With the driver R14B01Gal4 merged ABs dominated over bilateral ABs. In this

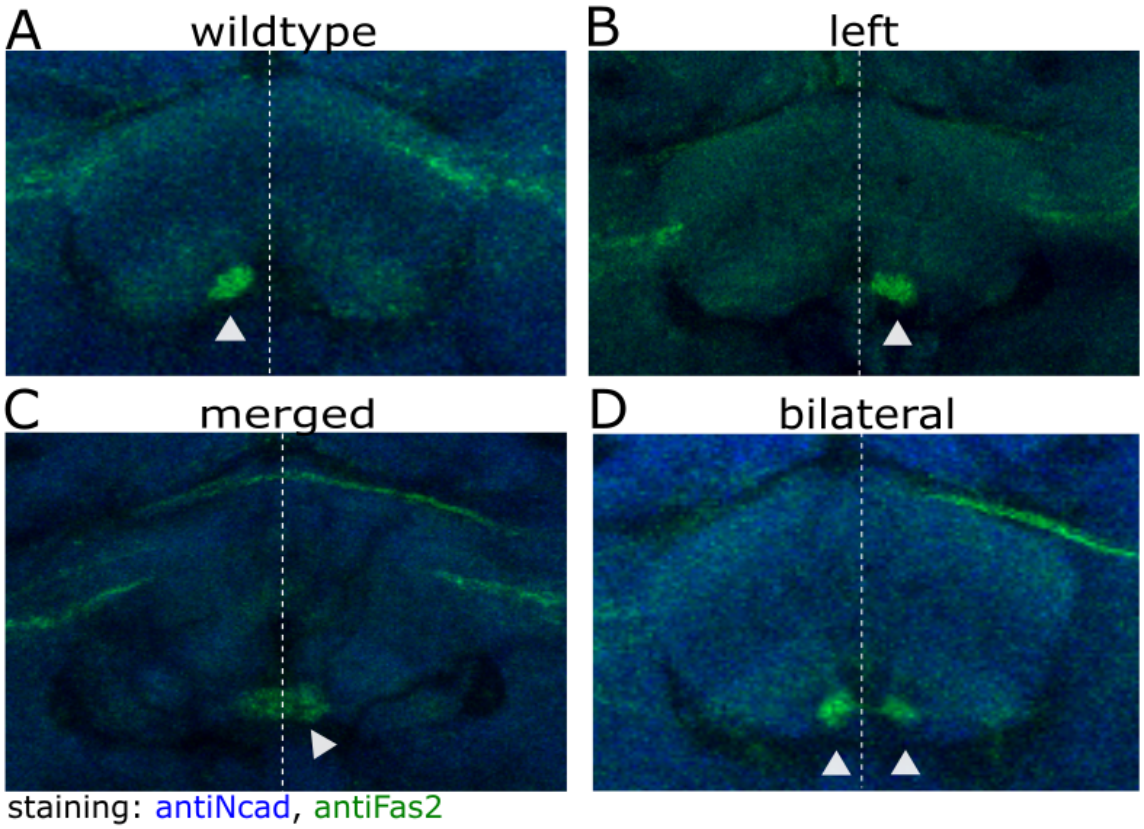

Figure 7. **AB phenotypes:** **A** displays the wildtype AB phenotype when stained against Fas2, only the right AB shows a Fas2 signal. **B** shows a unilateral AB but the Fas2 signal is on the left side which could be a result of a directional innervating or remodelling bias of the SA1/2 neurons. **C** shows once a merged AB where it seems that there was a fail in remodelling of the SA1/2 neurons and **D** shows a bilateral AB, where both ABs show a Fas2 signal and hence are innervated by the SA1/2 neurons. All brains were stained against Fas2 (green) and Ncad as reference staining (blue).

group, the Fas2 signal of one AB could not be detected at all. In the control group, for which flies of the stock that carried the RNAi construct over a TM6 balancer were dissected, a lot of abnormal phenotypes could be observed as well. Here, bilateral ABs and merged ABs were the most abundant abnormal phenotype classes.

The knockdown had a considerable effect on the development of the AB when performed in fly brains of the R14B01Gal4 line. It produced abnormal phenotypes in more than half of the brains, with merged ABs being the most dominant class. Surprisingly, the number of abnormal phenotypes in the control group was also high, reaching nearly 50%.

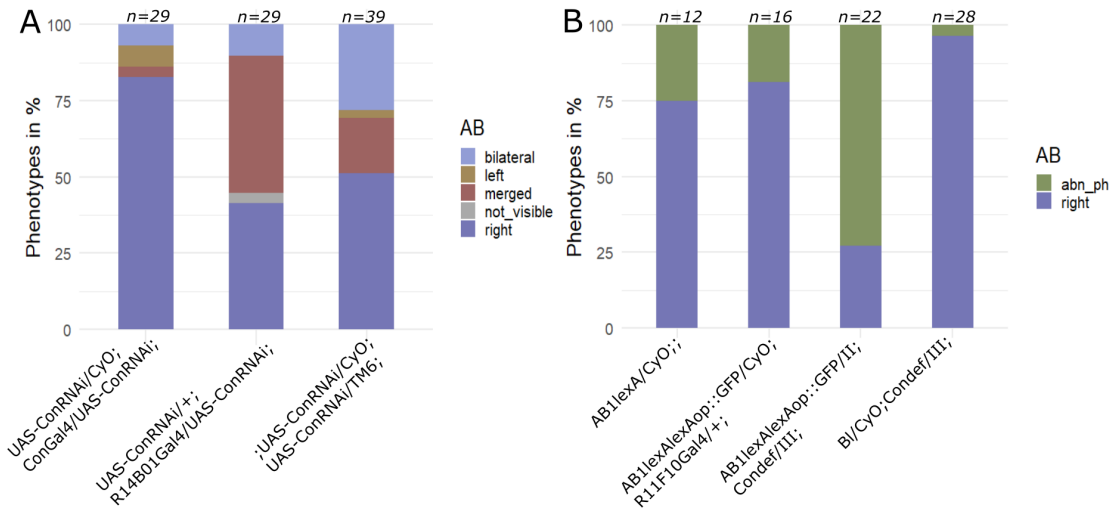

Figure 8. **Knockdown of Connectin and Connectin null mutants:** **A** depicts the knockdown of Connectin in two different driver-lines. The percentages of the different phenotypes are shown in different colours on the y-axis:dark blue represents the wildtype phenotype right;gray depicts the ABs that could not be seen at all; red represents merged ABs; ochre represents left ABs and blue bilateral ones. **B** shows the number of abnormal phenotypes in Connectin mutants and control groups. The percentages of abnormal phenotypes in Connectin null mutants and control groups are shown on the y-axis, the genotypes on the x-axis. The green colour represents abnormal AB morphology whereas the blue colour depicts wildtype ABs.

3.3 Investigation of Connectin mutants

In addition to the knockdown of Connectin via RNAi, Connectin null mutants were examined (Figure 8, B). Two different genotypes, both lacking a functional Connectin protein, and two control groups were investigated. One of the Connectin mutant genotypes carried a deficiency for the Connectin protein homozygous on the third chromosome. When these brains without the reporter-line (Bl/CyO; Condef/III) were stained against Fas2, a high number of abnormal phenotypes was expected but surprisingly all ABs showed wildtype morphology except for one brain (Figure 8, B).

However, the other genotype contained a reporter-line construct additionally to the deficiency (AB1lexAlexAop::GFP/II;Condef/III) on the second chromosome highlighting the SA1 and SA2 neurons. Three-quarters of the brains in this mutant showed abnormal phenotypes which are significantly more than without the reporter-line (Fisher's exact test, p=<0.001). This raised the question if the reporter-line itself influences the remodelling process of the SA1 and SA2 neurons out of the left AB. Therefore, two different control-genotypes were investigated, both with normally functioning Connectin proteins.

One of the control groups carried a part of the reporter-line construct on the second chromosome (AB1lexA/CyO;;) and the other one carried the full reporter-line construct over a balancer and a R11F10Gal4 construct (AB1lexAlexAop::GFP/CyO;R11F10Gal4/+;). None of the control groups had significantly more abnormal phenotypes than the plain Connectin null mutants, but both had significantly less abnormal phenotypes than the experimental group lacking Connectin but carrying the reporter-line (Fisher's exact test, p=0.012 and p=0.0025). Additionally, flies without functioning Connectin were dissected and stained with Connectin antibodies to prove its absence from the CX (Figure 4, C). The plain Connectin null mutant, the Connectin null mutant containing the reporter-line homozygous and heterozygous were also kept on 29 °C to see if higher temperatures affect the AB remodelling process. No significant influence could be detected (see appendix).

3.4 Fas2 loss of function in Con null background

These results indicate a complex synergistic effect between the loss of Connectin and additional stress factors like the reporter-line construct. For further investigations, another experiment was conducted in which a loss of function allele of Fas2 (Fas2^{EB112}) was introduced in a Connectin mutant background (Figure 9). The flies carrying a loss of function allele of Fas2 and the reporter-line in Connectin mutant background (Fas2^{EB112}/x;AB1lexAlexAop::GFP/+;Condef/III) showed the highest amount of abnormal phenotypes. Flies with the same genotype but a balancer (FM7a) instead of the Fas2 loss of function allele exhibited a slightly decreased number of abnormal phenotypes whereas brains of flies with the genotype Fm7a/y; AB1lexAlexAop::GFP/+; Condef/TM3; exhibited less than 25% of abnormal phenotypes. The loss of function allele of Fas2 alone causes, similar to the Connectin deficiency, hardly abnormal phenotypes. Taken together, a lot of abnormal phenotypes were observed when the Connectin null mutant was combined with a reporter-line, whereas the same Connectin mutants without this reporter line produced almost no abnormal phenotypes. Dissected flies that carried the reporter-line but had functional Connectin proteins did not show significantly higher abnormal phenotypes than the Connectin mutant. This indicates complex indirect interactions where it is more likely to observe abnormal phenotypes in a Connectin null mutant background when the system is intrinsically stressed with a genetic load like the reporter-line constructs or a Fas2 loss of function allele. A non-functional Connectin protein per se does not seem to be responsible for an increased number of abnormal phenotypes.

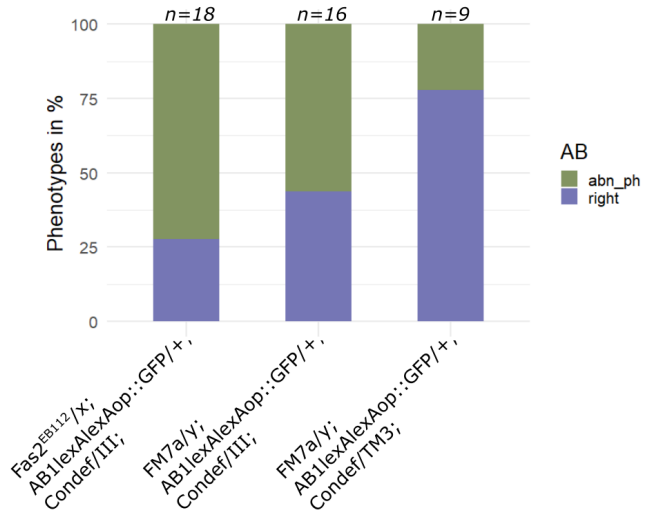

Figure 9. **Fas2 loss of function in Con null background:** The percentages of the different phenotypes are shown in different colours on the y-axis: green represents abnormal phenotypes and blue right ones. The first genotypes carry a loss of function allele of Fas2, the AB1 reporter-line in Connectin null background. The second genotype carries only the reporter-line in Connectin null background and the third genotype carries the reporter-line with a heterozygous Connectin deficiency.

3.5 Knockdown of Smog

The Fog pathway is a signalling pathway that, once activated, sets off a chemical cascade resulting in the activation of non-muscle myosin II. Non-muscle myosin II activation is crucial for changes in the morphology of cells and tissue (Kerridge et al., 2016a). In contrast to Connectin, Fog is an autocrine signal, indicating that it is expressed directly in the SA1 and SA2 neurons that are innervating the AB making it especially important to investigate its role in the AB remodelling process. To do so, systematic knockdown experiments of a GPCR (Smog) and the ligand (Fog), with various driver-lines were performed. The main goals were (1) to see if there is a significant effect on the AB remodelling when one of the elements of the Fog pathway is knocked down; (2) if there is an effect, to restrict it to a certain group of neurons.

To improve the visualization of the knockdown via different driver-lines, the genotypes were divided into groups. Graph A of figure 10 shows the genotypes without the reporter line. The biggest effect was detected in flies with the genotype 51705/+; AB1Gal4/+ showing significantly more abnormal phenotypes than the control group 51705/+;; (Fisher's Exact test, p=0.046). This group shows also significantly more

abnormal phenotypes than flies with the genotype 51705/CyO;AB4Gal4/+ (p=0.043), 51705/Fas2Gal4;; (p=0.032) and 51705/+;R11F10Gal4/+; (p=0.007). This indicates that the reduction of Smog has the biggest effect on the AB remodelling particularly in the SA1 and SA2 neurons. Interestingly, also flies with the genotype 51705/CyO;R11F10 Gal4/+ showed significantly more abnormal phenotypes than the group without the balancer on the second chromosome 51705/+; R11F10Gal4/+; (p=0.008) which could indicate a potential influence of balancer chromosomes. In graph B of figure 10, the genotypes with the reporter-line construct driven under R11F10Gal4 and control groups are shown. Flies where Smog was knocked down and carrying a reporter-line construct (AB1lexAlexAop::GFP/51705;R11F10Gal4/+) showed the highest amount of abnormal phenotypes, significantly more ($p<0.001$) than flies where Smog was knocked down as well, but without a reporter-line-construct (51705/+; R11F10Gal4/+) and the control group without a knockdown (51705/+;;) (p=0.0058). However, both experimental groups did not show a significant difference from an additional control group with the genotype AB1lexAlexAop::GFP/CyO; R11F10Gal4/TM6;. This indicates again a potential involvement of the reporter-line construct. The highest number of abnormal phenotypes overall was observed in flies where the Smog knockdown was driven with elavGal4 (elavGal4;AB1lexAlexAop::GFP/51705;;) which is displayed in the graph C of figure 10. This amount was significantly more than in flies without the RNAi construct (elavGal4; AB1lexAlexAop::GFP/CyO) (p=0.0057). Interestingly, half of the flies carrying the elavGal4 driver-line construct but lacking the reporter-line showed abnormal phenotypes, indicating an effect of the Smog reduction on the AB remodelling independent of the reporter-line construct.

When the receptor Smog was knocked down, additionally to the common abnormal phenotypes, two new phenotypes could be observed. In very few brains an innervation of the SA1 and SA2 neurons into the NO could be seen (Figure 11). This phenotype was hereafter called ”innervation into NO”. In some brains, an innervation into the left AB could be detected but these innervations were lacking a Fas2 signal. This abnormal phenotype was called ”Fas2 negative branches”. This new class accounts for about 15% of abnormal phenotypes in flies with the genotypes AB1lexAlexAop::GFP/51705;R11F10 Gal4/+ and 38% in flies with the genotype elavGal4;AB1lexAlexAop::GFP/51705;. Since the neuronal processes in the left AB were lacking a Fas2 signal it raised the question if it could be, that they are not caused by a failure of neuronal retraction but by reinnervation into the left AB again after the remodelling phase.

These experiments could show that the knockdown of the GPCR Smog affects the

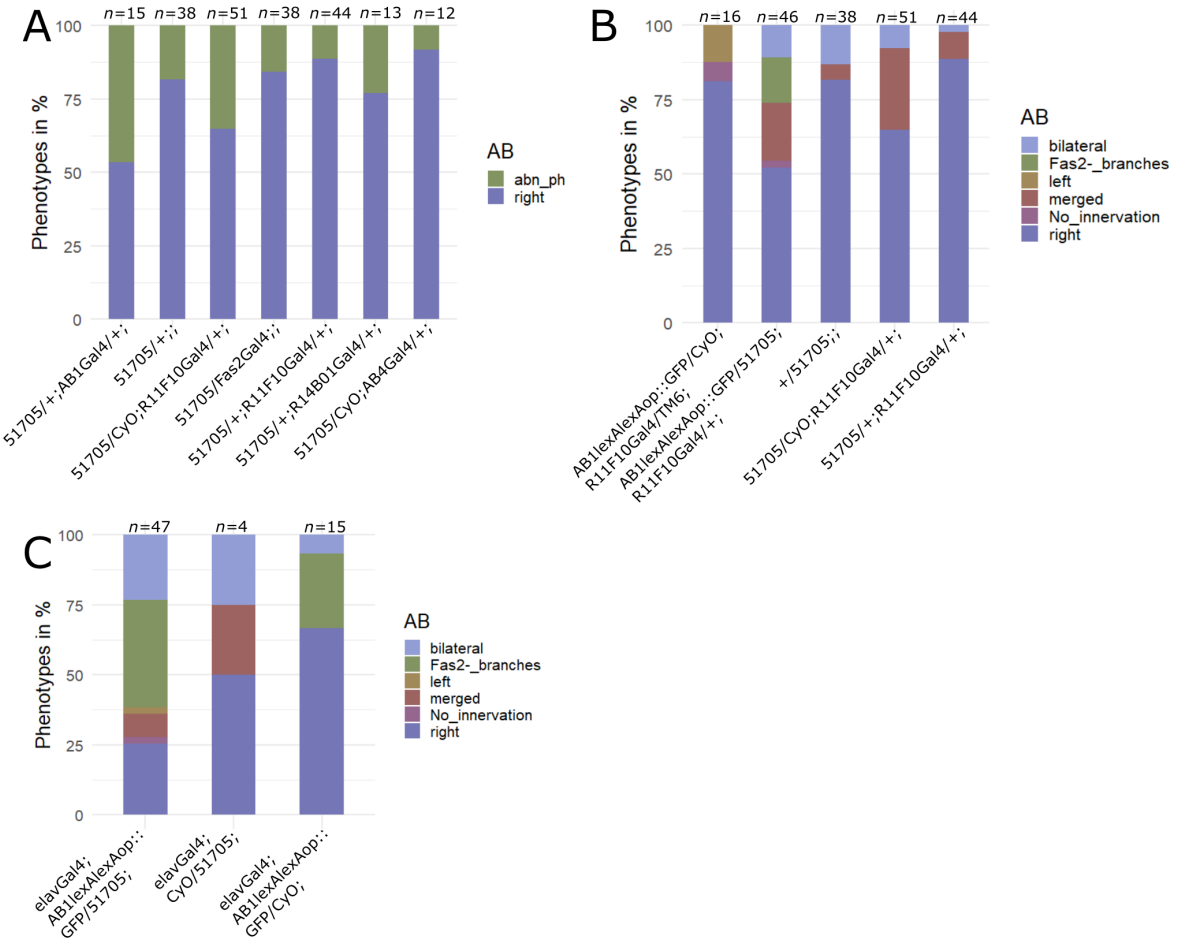

Figure 10. **Knockdown of Smog:** **A** shows the percentages of abnormal phenotypes in the brains where the receptor Smog was knocked down without the reporter-line construct. **B** shows the knockdown of Smog in flies with the reporter-line construct and driven with R11F10Gal4. **C** shows the knockdown driven with elavGal4 in flies carrying the reporter-line.

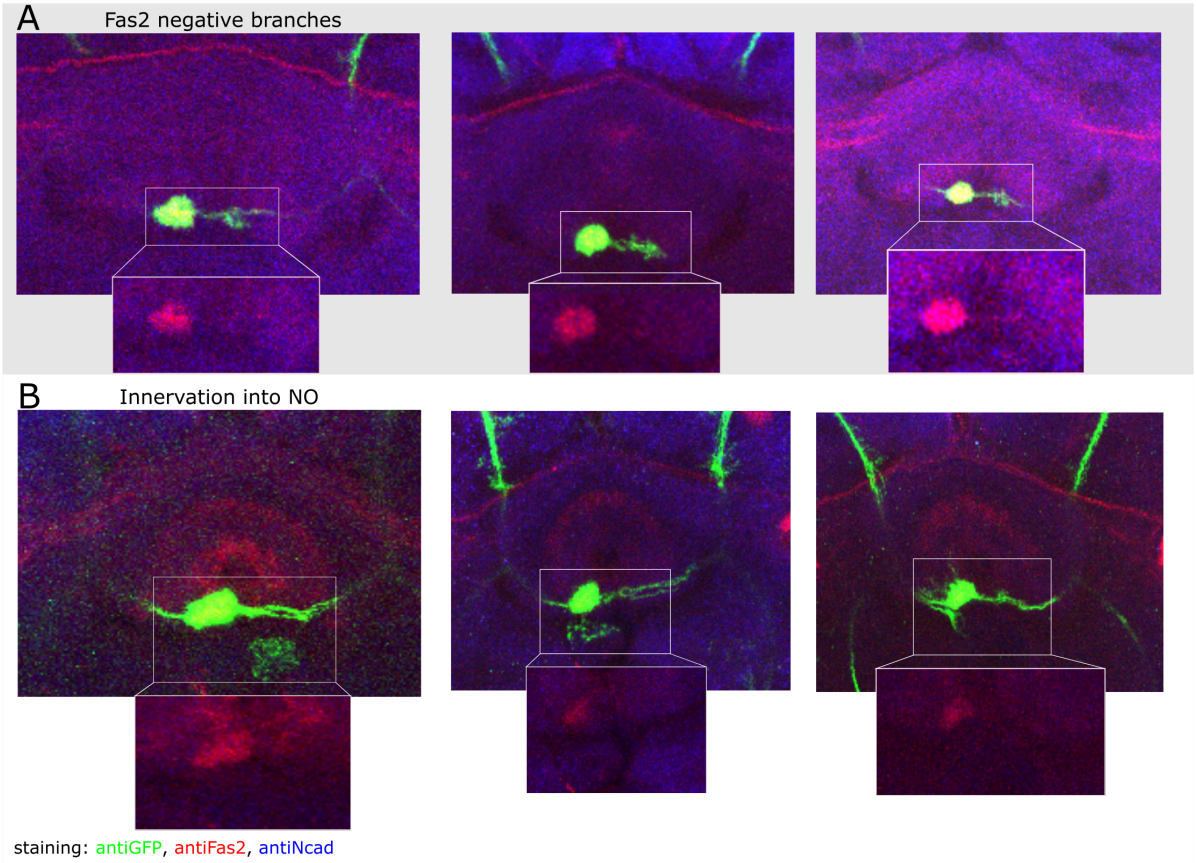

Figure 11. **New phenotypes in Smog knockdown: The confocal pictures** show new classes of abnormal phenotypes detected when the receptor Smog was knocked down. Pictures in row **A** show innervations of the SA1 and SA2 neurons into the left AB but lacking a Fas2 signal, hereafter called "Fas2 negative branches". The pictures in **B** show innervations into the NO, hereafter called "innervation into NO". The brains were stained against GFP (green) to strengthen the signal of the endogenous GFP expression, against Fas2 (red), and Ncad (blue) as reference staining.

remodelling process of the AB. Given that the highest number of abnormal phenotypes could be detected in crosses, using the driver line elavGal4 and AB1Gal4 or R11F10Gal4 indicates, that expression of Smog plays a role in the remodelling process, especially in neurons where the two last enhancer fragments are active. The occurrence of the novel phenotype classes raises the question of how the Fog pathway contributes mechanistically to the AB remodelling.

3.6 Knockdown of Fog

Similar to what was described in the chapter before, systematic knockdown experiments of the ligand Fog were performed (Figure 12). For better overview, the genotypes with and without the reporter-line construct are displayed in separate graphs. Graph A in figure 12 shows the effect of the Fog knockdown in genotypes without the reporter-line construct. The most abnormal phenotypes were observed in flies where the knockdown was driven with the R11F10Gal4 construct, whereas in flies of the driver line AB1Gal4 and AB4Gal4 no brains with abnormal phenotypes could be found. However, none of the experimental groups had significantly more abnormal phenotypes than the control group. Graph B in figure 12 shows the knockdown in groups carrying the reporter line construct and control groups. Here, the biggest effect could be seen in flies with the genotype AB1lexAlexAop::GFP/+;R11F10Gal4/36790; similar to the knockdown of Smog. This group showed significantly more abnormal phenotypes than flies where the knockdown was driven with R11F10Gal4 but without the reporter-line (;R11F10Gal4/36790;) ($p=0.014$), in flies without the knockdown (;R11F10Gal4/+;) ($p<0.001$) and in flies with the genotype AB1lexAlexAop:: GFP;/CyO;R11F10Gal4/+; ($p=0.007$). Also, when Fog was knocked down, the new classes of abnormal phenotypes described in the chapter before could be observed. These Fas2 negative branches appeared predominantly in flies with the genotype AB1lexAlexAop::GFP/+; R11F10Gal4 / 36790; and AB1lexAlexAop::GFP/+;442Gal4/36790;. The effect of the knockdown was stronger in flies carrying the reporter-line which is similar to the knockdown of Smog. This could again indicate a potential enhancement of abnormal phenotypes with this construct. But it must be considered, that in flies without the reporter-line the new abnormal phenotype classes cannot be detected because the abnormal innervations of the SA1 and SA2 neurons lack a Fas2 signal meaning it is not reasonable to directly compare the number of abnormal phenotypes of groups with the reporter-line and without the reporter-line.

To make this situation clearer, genotypes carrying a second reporter-line construct were tested (see appendix). Flies with this particular reporter-line construct, AB4lexAlex Aop::GFP and a knockdown of Fog had significantly more abnormal ABs than flies with the same genotype but lacking the reporter line construct. This leads to the presumption that indeed the reporter-line constructs additional to the knockdown of Fog or Smog have an impact on AB development. To see, whether only a reduction of the Fog pathway affects the AB remodelling, an overexpression experiment was performed. The Fog

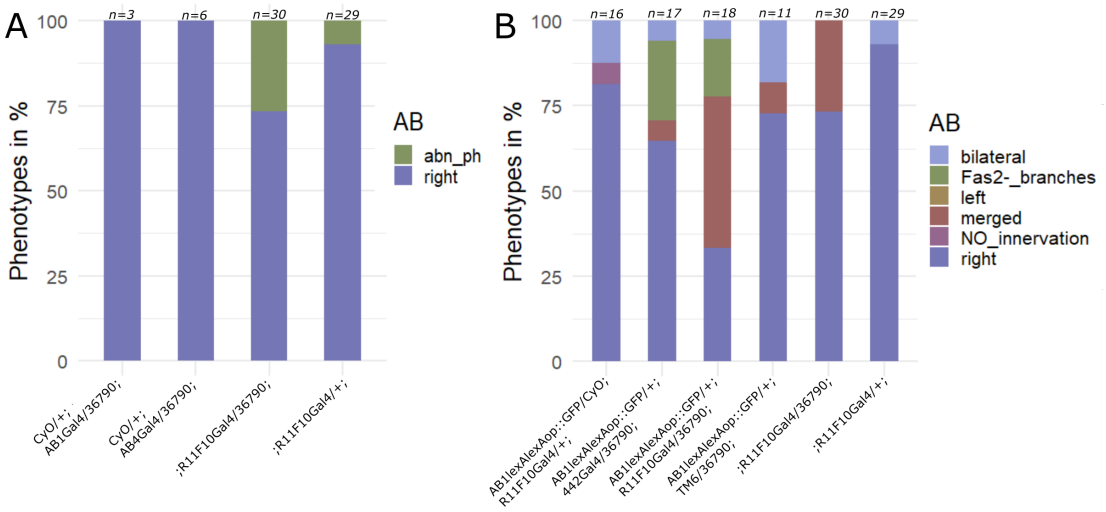

Figure 12. **Knockdown of Fog:** Both graphs show the effect of the knockdown of Fog in different driver- lines. **A** shows a group of genotypes without the AB1lexAlexAop::GFP reporter-line. In green are the percentages of wildtype ABs displayed and light blue indicates abnormal phenotypes. **B** shows genotypes carrying the reporter-line construct and control groups. Here the abnormal phenotypes are divided into the different observed classes. The violet colour stands for the wildtype morphology, red indicates the percentages of merged ABs, ochre depicts left ABs, purple depicts innervations into the NO, green Fas2 negative branches and light blue represents bilateral ABs.

gene is normally located on the X-chromosome, which means males that inherit only one X-chromosome from their mothers exhibit the amount of Fog protein of one allele. For the overexpression experiment flies were dissected that had a duplication of the Fog gene on the Y-chromosome mimicking an overexpression exclusively in males. Females were dissected as a control group. However, there was no significant difference between those groups and the number of abnormal phenotypes remained relatively low in both groups (see appendix).

Comparable to the Smog knockdown experiments described in the previous section, the driver-lines producing the most abnormal phenotypes were again AB1-Gal4 and R11F10-Gal4. This confirms the assumption that these neurons, or at least this lineage, is important for the AB remodelling process. However, similar to the experiments done with Connectin, the situation gains complexity when the impact of the reporter-line constructs is considered. It seems very likely that the reporter-line construct that is used to label the SA1 and SA2 neurons impacts the development of the AB as well, when combined with the simultaneous knockdown of the Fog protein.

3.7 Knockdown of Smog and Fog: developmental aspects

To find out more about the mechanistic role of the Fog pathway in the AB remodelling process, pupal brains were dissected (Figure 13). Three groups consisting of a control group, the Smog knockdown, and the Fog knockdown were dissected 48 hours apf and 72 hours apf. In brains opened 48 hours apf, the Fog knockdown produced the highest number of abnormal phenotypes (Figure 13, A). The phenotype classes in this group consisted mostly of bilateral ABs and merged ABs and some Fas2 negative branches. The percentage of observed abnormal phenotypes was in the group with reduced capacities of Smog the same as in the control group. Although the number of phenotypes was similar, they differed in the frequencies of observed classes. In the control group there was no bilateral AB, only merged, innervation in the noduli and Fas2 negative branches, each a third. In the group with reduced Smog, there was a larger amount of Fas2 negative branches and smaller parts of merged and bilateral ABs.

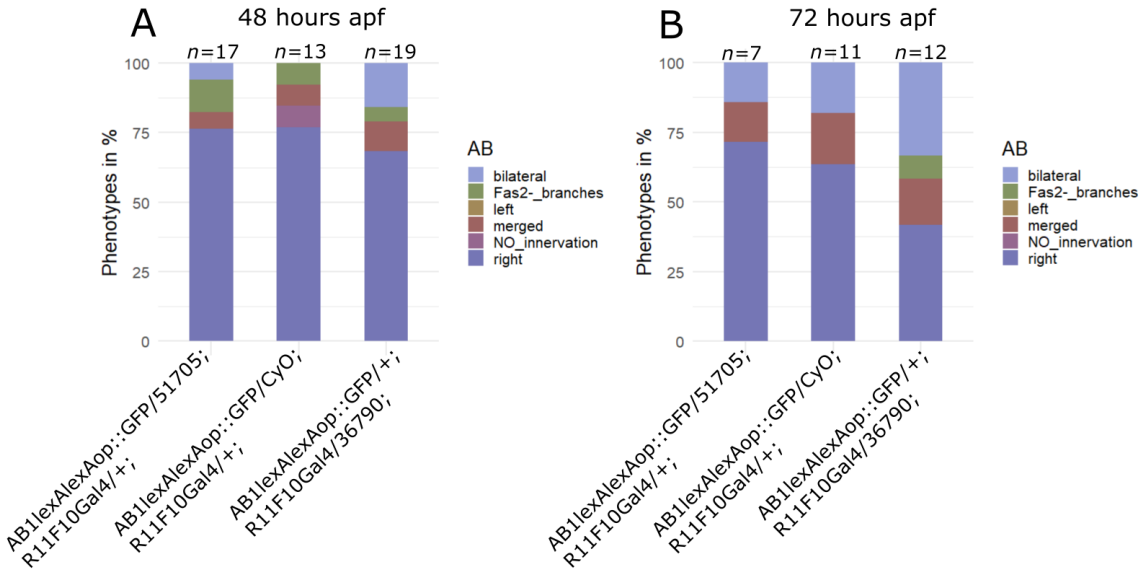

Figure 13. **Knockdown of Smog and Fog: pupal brains: A** shows the percentages of phenotypes when Smog and Fog were reduced via RNAi Knockdown in flies dissected 48 hours apf. **B** shows the effect of the knockdown 72 hours apf. The violet colour represents the wildtype morphology, red indicates the percentages of merged ABs, ochre depicts left ABs, purple depicts innervations into the NO, green Fas2 negative branches and light blue represents bilateral ABs.

Later in development, namely 72 hours apf, the number of abnormal phenotypes

increased in all three groups. Again, the highest number could be observed in the group where Fog was knocked down, with dominating classes being bilateral ABs and merged ABs. Taken together, the abnormal phenotypes in the brains with reduced Fog made up over 50%. The number of abnormal phenotypes in the control group exceeded the group with a knockdown of Smog. In both groups, the control group and the Smog knockdown, the phenotypes were merged and bilateral ABs. It is interesting, that the longer the pupae were exposed to a knockdown of those proteins, the more abnormal phenotypes were produced. The knockdown of the ligand Fog had a more severe effect on the flies compared to the Smog knockdown. None of the performed knockdowns produced a significant difference to the control group, neither when compared within the age-matched groups nor between the age-matched groups. Nevertheless, there is a tendency towards an increase in abnormal phenotypes the longer the pupae had low levels of those proteins. This raises the question of a reduction of those proteins and therefore a lower activity of the Fog pathway leads to instability allowing the SA1 and SA2 neurons to deviate from their normal routes.

4 Discussion

The asymmetric circuit of the SA1 and SA2 neurons in *Drosophila melanogaster* is a fascinating example of brain lateralisation in fruit flies. With the genetic tools and techniques available for *Drosophila melanogaster*, the AB serves as a good opportunity to study brain lateralisation and the contributing proteins as it was done in this thesis. The conducted experiments focussed on Connectin and the Fog signalling pathway and their role in the remodelling of the SA1 and SA2 neurons.

4.1 Interaction of AB1 reporter-line and the LRR protein Connectin

Leucine rich repeat molecules is a term for proteins that have the protein-protein interaction motive, the Leucine Rich Repeat domain, in common. This domain consists of 20 to 30 amino acids and usually, if arranged in multiple repeats, creates a very typical horseshoe shaped structure (Kobe and Deisenhofer, 1993). This curved structure, especially the concave side of the motive fulfills the function of a very efficient protein binding site which mediates cell-to-cell adhesion (De Wit et al., 2011; Ko, 2012). In processes like axon guidance, target formation, synaptogenesis, and homophilic and heterophilic binding of neurons, LRR proteins have been shown to play a particularly important role (Ko, 2012). For instance, in *Drosophila* the LRR protein called Capricious (Caps) was identified in the neuromuscular junctions. It is a transmembrane protein and is thought to mediate target finding in neuromuscular systems, as well as visual systems and olfactory systems. LRR proteins have a critical function in the nervous system which is why in case of malfunction they can compensate for each other, to ensure an undisrupted development of the nervous system (De Wit et al., 2011).

Connectin is a LRR protein that is, similar to Caps, expressed on the surface of neurons. It was previously reported that Connectin is expressed on embryonic muscle tissue and motor neuron growth cones (Nose et al., 1997). It recognizes itself (homophilic cell adhesion) and facilitates target finding in the neuromuscular system of *Drosophila*

(De Wit et al., 2011; Nose et al., 1997). Besides the neuromuscular system, Connectin is expressed in longitudinal and commissural axon pathways in the CNS (Nose et al., 1994).

In the previously mentioned RNAi screen with different LRR proteins, the effect of Connectin, Capricious, Tartan, Pandora, Artichoke and CG5819 on AB development was analysed. A knockdown of those proteins produced high numbers of abnormal phenotypes, which is why they were chosen to be studied further. Particularly a knockdown of Connectin turned out to produce continuously high numbers of abnormal phenotypes after which it was picked out for further experiments (Vokac, 2021). However, the experiments in this thesis that focussed on Connectin produced mixed results. The Connectin null mutants showed high numbers of abnormal phenotypes but only when the flies carried a reporter-line construct. When a Fas2 loss of function allele in flies with Connectin null mutant background was introduced the number of abnormal phenotypes increased even more. To confirm that there was indeed no Connectin expression in those mutant brains they were stained with Connectin antibodies. Given these high numbers of abnormal phenotypes that can be observed only in the combination of non-functioning Connectin and the reporter-line construct suggests (1) that there is a certain sensitivity of the AB remodelling process and (2) that there is a synergistic effect between the presence of the reporter-line construct and the loss of Connectin. Further, it could be the case that the loss of Connectin can be compensated through other LRR proteins since they are known to have a high degree of redundancy (De Wit et al., 2011). If there is an additional ”confounding factor”, the AB development seems to be disturbed. Such a disturbance could be indeed the reporter-line construct as it is built of a GFP tagged to mCD8 proteins of the neuronal membrane. Additionally, previous studies could report a weaker Fas2 expression in Connectin deficient flies (Vokac, 2021) which led to the hypothesis that Connectin expression has a synergistic effect on Fas2 expression. Here, the Fas2 expression in Connectin null mutants was not quantified but still, weaker Fas2 expression could add up to the AB1 reporter-line as an additional stressor.

Interestingly knocking down Connectin via RNAi did not produce such a strong disruption of AB development compared to the Connectin mutants. However, the control group that carried the RNAi construct on the second and the third chromosome but missed a driver construct showed a relatively high number of abnormal phenotypes. A possible explanation for that could be the leakiness of those RNAi constructs, meaning that it is expressed even in the absence of the Gal4 transcription factor. Another explanation could be that there is some additional environmental effect influencing the

development of the AB. Since all of the RNAi crosses were held on 29°C, the most obvious effect would be temperature. Testing and comparing different genotypes between 25°C and 29°C revealed that temperature does not influence the AB remodelling significantly. Taken together it seems that the sole loss of Connectin can be compensated by the organisms, but adding disruptions throws the system off balance resulting in higher amounts of abnormal phenotypes.

4.2 Fog pathway involved in retraction of SA1 and SA2 neurons

Similar to the LRR proteins also the folded gastrulation signalling pathway has been reported to play a role in CNS axon guidance (Ratnaparkhi and Zinn, 2007). With its downstream components the Fog pathway is thought to be involved in axonal midline crossing (Johnson and Van Vactor, 2003). Additionally, in the AB1 reporter-line, the regulatory element of the Smog gene was used, indicating activity of this receptor in these neurons. While the LRR protein approach focusses more on tissue specific interactions the Fog signalling pathway integrates a more direct approach examining the neurons directly innervating the AB.

The first event in multicellular organisms where the Fog pathway becomes important, is gastrulation. During this event, distinct tissue layers, also called germ layers, are formed in the early embryo. This process relies strongly on morphological tissue changes. Each of these layers will differentiate further and give rise to different organs. To form distinct layers, cells undergo irreversible morphological changes, for which four major processes have been defined: emboly, epiboly, convergence and extension (Solnica-Krezel and Sepich, 2012). In embryos of *Drosophila melanogaster* first cells of the ventral furrow (VF) invaginate to build the future mesoderm. Endodermal precursor cells are located more anterior and posterior and invaginate after the VF to form the posterior midgut (PMG) (Sweeton et al., 1991).

Although these two invaginations give rise to different tissues (mesoderm and endoderm) the cellular mechanisms underlying the shape change are similar and require contractile actomyosin networks (Munjal and Lecuit, 2014). In the case of *Drosophilas* gastrulation, the Fog signalling pathway was reported to stepwise activate non muscle MyosinII which leads to constriction of cells that results in the end in irreversible cell shape changes required for invagination (Costa et al., 1994). The signalling pathway is activated by Fog which is a protein that binds to different GPCRs that are called Mist

and Smog. The presence of the ligand Fog induces homoclustering of the receptor Smog on the apical surface of cells. The GPCRs activate the G protein Concertina (Figure 3) which in turn activates RhoGEF2-Rho-RhoKinase leading in the end to MyosinII activation and accumulation (Dawes-Hoang et al., 2005; Jha et al., 2018; Kerridge et al., 2016a).

This signalling pathway is not only essential in early embryogenesis but also plays a role in CNS development. Once gastrulation is completed, Fog does no longer induce apical constriction, however, it is expressed in longitudinal glia and neurons of proximity in the CNS. While the role of the Fog pathway during gastrulation was the subject of extensive studies, the knowledge of its impact on the development of the CNS is sparse. However, it has been shown that reducing the protein Fog in embryonic glia cells causes abnormal axon extensions and defects in glia ensheathment and a knockdown in neurons causes defects in axon guidance. Overexpressing Fog in turn led to ectopic axonal midline crossing as well as ectopic glial midline crossing (Ratnaparkhi and Zinn, 2007; Shweta et al., 2020). Furthermore, the phenotypes seen with Fog overexpression and reduction resemble those seen when reducing a transmembrane tyrosine phosphatase called ptp52F. When eliminating ptp52F in embryos with Fog overexpression, the phenotype could be suppressed which indicates a role of ptp52F downstream of Fog (Bugga et al., 2009; Ratnaparkhi and Zinn, 2007). The involvement of ptp52F is interesting because protein tyrosine phosphatases are crucial for axon guidance and synaptogenesis in *Drosophila* embryos (Johnson and Van Vactor, 2003; Tonks, 2006). Furthermore, ptp52F belongs to the type 3 receptor tyrosine phosphatase (RTP) which has been reported to be especially important in axon guidance across the midline of the CNS of *Drosophila melanogaster* (Johnson and Van Vactor, 2003).

The Fog pathway is studied here in the context of CNS development and in particular in AB development. Knocking down the ligand Fog and the receptor Smog led to an increased number of abnormal phenotypes. The biggest effect could be observed when the knockdown was driven with AB1Gal4 and R11F10Gal4. These driver-lines include neurons of the hemi lineage LALv1A which gives rise to the SA1 and SA2 neurons (Lee et al., 2020) highlighting the importance of these neurons. When the knockdown was performed in flies carrying the reporter-line, again even stronger effects could be observed, similar to the Connectin experiments. Interestingly, new classes of abnormal phenotypes could be detected in addition to the already known left AB, merged AB and bilateral AB. The new phenotype classes consisted of Fas2 negative branches innervating the left AB and innervations into the NO. Compared to innervations into the NO the

Fas2 negative branches made up, with up to 38% in some cases, a relatively high number of all phenotype classes.

Considering these results led to the hypothesis that the axonal retraction of the SA1/2 neurons depends on non-muscle MyosinII activation which is provided by the Fog signalling pathway. Since Fog is thought to act primarily as autocrine signal it can be assumed that Fog, as well as Smog, is expressed directly by the SA1/2 neurons or nearby neurons (Ratnaparkhi and Zinn, 2007). The MyosinII activation and thus retraction of SA1 and SA2 neurons is disrupted upon Fog or Smog knockdown which is why the knockdown experiments resulted in a higher number of abnormal phenotypes. Besides the mere increase of abnormal phenotypes, the issue of the origin of the so-called Fas2 negative branches raised questions.

To investigate the origin of the Fas2 negative branches, a second more speculative hypothesis was formed, namely MyosinII activation could be also important in maintaining the retraction of SA1/2 neurons. In a previous study, it was reported that a knockdown of Smog and Mist via RNAi resulted in MyosinII pulses, but the pulses did not persist as in control groups, which led to a disruption of stabilized cellular apical constriction (Kerridge et al., 2016b). Here, the underlying idea was that the Fas2 negative branches are growing after the actual retraction event. This would a least partly explain why the branches lost their Fas2 signal since the retraction event was already completed and the neuronal programme was set to a right hemispheric Fas2 expression. In contrast to that, the bilateral phenotype is a result of a failed retraction, thus the retraction was never completed and the Fas2 expression remained. Following this idea, it was expected to see higher amounts of Fas2 negative branches in older pupae, because the branches had more time to grow. That was not the case, in fact, the percentage of Fas2 negative branches even declined in pupae that were dissected at a later time point. Nonetheless, pupal brains showed a general tendency towards higher numbers of abnormal phenotypes when they were dissected later in development. This could mean that the initial activation of MyosinII leads to retraction but as it does not persist, the neurons would be able to grow out again and form innervations. These innervations could then build Fas2 negative branches, leading to the ”full” bilateral phenotype that is showing a detectable Fas2 signal or they could also be accountable for a merged phenotype. Given the results, the percentages of merged and bilateral phenotypes increased, the percentage of the Fas2 negative branches declined. The longer a pupal CNS is exposed to lower activation of the Fog pathway the more problems the SA1 and SA2 neurons have to properly retract. It is not likely that the Fas2 negative branches are the sole result of outgrowth after

the retraction event, it seems that they may be also a result of a failure of complete retraction. It remains unclear why exactly those small innervations are Fas2 negative, and how they are different from the bilateral innervations that still show Fas2 signal.

So far only a few studies concentrated on the function of the Fog signalling pathway in CNS development (e.g., Ratnaparkhi and Zinn, 2007; Shweta et al., 2020). These studies reported that the Fog signalling pathway plays an important role in axon guidance and glial morphogenesis. Nevertheless, there is a lack of knowledge about how this signalling pathway functions in CNS context, and how the exact mechanics work. In this thesis it was hypothesized that Fog binds also in the CNS on Smog leading to activation of non-muscle MysoinII. A knockdown of both proteins led to similar phenotypes, mostly ABs that were merged or bilateral or Fas2 negative branches in the area of the left AB, which supported the hypothesis that also in the CNS Smog serves as a receptor for Fog. A study conducted in 2020 proposed a different hypothesis. Here it was reported that an overexpression experiment with Fog resulted in ectopic embryonic axonal midline crossing. When downregulating Smog and simultaneously overexpressing Fog, they expected a suppression of this axonal midline crossing phenotype. Surprisingly the exact opposite was the case, they could observe an increase in axonal midline crossing. They propose that Smog functions as a negative regulator on the Fog pathway and developed a model of inactive Smog isoforms sequestering the Fog ligand and therefore leading to a restriction of the Fog pathway (Shweta et al., 2020). As mentioned above in the experiments conducted for this thesis, such negative regulatory effects could not be observed, which does not exclude a dynamic regulation of the Fog pathway through different isoforms of the Smog receptor. It would rather indicate that in the pupal and adult CX of *Drosophila* active Smog isoforms convey the signal leading to non-muscle MyosinII activation. However, various isoforms of the receptor Smog that differ in their ability to convey the signal that leads to NMII activation could be an interesting model because the retraction of the SA1 and SA2 neurons happens locally restricted. The SA1 neurons retract contralaterally whereas the SA2 neurons retract ipsilaterally which means that the activation of the Fog pathway happens in a site-specific, asymmetric, manner. Different Smog isoforms that are dynamically regulated could facilitate such a locally defined retraction event but there are of course numerous other possibilities how this could be achieved. In any case, the function and dynamic of the Fog pathway in the CNS of *Drosophila* needs to be addressed with further studies and experiments.

4.3 Is the AB1 reporter-line influencing AB remodelling?

The AB1 reporter-line construct is a very comfortable way to visualize the SA1 and SA2 neurons but in almost all performed knockdowns in this thesis, the presence of the reporter-line regardless of the genotypes yielded higher numbers of abnormal phenotypes. Concerning this, some aspects that have to be considered in greater detail are collected here: (1) In 28 brains of the Connectin null mutants, only one abnormal phenotype could be detected, whereas, in 22 brains of the same Connectin null mutants carrying a reporter line, 16 abnormal phenotypes could be observed. Connectin is a cell adhesion molecule expressed on the surface of the neuronal membrane. The GFP, which serves as fluorescent marker in the reporter-line is tagged to the membrane protein mCD8. The possibility of an synergistic interaction between those two membrane bound protein in the AB remodelling process cannot be excluded, but there are further experiments necessary to confirm this. When the same Connectin knockdown experiments were performed with a tmt protein instead of a GFP, the number of abnormal phenotypes decreased significantly, highlighting the specific role of GFP. (2) If an interaction of Connectin and the reporter-line is assumed, it could account for higher numbers of abnormal phenotypes in the Connectin experiments, but it also has to be considered that the number of abnormal phenotypes increased as well when Smog or Fog was knocked down. Although Smog is expressed on the membrane as well it is functionally different compared to Connectin. This means that a concept of a potential interaction between a membrane bound protein like Smog or Connectin and the GFP has to be handled with caution as it would mean that the GFP ”acts” on two functionally different proteins leading to similar results. (3) In the aspects above it is stated that with the reporter-line construct higher numbers of abnormal phenotypes are reached. In the Smog/Fog knockdown experiments also new classes of phenotypes were detected. It has to be pointed out that if the new classes of phenotypes (Fas2 negative branches and innervations into the NO) are taken into account, they are only detectable in brains carrying the reporter-line. This means that the number of abnormal phenotypes in brains without the reporter-line and with reporter-line cannot be directly compared, which makes it hard to assess the influence of the reporter-line construct.

To conclude briefly: (1) There might be an interaction of Connectin and the membrane tagged GFP reporter protein; (2) If there is an interaction it also influences the Fog pathway because there was also an increase of abnormal phenotypes when the reporter-

line was involved; (3) Brains with and without the reporter-line cannot be compared directly, since some of the abnormal phenotypes can only be detected when the reporter-line is present.

It was remarkable that either way (with or without the reporter-line) the driver-lines R11F10Gal4 and AB1Gal4 in the Smog knockdown experiment and R11F10Gal4 in the Fog knockdown produced the highest number of abnormal phenotypes. This indicates that this neuronal lineage that gives rise to the SA1 and SA2 neurons is involved in the Fog pathway and the AB remodelling.

4.4 Symmetry breaking of bilateral symmetric structures

Looking at brain lateralisations of other organisms may help to understand the AB and its asymmetry better. For instance in the epithalamus of the zebrafish *Danio rerio* some structures are reminiscent of the AB, the parapineal gland, and the habenular nuclei. The pineal gland lies in the midline of the fish whereas the parapineal gland differentiates in 95% of the fish to the left side. The habenular nuclei, bilateral structures in each hemisphere that are also part of the epithalamus of the fish, exhibit an asymmetry as well. The left habenular nucleus which is also densely connected with the parapineal gland is 20% bigger than the right habenular nucleus. Some features seem to be similar to the AB, for instance, there is also a bilateral symmetric ground state out of which asymmetries develop. Nevertheless, the mechanism seems to be different, in the zebrafish, neurons migrate to form the parapineal gland, but in the AB the SA1 and SA2 neurons retract previously formed connections. Furthermore, in *Danio rerio* it could be shown that the nodal signalling is involved in the migration of the parapineal gland to the left side (Corey D. Snelson and Joshua T. Gamse, 2009), which does not play a role in *Drosophila*. However, these two asymmetric circuits have in common, that they do not lead to directly lateralized behaviour. Instead, the habenular circuit in the zebrafish is thought to play a role in the fear response (escaping or freezing) (Ichijo et al., 2017). In *Drosophila*, the only reported function of the AB we know about is the role in long term memory (Pascual et al., 2004b). It is still unclear if disturbed long-term memory is the only behavioural implication that can be assessed when flies exhibit a bilateral innervated AB. To clear this up further behavioural experiments are necessary. Despite having only a small number of neurons, another neuronal asymmetry lies in the nematode *Caenorhabditis elegans*. The gustatory ASEL (on the left side) and ASER

(on the right side) neurons, are symmetric in their morphology but exhibit asymmetry in the expression of some chemoreceptors which leads consequently to a functional asymmetry. Differently expressed receptors lead to differentiation into ASEL whereas expression of other receptors leads to ASER neurons. With those different neurons, worms can distinguish different chemosensory inputs (Poole and Hobert, 2006). The asymmetric structures mentioned here are an interesting example of how diverse asymmetric features can be. Lateralisation in brains is not always detectable at first glance and goes far beyond morphological traits. The asymmetric expression of the chemoreceptors of *C. elegans* show that asymmetric features can define neuronal fate. It is now important to find out if there could be a similar asymmetric expression in *Drosophila melanogaster*, for instance, of the Smog receptor in the SA1 and SA2 neurons. The next important steps which are not in the slightest small steps are, to find out where the lateralisation of the AB circuitry starts.

4.5 Conclusion

Taken together, the remodelling of the SA1 and SA2 neurons out of the area of the left AB is a very complex process, which is far from being solved. However, it was already shown that Connectin patterns the CX of *Drosophila* during development in a very distinct manner. The columnar neurons expressing Connectin are also involved in the AB formation (Vokac, 2021). However, it seems that a mere loss of Connectin, can be compensated through other proteins, eventually other LRR or at least cell adhesion molecules. Adding a confounding factor to flies already experiencing a loss of Connectin resulted in an increase of abnormal phenotypes. Such an increase in abnormal AB phenotypes could be observed as well when Fog or Smog was knocked down. The knockdown of these proteins leads to a decreased non-muscle MyosinII activation (Kerridge et al., 2016a). This leads to the assumption that the AB remodelling process depends at least partly on non-muscle MyosinII activation. The experiments conducted for this thesis are more of descriptive nature while the even more complicated topic of the underlying mechanisms stays left open. Nevertheless, this thesis could hopefully make a small contribution to unraveling the mystery of the AB.

5 Outlook

Connectin and the Fog pathway play a role in the remodelling process of the SA1 and SA2 neurons, but a lot of aspects are still unclear, and it can only be speculated about the underlying mechanisms. Therefore, further experiments are necessary to firstly confirm the results presented here and secondly to collect new insights on the AB development and the asymmetric innervation of the AB neurons.

5.1 Connectin

To evaluate whether the low numbers of phenotypes in the Connectin null mutants is an effect of the ability of LRR proteins to compensate for each other it could be interesting to knock out simultaneously multiple LRR proteins. This has been already tackled, with highly significant results, when a knockdown of Capricious and Pandora was combined and Capricious and Connectin, indicating a crucial role of Capricious and Connectin (Vokac, 2021). Given that a knockdown is only reducing the amount of protein expressed and not completely removing it, it would be interesting to see if a knockdown of Capricious in Connectin null mutant background yields a high number of abnormal phenotypes as well.

5.2 Fog pathway

To target the Fog pathway, the next interesting steps could be to find out where exactly the receptor Smog is expressed. Given the results collected in this thesis and the hypothesis that the remodelling process is depending on non-muscle MyoII activation in the SA1 and SA2 neurons, it would be interesting if the Smog receptor is indeed expressed in these innervations that experience the retraction. This would implicate an asymmetric expression pattern of Smog, although, if there is no such asymmetric distribution of Smog, it would not be in conflict with the stated hypothesis, since a unilateral mode of action can be achieved otherwise as well, for instance with different Smog isoforms, as

mentioned above. Nevertheless, it could be very interesting to assess the distribution and dynamics of Smog in the adult fly as well as during development. Equally interesting is the question of where and when the ligand Fog is expressed, to get a grasp of how the Fog pathway functions in the CNS of *Drosophila*. For this purpose, a convenient way would be to use antibodies which was already done in fly embryos Kerridge et al., 2016a. Kerridge and colleagues also used anti-Mist antibodies which are staining the second GPCR that binds Fog. If it is assumed that Mist also binds Fog in CNS context it would be interesting to perform a combinatorial knockdown of Mist and Smog to see if it results in even higher numbers of abnormal phenotypes. However, it was already reported that Mist does not play a role in the activation of the Fog pathway in the CNS context in embryonic stages of *Drosophila* (Shweta et al., 2020). Further players of the Fog pathway which are holding in my opinion great potential are Concertina, the Gprotein that acts downstream of Fog, and non-muscle MyosinII. To test those proteins for their role in the AB remodelling process knockdown experiments could be performed and perhaps confirm the results presented here.

5.3 Additional ideas

Given the influence of the AB1 reporter-line on the remodelling process, one obvious and important step is to target this problem. In my opinion, one way to do so is to test the AB1 reporter-line and all sub-constructs again with higher sample size. Additionally, different AB1 reporter-lines could be introduced. Although in the experiments mentioned above, temperature had no significant influence on AB development it would be interesting to assess other possible environmental factors that could have an influence, for instance, food quality.

Additionally, very interesting further steps could focus on the behavioural implications of the asymmetric innervation of the AB. The only study that investigated possible behavioural implications of the AB circuitry, that I am aware of, reported that flies exhibiting a bilateral Fas2 signal in the area of the AB are lacking long term memory (Pascual et al., 2004a). For a better understanding of functional aspects of the AB it would be necessary to conduct experiments that test the hypothesis of the AB circuit being involved in memory formation.

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Appendices

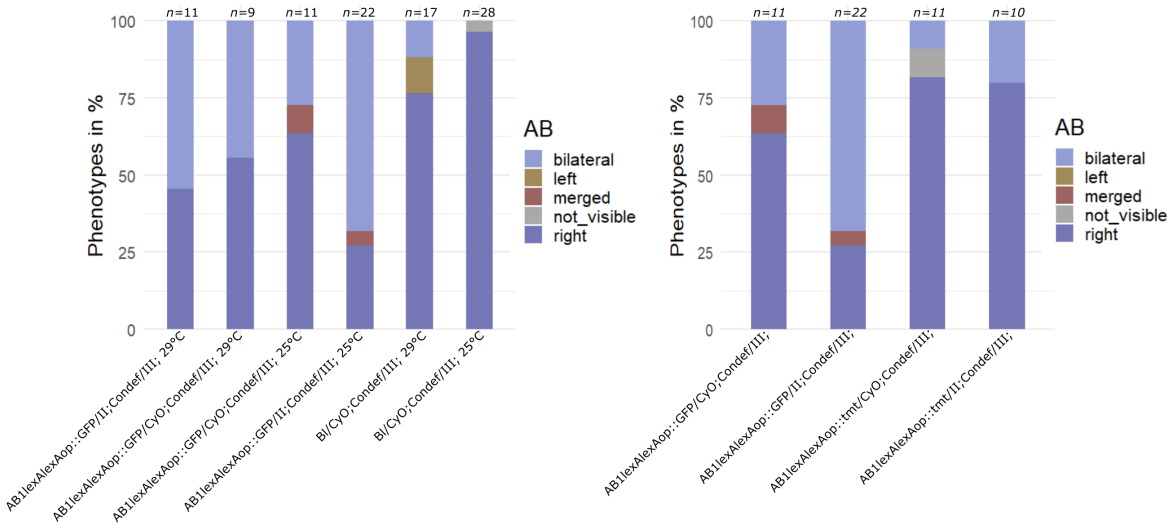

Figure 14. **Additional Connectin mutants: The left graph** shows the % of abnormal phenotypes in Connectin mutant brains raised at 29°C and control groups. None of the genotypes showed a significant difference to the corresponding genotype raised at 29 °C. **The right graph** shows the percentages of abnormal phenotypes in flies carrying a different reporter-line construct (AB1lexAlexAop::tmt) than the one usually used (AB1lexAlexAop::GFP). Brains of flies with the genotype AB1lexAlexAop::tmt/II;Condef/III; had significantly less abnormal phenotypes than flies with the same genotype but carrying instead of the tmt a GFP reporter protein (Fisher's Exact test, p=0.0084), all other groups were not significantly different.

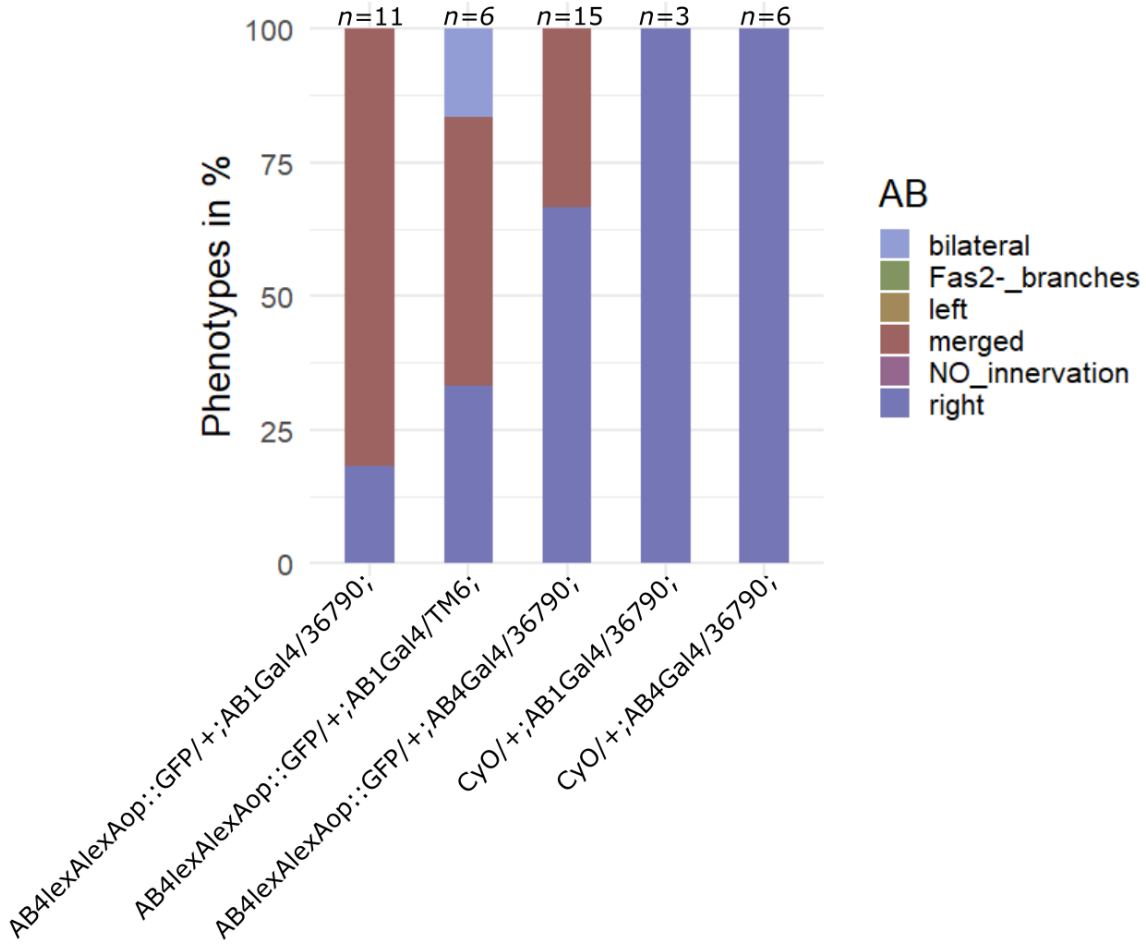

Figure 15. **Knockdown of Fog with additional reporter line:** The x axis shows the % of abnormal phenotypes in the brains where the ligand Fog was knocked down with the AB4lexAlexAop::GFP reporter line and control groups. Flies with the genotype AB4lexAlexAop::GFP/+;AB1Gal4/36790; had significantly more abnormal phenotypes than flies lacking the reporter line (CyO/+;AB1Gal4/36790). Interestingly in this group the abnormal phenotypes observed were exclusively merged ABs.

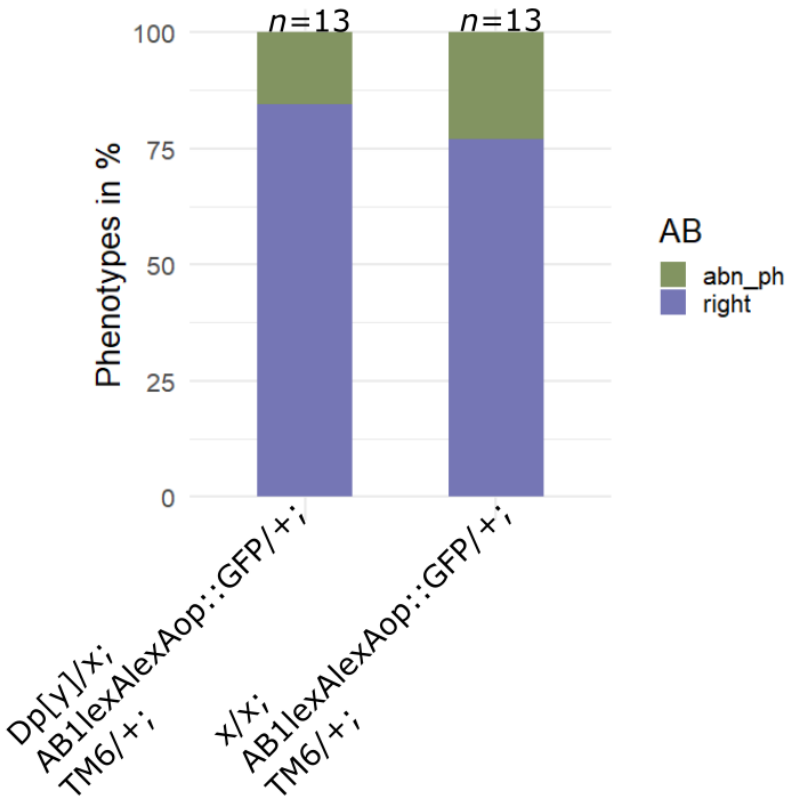

Figure 16. **Overexpression of Fog:** The x axis shows the % of abnormal phenotypes in the brains where the ligand Fog was overexpressed. Males had a duplication of the Fog gene on the Y-chromosome which mimics an overexpression, since the Fog gene is normally located on the X-chromosome. The flies carried additionally a AB1lexAlexAop::GFP reporter line. There was no significant difference between males and females.