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"The function of Netrin signaling in Drosophila brain lateralization"

verfasst von | submitted by

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Abstract

Despite the overall bilateral symmetric organization of animal nervous systems, differences in brain regions and neuronal activity have been described between the left and right hemisphere. In the Central Complex of *Drosophila melanogaster* there are bilateral neuropils called the Asymmetric Body (AB) that first develops symmetrically, but later becomes lateralized via axonal remodeling and remain so in the adult fly brain. Previous studies have shown that the conserved Netrin receptor UNC5 plays a critical role in the remodeling process, but the cellular context of Netrin release could not be identified. The aim of this study the aim was to pinpoint the developmental source of the Netrin signal by targeted RNAi using a collection of Gal4-lines for different AB neuron classes. A combination of tools for the asymmetric SA^{uni}-neuron class including a transgenic reporter line and the molecular marker Fasciclin 2 was used to measure the effect on AB lateralization. While no change in AB asymmetry could be observed following NetrinB knock-down in SA^{uni}-neuron itself, a reduction of NetrinB in 2 classes if bilateral AB afferent neurons (SA^{bi} and FB1B) phenocopies the loss of UNC5 in SA^{uni}. As targeted knockdown of UNC5 in SA^{bi} and SA^{FB} did not show any effect on SA^{uni} remodeling, these results suggest a directed Netrin signaling among lineage-related AB afferents, in which axons of early-projecting bilateral neurons release a repulsive signal for later-arriving SA^{uni} neurons to trigger a lateralized remodeling process.

Abstrakt

Tierische Nervensysteme sind bilateral symmetrisch organisiert. Jedoch wurden Unterschiede in Gehirnregionen und neuronaler Aktivität zwischen der linken und rechten Hemisphäre beschrieben. Im Zentralen Komplex von *Drosophila melanogaster* befinden sich bilaterale Neuropile, die als „Asymmetrischer Körper“ (Asymmetric Body, AB) bezeichnet werden. Diese entwickeln sich zunächst symmetrisch, erfahren jedoch später durch axonales Remodelling eine Lateralisation und verbleiben in dieser Form im Gehirn der adulten Fliege. Frühere Studien haben gezeigt, dass der konservierte Netrin-Rezeptor UNC5 eine zentrale Rolle in diesem Remodelling-Prozess spielt. Der zelluläre Ursprung des Netrin-Signals konnte jedoch bislang nicht bestimmt werden. Ziel der vorliegenden Studie war es daher, die entwicklungsbedingte Quelle des Netrin-Signals zu identifizieren, und zwar mithilfe gezielter RNA-Interferenz unter Verwendung einer Sammlung von Gal4-Linien für verschiedene AB-Neuronentypen. Für die asymmetrische SA^{uni}-Neuronenklasse wurde eine Kombination aus einer transgenen Reporterlinie und dem molekularen Marker Fasciclin 2 verwendet, um den Effekt auf die AB-Lateralisierung zu analysieren. Während ein Knockdown von NetrinB in SA^{uni}-Neuronen selbst keine Veränderung der AB-Asymmetrie zeigte, führte die Reduktion von NetrinB in zwei bilateralen afferenten Neuronentypen des AB (SA^{bi} und FB1B) zu einem ähnlichen Phänotyp wie der Verlust von UNC5 in SA^{uni}. Da ein gezielter Knockdown von UNC5 in SA^{bi}- und SA^{FB}-Neuronen keinen Einfluss auf das Remodelling der SA^{uni}-Neuronen hatte, deuten die Ergebnisse auf ein gerichtetes Netrin-Signal unter abstammungsverwandten afferenten AB-Neuronen hin. Dabei setzen Axone früh projizierender bilateraler Neuronen ein abstoßendes Signal frei, das bei später eintreffenden SA^{uni}-Neuronen ein lateralisiertes axonales Remodelling auslöst.

Table of Contents

1. Introduction

In animals with bilateral symmetry, such as humans, having a body that's the same on both sides helps with survival (Toxvaerd, S., 2021). Darwinian natural selection plays a vital role in preserving the stability of bilateral symmetry in animals. Having organs such as ears, eyes, and limbs positioned symmetrically in relation to the symmetry plane offers advantages across various contexts (Toxvaerd, S., 2021). But there are numerous examples of asymmetric morphology in animals, which have been established through genes, environment, and chance (Toxvaerd, S., 2021). One fascinating example of an asymmetric development are the flatfish (Pleuronectiformes), which are the most asymmetrically shaped and behaviourally lateralized of all the vertebrates (Schreiber, A. M., 2013). These fish undergo a remarkable transformation during their development where one eye migrates to the opposite side of the head, resulting in both eyes being located on the same side (Friedman, M., 2008). This adaptation allows flatfish to effectively camouflage themselves on the ocean floor. The asymmetric development of their eyes is a great example of how animals can undergo morphological changes during their development to better suit their environment (Friedman, M., 2008).

The asymmetric development is not limited only to body structure. Differences in how our brains are organized and in behaviours such as hand preference add variety and specialize our skills and tasks (Corballis, M. C., 2020). In *C. elegans* sensory neurons (AWC) a development of left- right differences in gene expression occurs. Asymmetric calcium signalling is established through axonal contact, ultimately leading to the expression of an unidentified transcription factor (Hobert et al., 2002). There are also a pair of gustatory neurons called ASE. Like the AWC odour-sensory class, the ASE neuron class consists of two bilaterally symmetric cells, called ASEL (ASE left) and ASER (ASE right) which exhibit a non-autonomous development of L/R differential gene

expression subsequent to the establishment of symmetrical organization (Goldsmith et al., 2010; Troemel et al., 1999).

In zebrafish asymmetric nodal signalling induces left-sided migration of the parapineal organ, which is s a midline-derived epithalamic structure, thereby inducing further asymmetries (Gamse et al., 2003). Although asymmetry is also established on an initially symmetric organization in this context, the mechanism for establishing asymmetry in the zebrafish diencephalon appears markedly different, where asymmetric innervation is achieved through neural migration.

In humans there are also asymmetries in brain lateralization. Eighty-five percent of people have left-hemisphere language dominance, according to functional magnetic resonance imaging (fMRI) (Kong, X. Z et al., 2022). Asymmetries on a cortical level are also crucial for a healthy functioning brain and when changes occur and symmetry does not break during development, it can lead to various psychiatric diseases such as dyslexia, schizophrenia and autism (Wang, B. et al., 2023; Ribolsi, M., Daskalakis, Z. J., Siracusano, A., & Koch, G., 2014; Oertel-Knöchel, V., & Linden, D. E., 2011.). In some diseases like schizophrenia studies have shown that individuals with schizophrenia exhibit decreased efficiency in structural brain networks, particularly in the left hemisphere, possibly due to genetic risk factors (Sun Y., et al., 2017). In other diseases like Alzheimer the brain network connections have slowly been disrupted most likely due to the degenerative nature of the disease and have become more asymmetrical (Daianu M., et al., 2013). These interesting insights show that abnormal amount of both asymmetry and symmetry in the brain can cause (or can be caused) by neuropathological developments and thus research in the topic of brain symmetry and asymmetry is an important one.

The asymmetry of the nervous system is widespread among bilaterians. It appears in different forms such as overall morphology, neuron quantity or activity, connectivity, and gene

expression (Salam et al., 2021; Yu et al., 2022). During development the posterior-anterior axis formation in vertebrates is a crucial period in which both left and right body sides split almost perfectly identical (Mongera, A., et al., 2019), but in the finished and fully-developed individual asymmetries are important for normal functioning. According to Concha et al. (2012) left and right asymmetries of the nervous system can be divided into two main classes: asymmetric circuits with common components, where circuits on the left and right contain similar types of neurons and patterns of connectivity and unilateral circuit components. Examples of this type of asymmetry are the male fiddler crabs, where the first thoracic ganglion adjacent to the major claw is bigger. Additionally, the enlarged nerve root accommodates a greater number of sensory axons compared to the contralateral nerve root, which serves the minor claw. In humans, there is a left–right discrepancy in the size of the arcuate fasciculus, a significant association tract linking perisylvian regions of the frontal, parietal, and temporal lobes. The tract on the left side connects the language centres of Broca and Wernicke, and undergoes asymmetric enlargement in correlation with left hemispheric dominance for language (Concha et al., 2012).

The second class of asymmetry represents a unilateral asymmetry, in which a particular circuit component is present on only one side (Concha et al., 2012. Such is for example the asymmetric body (AB) in the Central Complex in Drosophila (see Figure 1.1 and 1.2), which first develops on one side and then later during pupation retracts on the left side and remain in 95% of wild type flies only on the right side (Wolf & Rubin 2018; Janett & Rubin, 2012; Pascual 2004). The Central Complex is compromised by a group of neuropils which are involved in sensory integration, memory and the generation of different locomotory behaviours (Wollf & Rubin 2018). The AB was discovered by staining the cell adhesion molecule FasII. Here a directional asymmetry at the

level of protein localization was found, with only the right neuropil of the AB expressing FasII

(Pascual et al., 2004). The AB has a cluster of about 10 bilateral SA^{uni} neurons per hemisphere which are characterized by an asymmetric innervation pattern (see Figure 1.1), where neurons from both hemispheres innervate only the right AB neuropil. SA^{uni} neurons developed symmetrically by innervating both sides of the central complex and later in pupa stage retract and remain only on the right hemisphere of the fly brain (Markovitsch, 2019) (see Figure 2.2).

One significant finding in axon guidance research revealed that growth of axons, influenced by a previously unidentified extracellular cue, exhibited a directional bias toward the source of the cue (Serafini et al., 1994). Through the isolation of key factors from the chick brain, known to facilitate axon growth in embryonic rat spinal cord explants, researchers identified two proteins akin to the

C. elegans UNC-6 gene, which are crucial for axon guidance (Hedgecock et al., 1990). These proteins are called Netrin-1 and Netrin-2. The names come from the Sanskrit term "netr," signifying "one who guides" (Serafini et al., 1994). Netrins function as extracellular proteins and guide cell and axon migration during embryogenesis, acting as attractants or repellents (Duncan et al., 2021). They regulate synaptic interactions through binding to transmembrane receptors, primarily in axon guidance during nervous system development. Netrins also influence cell adhesion, cell morphology maturation, cell survival, and tumorigenesis (Rajasekharan et al., 2009; Duncan et al., 2021).

In Drosophila there is a similar repulsive receptor - the UNC-5 receptor, which is localized in the axons and act as a guide during axon movement in the developmental stage. They also act as a signal for retraction, meaning the already established axon can be remodelled when UNC-5 signal is being sent through the Netrin pathway (Boyer & Gupton et al., 2018). The attraction receptor Frazzled (Fra) plays a role in growth cone attachment in the Drosophila visual system, potentially through adhesion to substrate-bound Netrin (Akin & Zipursky et al., 2016). According to Boyer &

Gupton (2018) both of the receptors act as heterodimers and suggest that the Netrin-1 receptors DCC and UNC5 can act together to mediate both attraction and repulsion in axon guidance, and that disruption of one receptor can affect the function of the other, meaning that UNC-5 deletion or loss of function can cause a disruption of the Frazzled receptor.

Aim of study

A recent publication by Lapraz et al. (2023) showed that the conserved Netrin-B pathway is critical for the AB asymmetry development. According to their findings the expression of the repulsion receptor - the UNC-5 receptor - in bilateral SA^{uni}-neurons induces their asymmetric retraction. The knockdown of the attraction receptor Frazzled also had an effect and its knockdown produced a bilateral phenotype. Lapraz (2023) also found that Netrin-B on the right side is needed for symmetry breaking, but not on the left side. A main critique on the study is that it lacks sufficient description of the exact source of the Netrin-B signaling. This aspect of Netrin signalling in AB development seems critical to understand its role in lateralized axon retraction.

Here I am aiming to determine in which cells of the developing AB neuropil the expression of Netrin is required to trigger UNC5-mediated retraction of SA^{uni} axons (see Figure 2.1). I used these neurons from the lineage in order to see, if these neurons have an effect on the SA^{uni} development. In my Master project I seek to localize the function of Netrin within the LALV1-lineage neurons. Using a collection of transgenic lines, which allow the neuron-type specific knock down of NetrinB or the candidate receptors Frazzled or UNC-5 I will determine the effects on AB innervation with the help of two molecular markers, a transgenic SA^{uni} reporter and the SA^{uni}- specific expression of the cell adhesion molecule Fasciclin-II, in order to observe changes in bilateral symmetry.

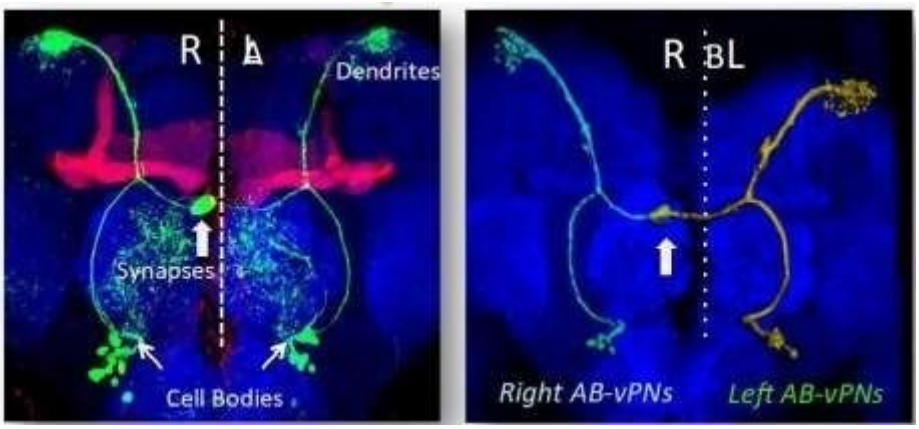


Figure 1.1. Unilateral SA^{uni} neuron (in green, antibody used for staining is anti-GFP rabbit, NCAD and FasII staining) in an adult fly brain.
Asymmetry of bilateral SA^{uni} neurons which display unilateral innervation in the AB on the right hemisphere. Left picture shows a 3D model.

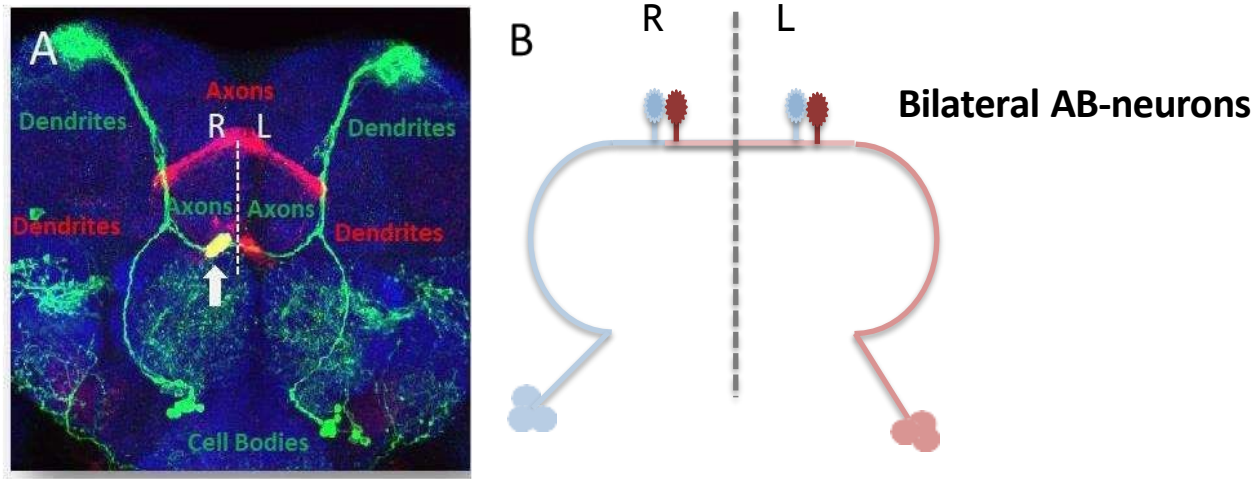


Figure 1.2. Organization and Development of theAB Circuit. **A:**
Class II asymmetry of bilateral SA^{uni} neurons unilaterally innervating postsynaptic FB-AB neurons, which are class I asymmetry, showing a bigger innervation on the right than on the left side (adapted from Markowitsch (2019)). **B:** Model illustrating the bilateral AB in adult flies; in wild type it is only found in 5% of individuals and in the current experimental studies this is how it looks when there is a bilateral phenotype. Compared, the axons of left AB-neurons innervate only on one side of the hemisphere, neurons from the right hemisphere cross the midline to innervate contralaterally.

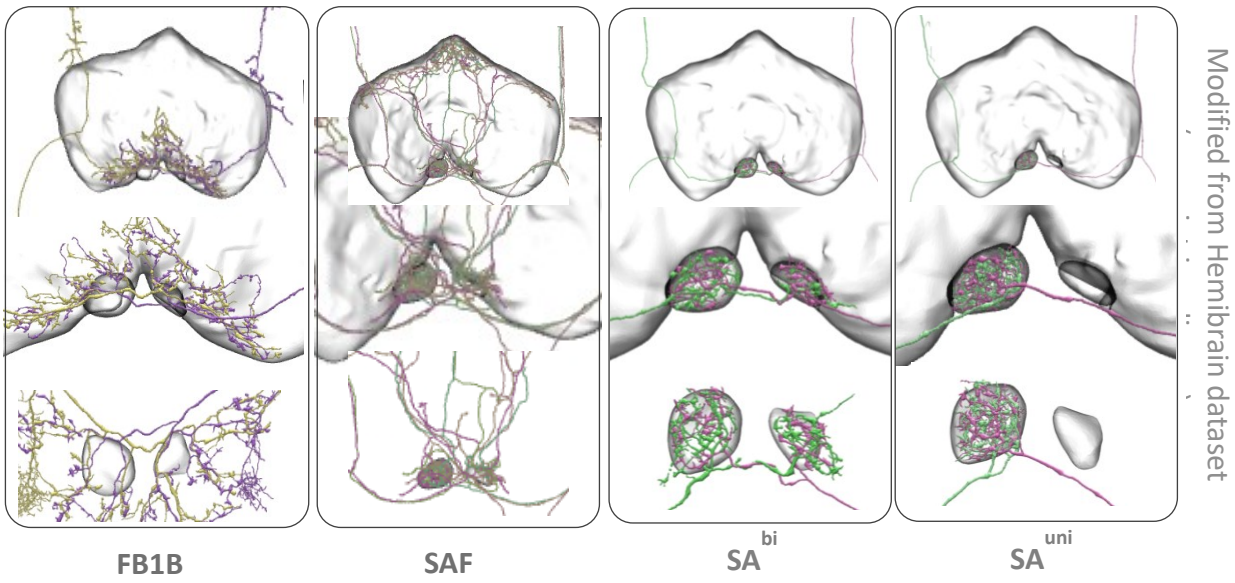


Figure 2.1. The four key neurons innervating in the Asymmetrical Body (AB).
From left to right: FB1B-neurons which innervate in the Fan-shaped body (FB) and the AB; have a dynamic development and are used in the current study to research cell-nonautonomous effect; SAF which also innervate in both ABs; SA^{bi} which develop almost simultaneously with the SA^{uni} and remain bilateral after pupal stage; SA^{un}i which are the only unilateral neurons and remain asymmetric.
For all of these neurons we used a corresponding Gal4-line to study the knockdown effect.

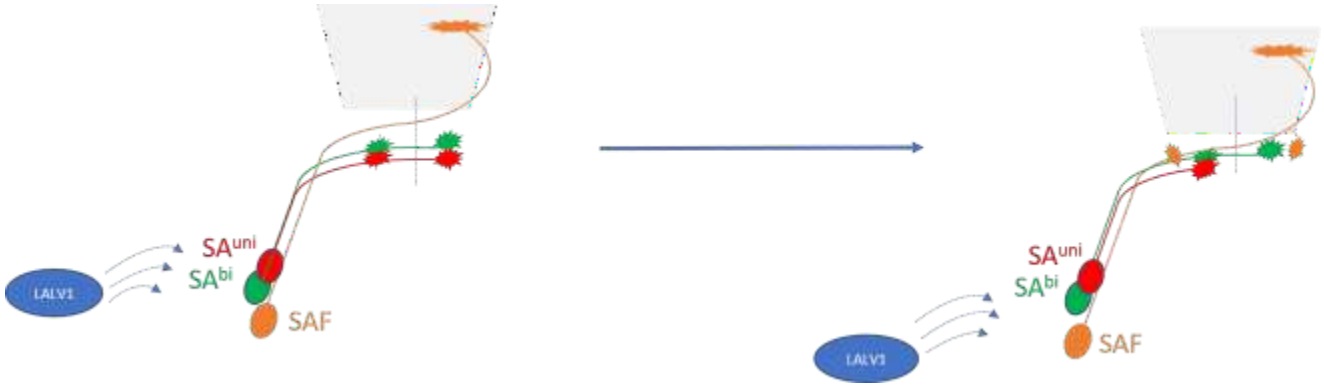


Figure 2.2. Development of AB afferent neurons in Drosophila according to Lapraz et al. (2023).
LALV1-lineage neurons which start to develop at almost the same time in the brain. All three neuron classes initially innervate in the AB neuropil in a bilateral fashion. SA^{bi} neurons remain in a bilateral state, whereas the SA^{uni} reorganize their axon processes. SA^{uni} neurons are the only ones from the lineage that go through a remodeling process and after developing on both sides retract from the left side and remain only on the right side.

2. Methods

2.1 Fly stocks and genetics

Flies were raised on standard fly food at 25°C. RNAi and overexpression experiments were conducted at 18°C and 29°C. For the knockdown and overexpression experiments, the following strains were used: R72A10-Gal4, R66A02-Gal4, R16E08-Gal4, VT019728-Gal4.DBP, UAS- UNC-5-RNAi, UAS-NetB-RNAi, UAS-Fra-RNAi.

Table 1. List of Fly Lines

Genotype	
R72A10-Gal4	Bloomington Drosophila Stock Center
R66A02-Gal4	
R16E08-Gal4	
VT019728 -Gal4.DBP	
10x lexAop mCD8::GFP	
UAS-UNC5-RNAi	
UAS-Fra-RNAi	
UAS-NetB-RNAi	Vienna Drosophila Resource Center

2.2 Immunohistochemistry

After dissection in PBS (phosphate-buffered saline), brains were fixed in 2% PFA (paraformaldehyde) in PBS for 1h. After washing the samples (4x15m) in 0.3% PBT (PBS containing 0.3% TirtonX-100) the brains were blocked with 10% Goat Serum in PBT. After adding the primary antibodies, samples were incubated overnight at 4°C. Subsequently, the brains were washed again in PBT and the secondary antibody (diluted in Goat Serum) was added followed by overnight incubation at 4°C and washing in PBT. For mounting whole brains were placed in VECTASHIELD (Vector Laboratories).

Table 2. List of Antibodies used for Staining.

Type	Antibody	Origin	Source
Primary	α-NCadherin (Ncad)	Rat	DSHB
Primary	α-Fasciclin II (FasII)	Mouse	DSHB
Primary	α-GFP	Rabbit	DSHB
Secondary	α-Rat Alexa 568	Goat	Invitrogen
Secondary	α-Mouse Alexa 647	Goat	Invitrogen
Secondary	α-Rabbit Alexa 488	Goat	Invitrogen

2.3 Imaging and processing

Brains were imaged using a Leica Confocal Microscope TCS SP5II and scanning was performed with a resolution of 512 x 512 pixels (400 Hz) employing a space of 1.5µm between the single optical sections. Processing of the acquired images was done with the image processing program ImageJ®. Statistical analysis was conducted using Python.

3. Results

3.1 Knockdown of UNC-5 in SA^{uni} results in an increased level of bilateral SA^{uni} innervation

The first aim was to determine whether the bilateral FasII staining was present, when we cross the transgene SA^{uni} Gal4-lines the UAS-UNC-5 transgenic flies. SA^{uni} neurons were visualized using R72A10-mCD8::GFP. A strong increase in bilateral SA^{uni} innervation indicate a cell- autonomous function in axonal remodeling. Developmental UNC5 knockdown at both 29°C and 18°C slightly increases the frequency of bilateral SA^{uni} innervation (see Figure 4). Example data is shown in Figure 5.

3.2 Knockdown of UNC-5 in SA^{bi} and SAF has little impact on the unilateral remodeling of SA^{uni}.

To determine whether or not the repulsive receptor signaling is coming from a different source we also looked at the neurons from the same linage who also innervate in the AB neuropil. Results show that SA^{bi} neurons have a significant bilateral effect at the 29°C condition (see Figure 4).

3.3 Downregulation of UNC-5 within FB1B leads to increased number of bilaterally innervating SA^{uni} neurons

Results show that the FB1B has a significant number of bilateral brains in both the normal condition (61% bilateral AB-stainings, n=31, 25°C) and in the increased temperature condition (100% bilateral AB-stainings, n=31, 29°C) indicating a cell-non-autonomous influence on the SA^{uni} neurons (see Figure 4). Example data is shown in Figure 5.

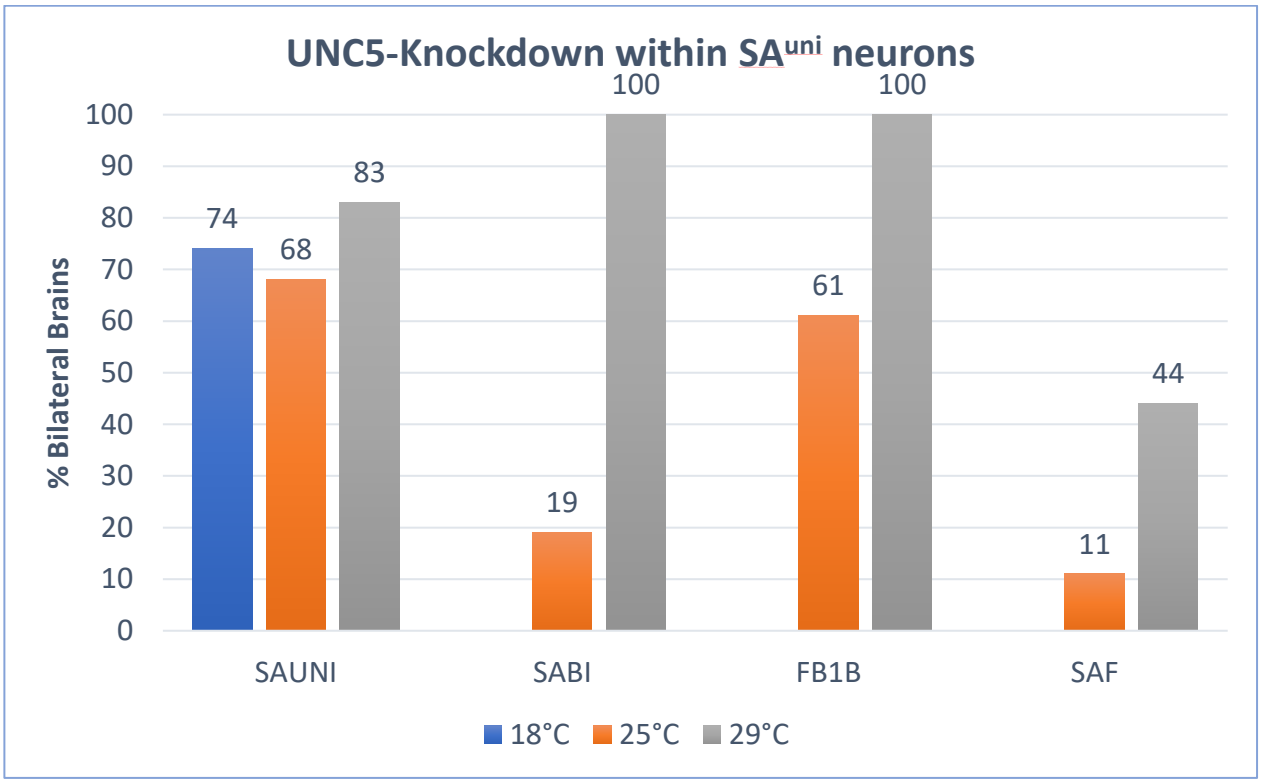
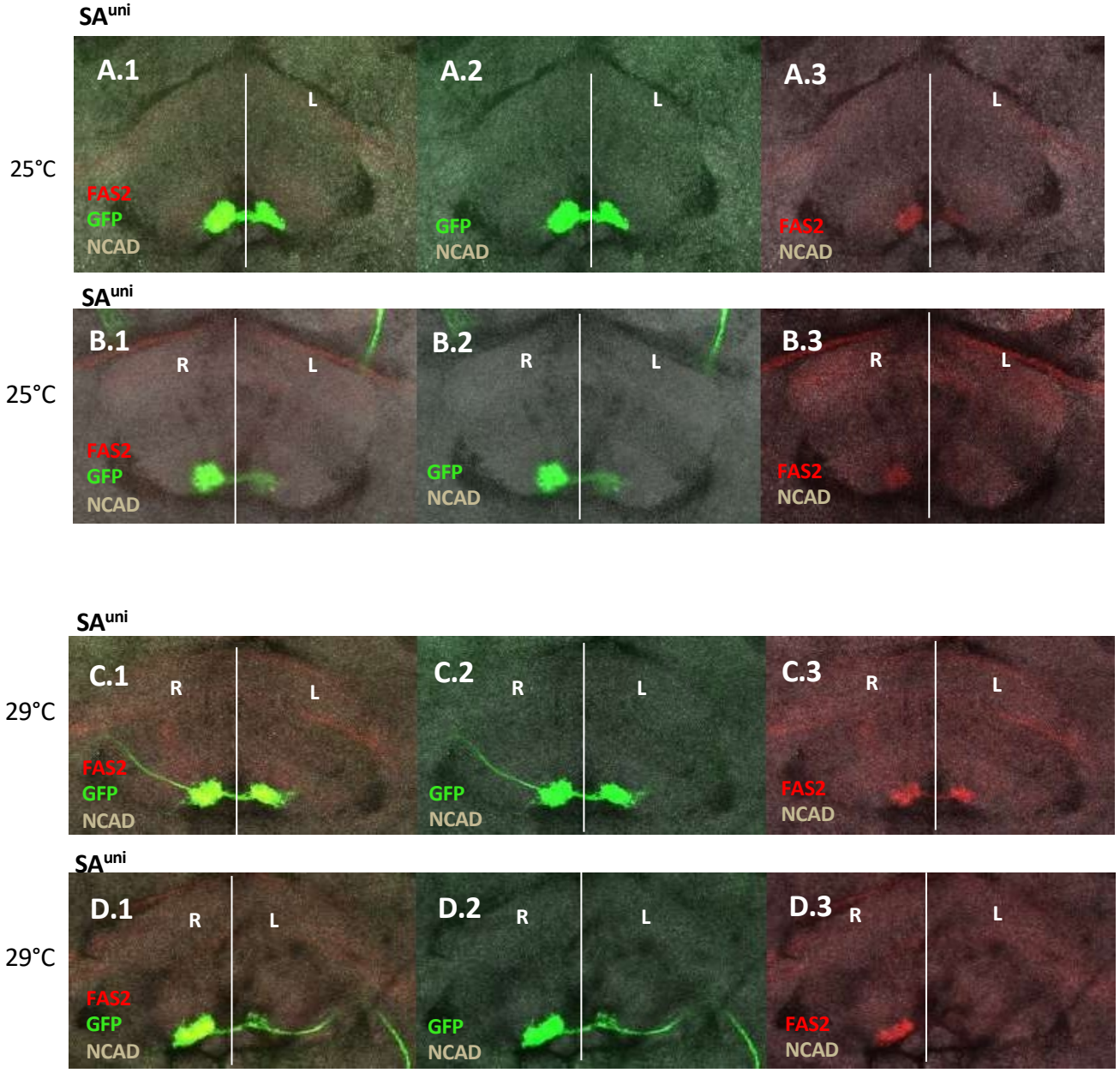


Figure 4. Knockdown of the repulsive receptor UNC-5 within the AB-neurons in the 25°C and the 29°C environmental conditions in all four neuron types.


SA uni
25°C
A.1
L
FAS2
GFP
NCAD
A.2
L
GFP
NCAD
A.3
L
FAS2
NCAD
SA uni
25°C
B.1
R
L
FAS2
GFP
NCAD
B.2
R
L
GFP
NCAD
B.3
R
L
FAS2
NCAD
SA uni
29°C
C.1
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FAS2
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NCAD
C.2
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NCAD
C.3
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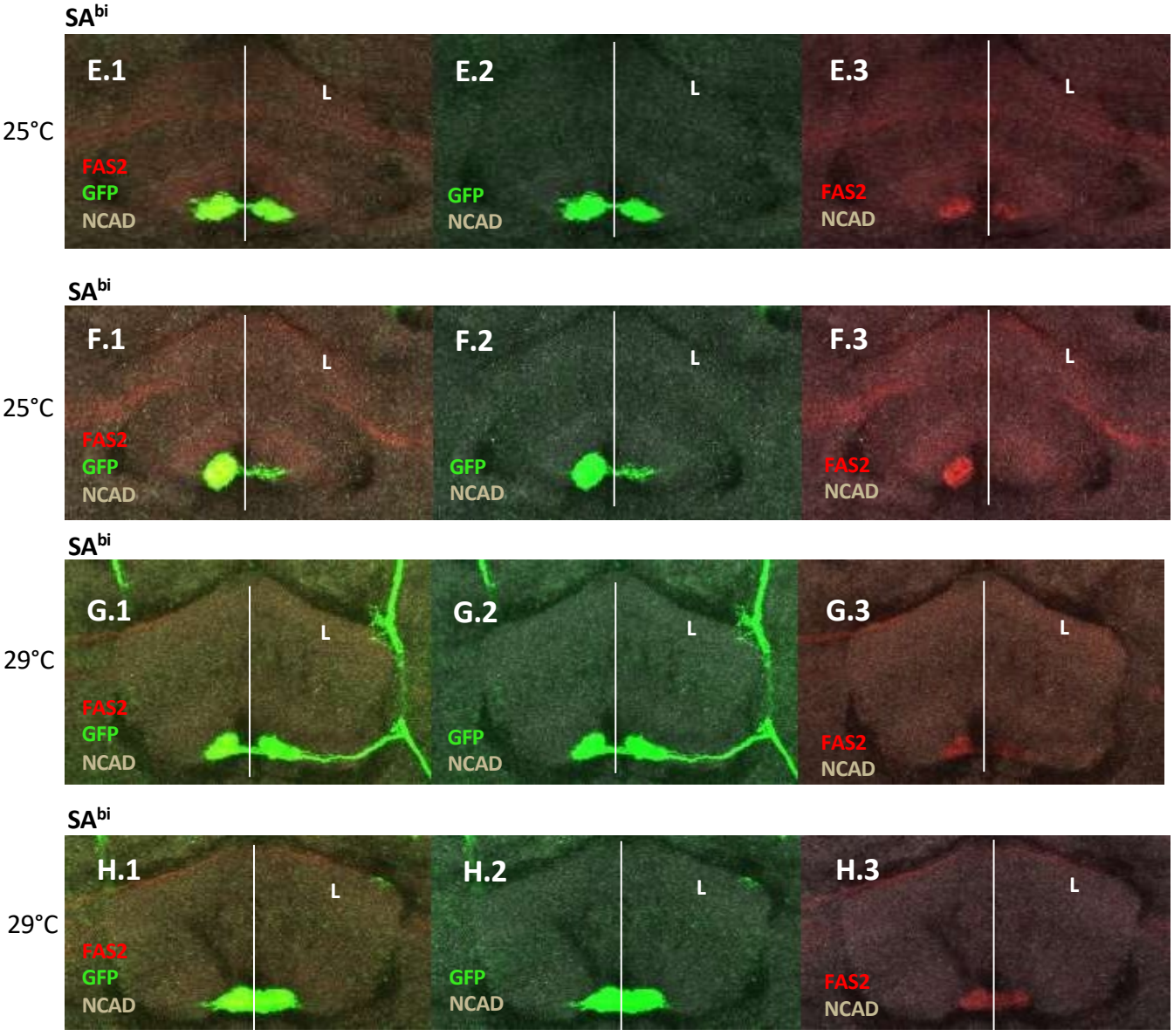
SA^bi
25°C
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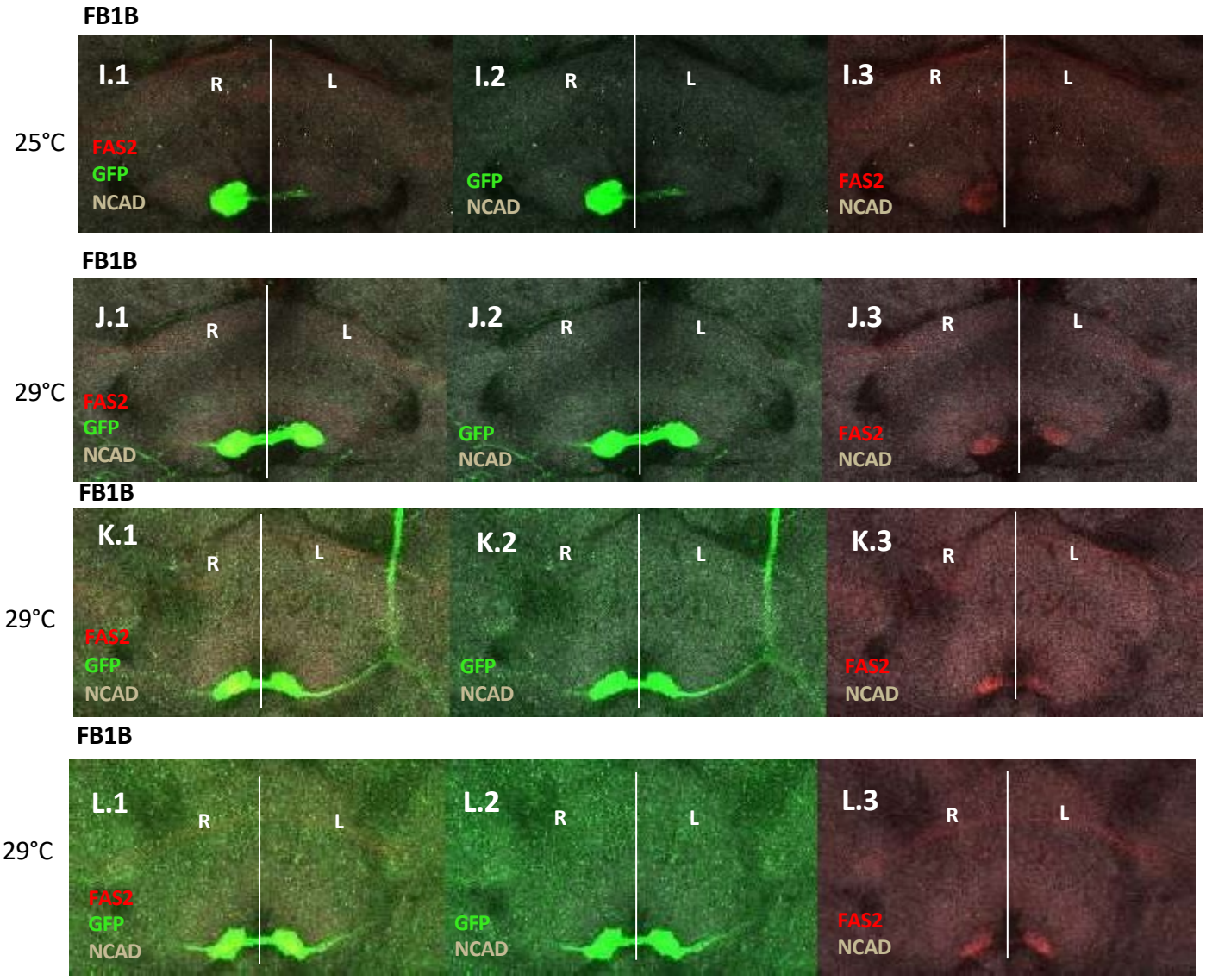

Figure 5. Knockdown of UNC-5 in AB afferent neurons.
Knockdown of the repulsive receptor within the SA^{uni}-neurons (A, B, C & D), SA^{bi}-neurons (E, F, G & H) and FB1B-neurons (I, J, K & L) in 25°C and 29°C.
Note that there are no unilateral SA^{bi} and FB1B phenotypes in the 29°C eviromental condition. In the 29°C condition a merged phenotype was often observed.
Staining: antibodies anti-GFP rabbit (in green), Ncad rat (in grey) and FasII mouse (in red). Images processed in Fiji.

3.4 FasII expression in SA^{uni} disrupted with UNC-5

The RNAi-mediated knockdown of UNC-5 in SA^{uni} causes a peculiar effect – there was a missing left AB in the FasII staining, even though it was clearly there and visible in the other channel. This phenomenon was observed only in the SA^{uni} knockdown of the repulsive receptor UNC-5 (cell-autonomous effect) and in all three temperatures there was a missing FasII staining, according to fluorescence analysis. Example data is shown in Figure 7. Figure 6 shows the quantified data in the 18°C and 25°C. The most instances of missing FasII- stainings were found in the 18°C environmental condition. We used the mean Lateralization Index (LI), which quantifies the dominance of one hemisphere over the other for a specific function, calculated as LI = (A - B) / (A + B), where A and B represent activation levels in each hemisphere. In this case the left and right AB-fluorescence was quantified and then the above formel was used to estimate the mean LI for both the FasII and GFP channels. Our quantifications show that the signal in the FasII expression is stronger than in the GFP expression, indicating a higher lateralization (hemispehere preference).

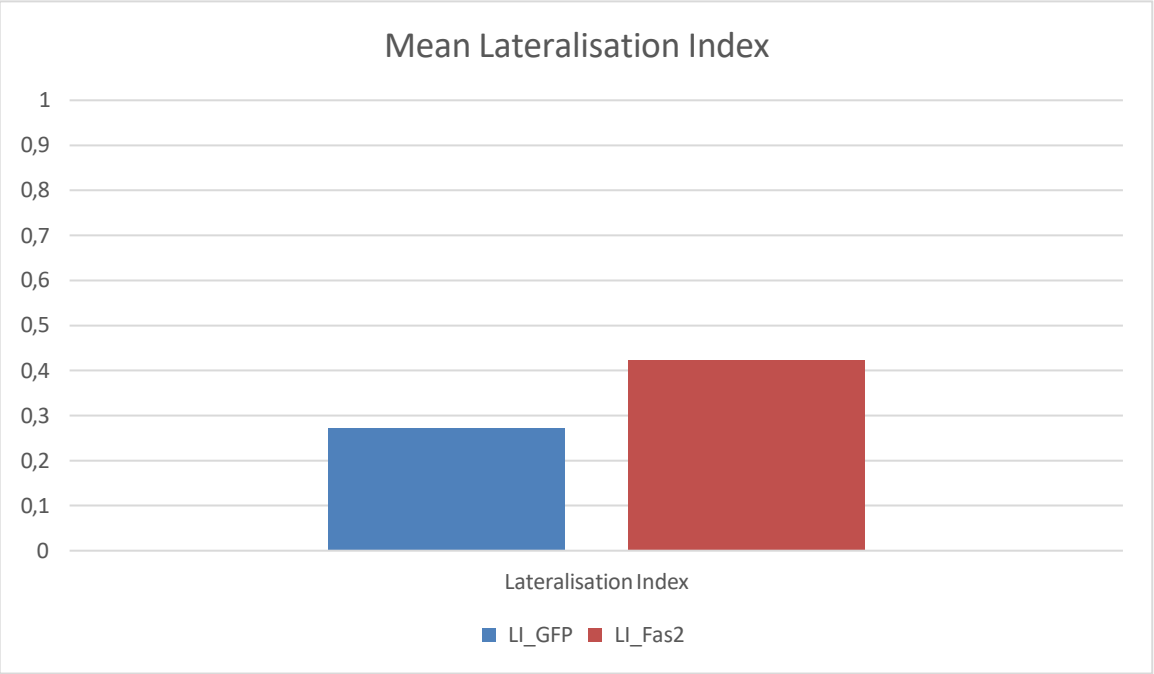

Figure 6. Differences in the Latralisation Index in the SA^{uni} in GFP and FasII channel. Measured staining intensity in Fiji.
N: - fly number; size_left – the measured staining intensity in left AB in the 1st channel (antibody used: anti-GFP rabbit); size_right – the measured staining intensity in right AB; fas2_intensity left/right – 3rd channel staining intensity; background – measured background staining intensity as control (antibody: Ncad rat); Temp in °C – temperatures, in which the flies were incubated/hatched; Driver – the driver gene used, in this case it was to knockdown the UNC-5-receptor in the SA^{uni} neurons.

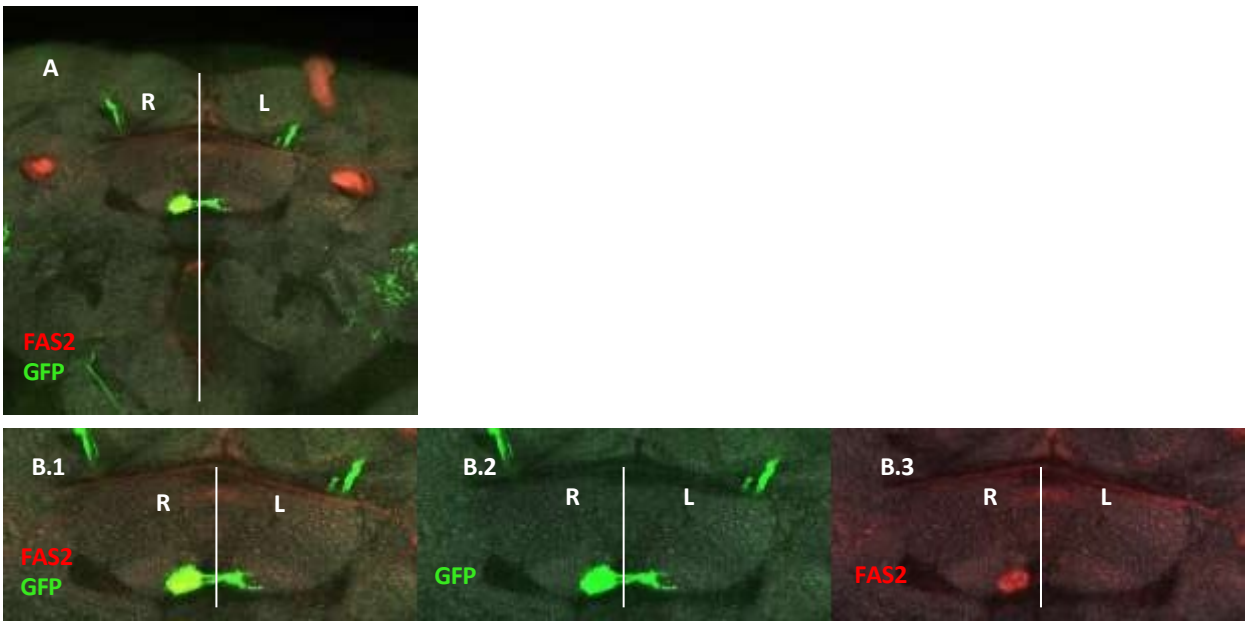

Figure 7.1. An example of a bilateral SA^{uni} in the context of UNC-5-Knockdown missing on one sid e in the FasII staining (25°C).
A - Bilateral SA^{uni} with background staining
B: B.2 - Bilateral SA^{uni} in the GFP-channel; B.3 – Unilateral SA^{uni} in the Fas2-channel
Staining: antibodies anti-GFP rabbit (in green), Ncad rat (in grey) and FasII mouse (in red). Image processed in Fiji.

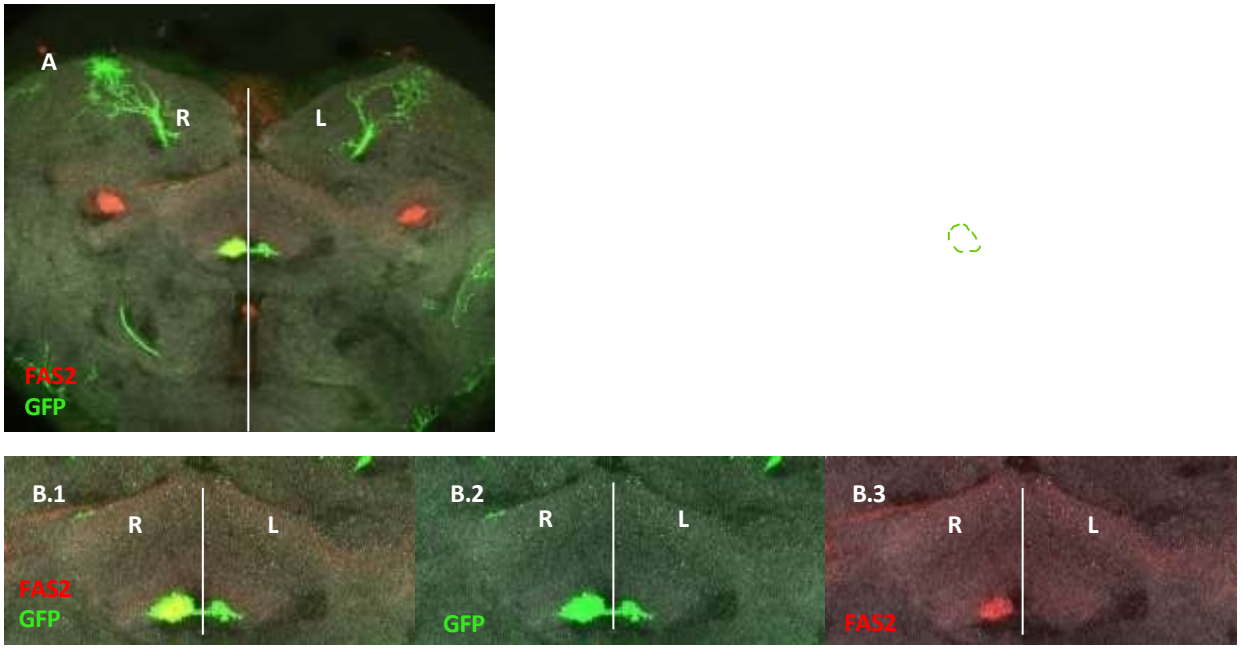

Figure 7.2. An example of a bilateral SA^{uni} in the context of UNC-5-Knockdown missing on one side in the FasII staining (25°C).
Staining: antibodies anti-GFP rabbit (in green), Ncad rat (in grey) and FasII mouse (in red). Image processed in Fiji.
B: B.2 - Bilateral SA^{uni} in the GFP-channel; B.3 – Unilateral SA^{uni} in the Fas2-channel
Staining: antibodies anti-GFP rabbit (in green), Ncad rat (in grey) and FasII mouse (in red). Image processed in Fiji.

3.5 Knockdown of NetB in SA^{bi} and FB1B results in an increased level of bilateral SA^{uni} connections. Knockdowns in SAF neurons has little impact on remodeling.

Net-B knockdown in the SA^{uni} neurons (Genotype: R72A10-Gal4) does not cause a disruption in the asymmetric development (29% bilateral AB-stainings, n=31, 25°C). However, knocking down NetB in the SA^{bi} neurons (Genotype: R66A02-Gal4) (65% bilateral AB- stainings, n=30+, 25°C). Knockdown in the FB1B neurons also leads to an increased number of bilaterally innervating SA^{uni} neurons (75% bilateral AB-stainings, n=20, 25°C). (see Figure 3). There is no significant effect when NetB is knock downed in the SAF neurons (21% bilateral AB-stainings, n=30+, 25°C). This means that NetB is not required in SAF neurons (Figure 8). Controls used were wild type flies (Canton S), which produced a 20% bilateral FasII-staining. This means that the SA^{bi} and the FB1B neurons influence the lateralized development of the SA^{uni} neurons through Netrin secretion.

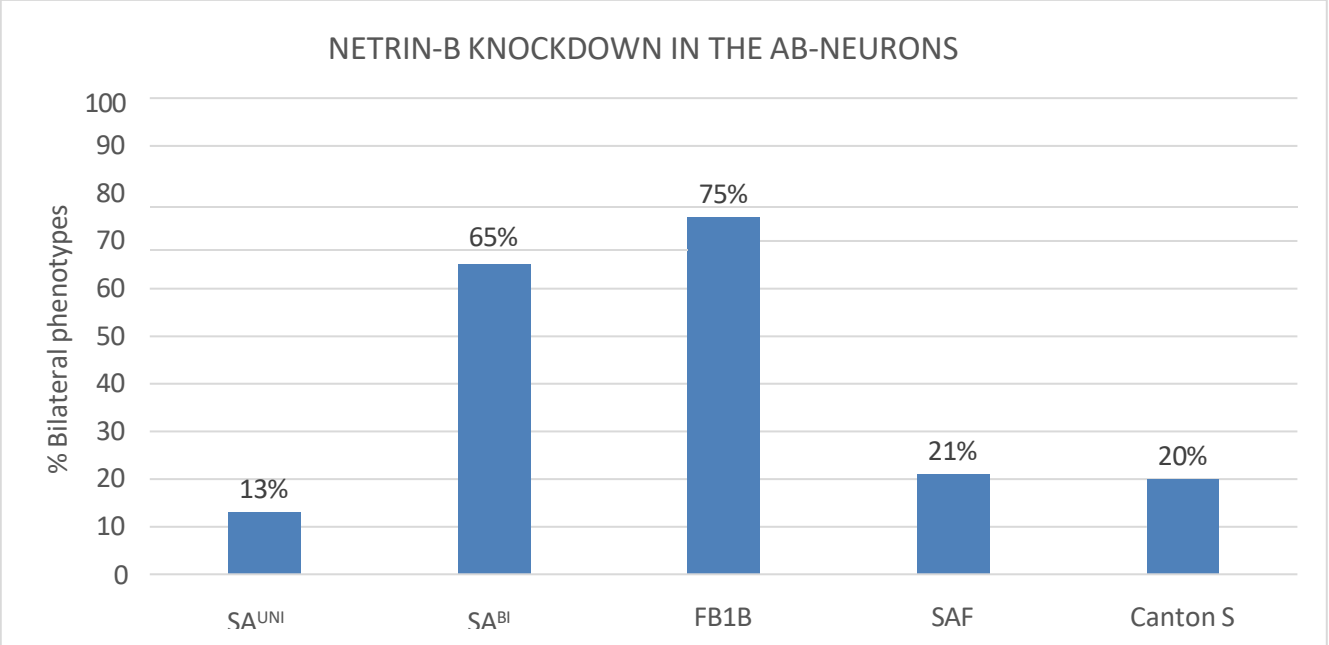

Figure 8. Knockdown of the NetB-pathway in AB afferent classes.
The bar graph illustrates the percentage of bilateral AB-phenotypes observed following Netrin-B knockdown in different neuron types. Among the tested neurons, FB1B showed the highest proportion of bilateral AB-phenotypes at 75%, followed by SA^{bi} at 65%. In contrast, SA^{uni}, SAF, and control group (Canton S) exhibited considerably lower percentages, at 15%, 21%, and 20% respectively.

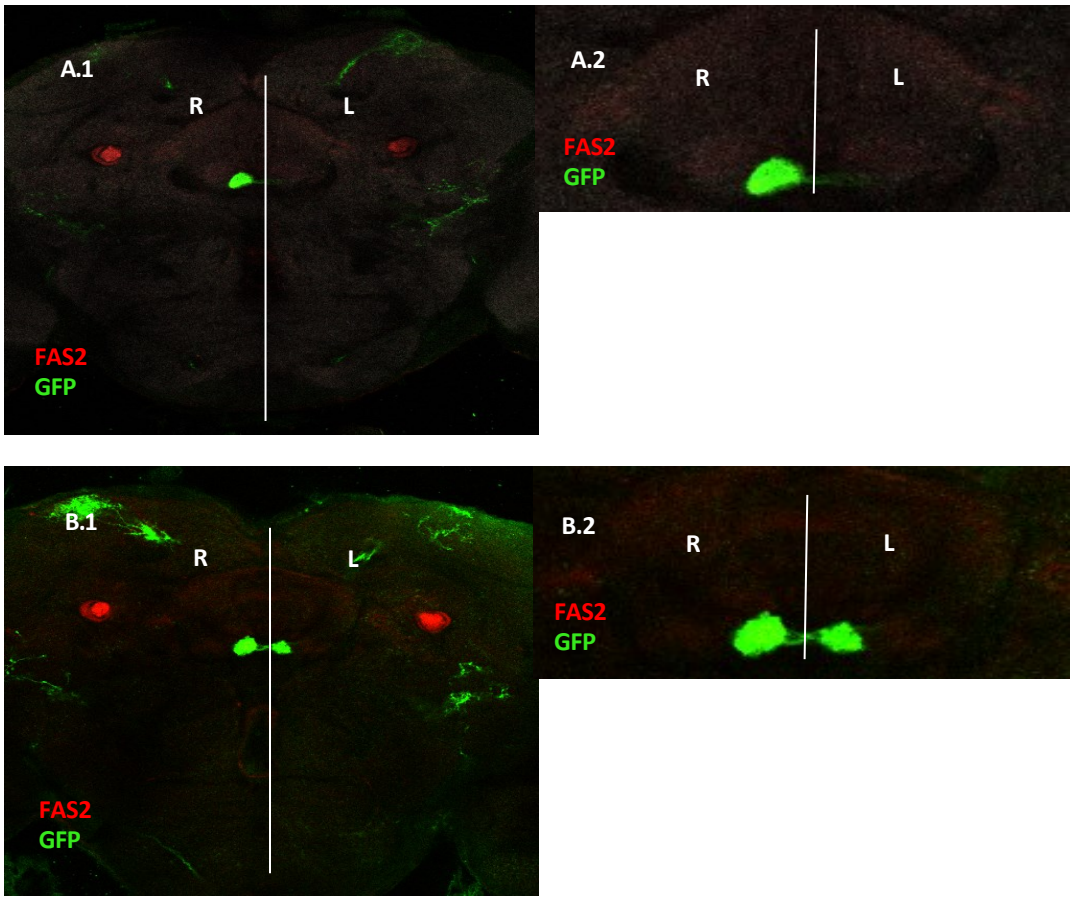

Figure 9. Knockdown of the NetB-pathway in FB1B neurons.
A1. & 2 - Uniilateral SA^{uni} with background staining; B1 & 2 - Bilateral SA^{uni} with background staining. Staining: antibodies anti-GFP rabbit (in green) and FasII mouse (in red). Image processed in Fiji.

3.6 Attraction receptor Frazzled knockdown in AB-afferent neurons.

Knockdown of the attraction receptor Frazzled in both a cell-autonomous (SA^{uni}) and non-cell-autonomous (afferent AB input neurons) context does not lead to a strong increase in bilateral AB phenotypes. In the SA^{uni} neurons, only one out of 18 showed bilateral innervation (5% bilateral AB-stainings, n = 18, 25 °C), indicating that Frazzled is not critically required for the lateralization of these projections. Similarly, knockdowns in afferent neurons had only mild effects: in SA^{bi} (16% bilateral AB-stainings, n = 25, 25 °C), in FB1B (14% bilateral AB-stainings, n = 14, 25 °C), and in SAF neurons, there were no bilateral phenotypes observed (0% bilateral AB-stainings, n = 31, 25 °C). These consistently low percentages suggest that Frazzled does not play a major role in directing AB asymmetry, either directly within SA^{uni} or via their synaptic partners (see Figure 9).

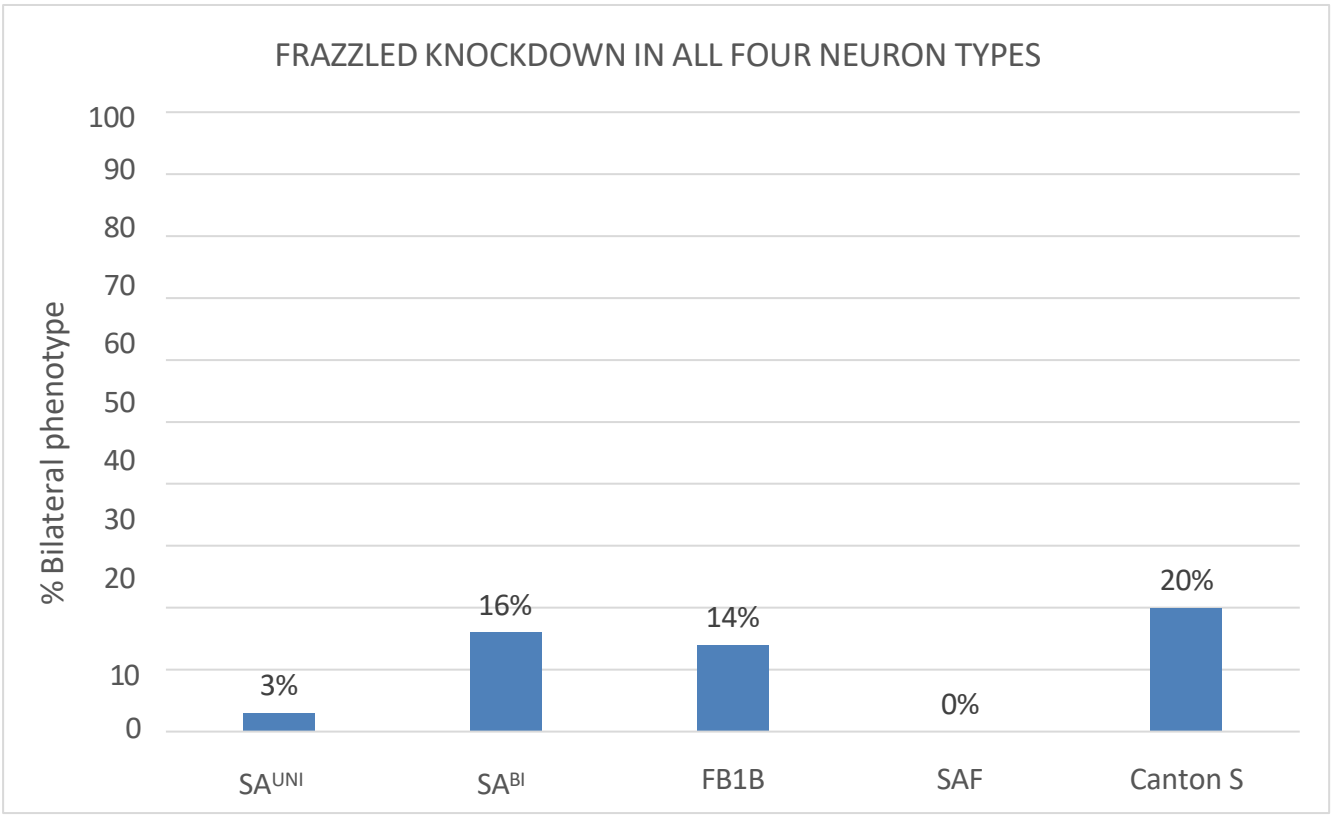

Figure 9. Knockdown of the attraction receptor Frazzled in in all four neuron types (25°C).
Knockdown of the repulsive receptor Frazzled has no significant effect on the remodeling of the SA^{uni} neurons.

3.7 Chi-Quadrat test

In order to estimate the difference in means between the observed number of bilateral brains in the transgenic flies with the results from the controls (wild type) a Chi-Square test was used. According to results from the Chi-Square statistic only the UNC-5 has a statistically significant difference in means (χ^2 (1, N = 31) = 9.25975, p = 0.00234). For the NetB the test wielded the following results: χ^2 (1, N = 33) = 0.1450, p = 0.70331; and for Frazzled: χ^2 (1, N = 31) = 2.20389, p = 0.13766 which were not significant (with α = 5%).

4. Discussion

In my research, I found that NetB plays a key role in asymmetric development in the *Drosophila* central brain. It guides certain axons away from one brain hemisphere, which points to a more structural, signal-driven form of lateralization. That makes it different from other known mechanisms in flies, where asymmetry often comes from functional differences or timing. One interesting contrast comes from the olfactory system. Gaudry (2012) showed that even though the structure of the olfactory circuit is symmetrical, flies can process smells more strongly on one side. That means the asymmetry comes not from wiring differences, but from how strongly neurons respond on each side—suggesting a functional lateralization rather than a structural one, like we see with NetB. Another approach to symmetry or asymmetry is about timing. Prieto-Godino et al. (2019) found that early-born interneurons help guide axons in a way that supports bilateral development. This means the order in which neurons grow and connect can decide whether the brain ends up symmetrical or not. Compared to NetB this is more about preserving symmetry than breaking it. And Buchanan et al. (2015) looked at something that involves behavior: how flies tend to turn more in one direction than the other. They linked this "handedness" to activity differences in the Central complex, so here lateralization isn't just about how neurons grow, but also about how they behave once circuits are already built. In the case of the current study the lateralization process involves the SA^{uni} neurons, which send projections exclusively into the right-sided AB. This lateralized wiring pattern is disrupted in flies lacking NetB or its receptor UNC5, resulting in symmetrical, bilateral projections instead of the typical right-sided targeting. Interestingly, NetB is not produced by the SA^{uni} neurons themselves, but is secreted by two earlier-developing, bilaterally projecting neuron classes: SA^{bi} and FB1B. These afferent neurons release NetB before the SA^{uni} neurons project, providing a protein cue that repels SA^{uni} axons through UNC5-mediated signaling. This neuronal regulation ensures the correct one-sided innervation of the AB. Afferent neurons play a central role in organizing and refining the

function of neural circuits. In the spinal cord, inhibitory interneurons located in the substantia gelatinosa (lamina II) receive input from one group of sensory neurons and can suppress the activity of other neurons receiving signals from different afferents. This enables cross-communication and modulation between sensory pathways, primarily through GABAergic mechanisms and reciprocal inhibition (Zheng, Lu, & Perl, 2010). In the developing cerebellum the axons from the brainstem afferent neurons not only interact with target neurons, but also with other afferent fibers (Baird, Hatten, & Mason, 1992). While contact between afferent axons can support growth, interaction with specific target neurons acts as a stop signal, highlighting how afferent-afferent communication helps guide wiring decisions during development (Baird, Hatten, & Mason, 1992). Together, these examples demonstrate the diverse roles of afferent neuron interactions—from presynaptic inhibition and reciprocal feedback to regulating growth signals, all of which contribute to the adaptability of the nervous system. Functionally in the case of the fruit fly and the current study this neural asymmetry is not just anatomical, but its disruption can lead to impairments in long-term memory, suggesting that lateralized circuits in the central complex contribute to higher-order cognitive processes in the fly (Lapraz et al, 2023).

In this study the remodeling process in the SA^{uni} neuron in context of the Netrin-pathway was examined. The role of Netrin and its repulsive receptor UNC-5 on axon guidance in a cell- autonomous and cell-nonautonomous context was shown to significantly influence the symmetry breaking in the SA^{uni} neuron. Its function as a short-range guidance cue (communication through nearby neurons along the NetB pathway) was explored through the different neuron types from the LALV1-lineage. The bilateral SA^{bi} neuron has a significant influence on the asymmetrical development of the SA^{uni}, but only when the NetB function is influenced. Knockdown of UNC- 5 in the SA^{uni} neuron causes a disruption in the remodeling process indicating a cell-autonomous function. This does not apply however for the attraction receptor Frazzled. Knockdown of NetB had also no effect in the knockdown in SA^{uni}

remodeling. This finding indicates a cell- autonomous function and highlights the importance of the repulsive receptor in the development in the SA^{uni} neuron and are shown schematically in Figure 9. Knockdown of both receptors UNC-5 and Frazzled does not significantly influence the remodeling of SA^{uni} where the knockdown took place. What makes the knockdown of the UNC-5 receptor in FB1B neurons particularly interesting is that under 29 °C conditions, the effect was fully bilateral in every case. This suggests a strong influence of the environment on how the knockdown works. One possible explanation is that the Gal4/UAS system becomes more active at higher temperatures. At 29 °C, Gal4-driven expression might simply be stronger, which could lead to a more efficient UNC-5 knockdown. That doesn't mean the gene itself behaves differently, but rather the degree in which the environment influences the knockdown. The Frazzled-knockdown also yielded a similar effect in the 29°C temperature condition in the FB1B neuron (70% bilateral AB-stainings, n=20, 29°C), but caused almost no effect in the 25°C (14% bilateral AB-stainings, n=14, 25°C). This insight helps us better understand how attractive and repulsive receptors work together through heterodimer formation (Hong et al., 1999). When two different receptors—like the attractive receptor DCC and the repulsive receptor UNC5—come together, they can change how a cell responds to the same signal. Instead of being attracted, the cell can become repelled, depending on which receptor pair is active. This switch is important because it allows neurons to coordinate their response to guidance cues, especially when direction is needed. By forming heterodimers, these receptors enable short-range repulsion, helping growing axons avoid certain areas while still being attracted to others. Only the long-range repulsion needs Frazzled and that is why Frazzled has no effect, because Netrin communication along the pathway is short range.

4.1 This study and its role in current research

The finding that AB asymmetry develops from a bilaterally-symmetrical state has been presented in previous studies in Drosophila. Lapraz et al. (2023) examined the Netrin protein and its receptors in context of AB asymmetric development in the LALV1-lineage as a whole, but didn't point out the different neurons from the lineage. The present study addresses this gap by focusing on specific neurons of the LALV1 lineage, examining the molecular pathway and receptor dynamics involved in symmetry breaking in the adult fly. This is the first study to characterize the influence of SA^{bi}, FB1B, and SAF neurons on the development of SA^{uni} neurons. Notably, SA^{bi} and FB1B neurons modulate SA^{uni} neurons through the NetB signaling pathway, transmitting NetB signals that activate the UNC-5 receptor in SA^{uni} neurons. This receptor activation triggers a repulsive response to NetB, thereby initiating the neuronal remodeling processes essential for AB asymmetry. A model based on the current findings is presented in Figure 9.

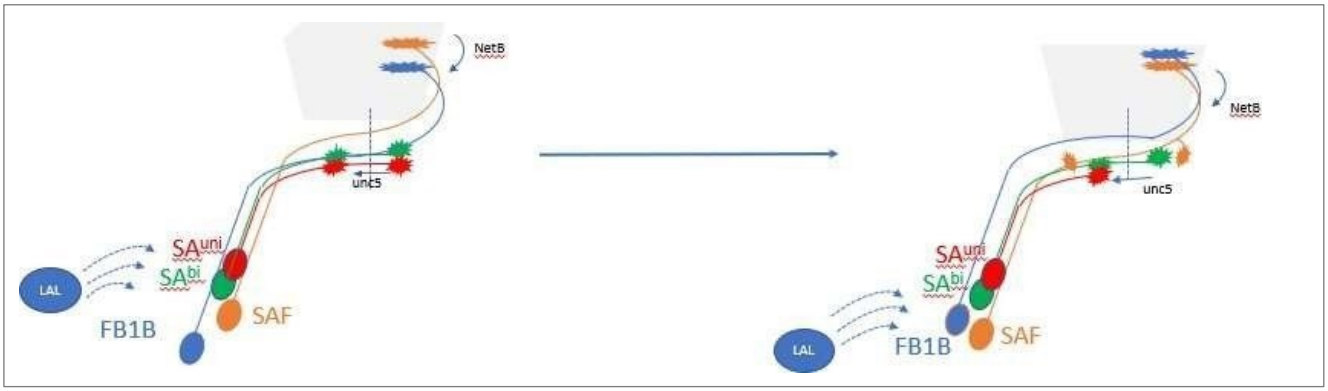

Figure 9. Schematic representation of the proposed model for AB asymmetry development in Drosophila, highlighting the roles of SA^{bi}, FB1B, and SAF neurons in regulating SA^{uni} neuron remodeling through Netrin-B signaling and UNC-5 receptor-mediated repulsion.

4.2 Future research

This study and also the study from Lapraz et al. (2023) showed that Netrin and its repulsive receptor UNC-5 is necessary for asymmetric remodeling of AB neurons and that the actual signal leading to the establishment of asymmetry comes from various sources and there is an interplay between cell-autonomous and non-cell-autonomous signals. NetB knockout on the right side produces a bilateral SA^{uni} innervation (Lapraz et al., 2023), but not on the left side. It is not fully understood why this evolutionary phenomenon occurs. The complex relationship between the LALV1-neurons has been explored, but does not explain why does the remodeling process happen only on one side and how is that important for the fly brain circuit. The Netrin molecule secretion in the SA^{bi} and FB1B is crucial for the remodeling in SA^{uni}, but not from the SAF or SA^{uni}.

Building upon these findings, the new model (in Figure 9) has been proposed to illustrate the molecular mechanisms underlying AB asymmetry, emphasizing the specific roles of SA^{bi}, FB1B, and SAF neurons in modulating SA^{uni} neuron development through NetB signaling and UNC-5 receptor activation. According to our findings the NetB signal, originating from SA^{bi} and FB1B neurons, suggests that these neurons serve as key regulators or organizers of AB asymmetry. NetB is transmitted through the signaling pathway and is subsequently repelled by the UNC-5 receptor located on the SA^{uni} neuron cell membrane, triggering neuronal remodeling. Future experiments could include selective ablation of either SA^{bi} or FB1B neurons to assess, if their absence induces a bilateral phenotype. Another potential experiment would involve generating a NetB null-mutant and crossing it with *Drosophila* lines that express NetB exclusively in either SA^{bi} or FB1B neurons, but not elsewhere in the brain. This approach would serve as a reverse experiment to that conducted by Lapraz et al. (2023), providing further insights into the specific contributions of these neurons to AB asymmetry.

5. Conclusion

This study aimed to delve deeper into the potential of utilizing the Drosophila AB system as a robust platform for investigating brain asymmetry. It sought to elucidate not only the molecular mechanisms that trigger the remodeling of the SA^{uni} neuron, but also to unravel the cell-autonomous and nonautonomous functions influencing this asymmetry. By looking into these aspects, the study aimed to shed light on the intricate processes governing brain development and neuronal circuit, thereby contributing to our broader understanding of neurodevelopmental phenomena.

This thesis identified a connection between the NetB system and its receptors with the different protein molecules that play a key role in axonal remodeling, leading to asymmetry in the adult brain. While also confirming previous findings, this is the first time that the exact neurons from the LALV1-lineage have been identified to influence the asymmetrical remodeling of the AB. The findings are one of a kind deep dive into a conserved across species developmental feature which remains an interesting mystery for which the left and right hemisphere remain perfectly imperfect.

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