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Master thesis

Wnt Signaling in neural asymmetry formation in the Central Brain of Drosophila melanogaster

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Contents

1 Abstract

Asymmetry is a fundamental biological feature, leading to a wide range of structural functional and behavioral differences in the animal kingdom[1]. Lateralization reflects different aspects like development, evolution, experience, pathology and hereditary [1]. It is known that the brain hemispheres in humans process different information independently, leading to a lateralization of information processing. This leads to an increase of capacity of cognition [2] as well as to more "neural space" [3]. In the concept of neuronal diversity, for example in autism spectrum [4] and schizophrenia spectrum [5] – it is known that in those spectra less lateralization of the brain is a common feature on the morphological [4, 5] and the genetic level [6, 7].

It is not exactly known which genetic developmental programs leading to the lateralization of neural circuits. The question that therefore emerges is what genetic developmental factors lead to the lateralization of brain networks.

Numerous traits are shared among a variety of species as a result of evolution. The establishment of model organisms resulted from these similarities between humans and other organisms. We can address the questions by demonstrating which molecules may be relevant to humans based on the results in model organisms. Several genetic tools are available for *Drosophila melanogaster* (*D. melanogaster*), enabling us to ask intricate questions and gain insight into the molecular origins of certain molecules and pathways. I decided to use *D. melanogaster* as the model organism for the studies in order to answer the questions.

The central brain of *D. melanogaster* is used as a model to study this question. More specifically, the asymmetrical body (AB) is used. The AB is a lateralized neuropil, which is larger in size and connections on the right side [8]. It is known, that Fas2 expression in SA_{uni} is lateralized in the adult fly, while these neurons develop from a bilateral ground state [9].

Wnt proteins are known to act in different signaling pathways as secreted proteins, which bind to Frizzled (Wnt receptors) to activate those pathways. In the canonical Wnt pathway a binding of Wnt proteins to the Fz receptor leads to an activation of genes, which are essential for cytoskeletal rearrangements [10]. In the Wnt/planar cell polarity (Wnt/PCP) pathway the role of Wnt proteins is controversially discussed [11], but the activation of this pathway leads to cytoskeletal rearrangement – which then leads to a specific polarity of all cells in this planar, for example in the wings [12], the ovarian [13] or the eyes [14]. More specific to Wnt4 it is already known that Wnt4 plays a role in axon guidance in the visual system along the ventral-dorsal axis [15], regulates the morphogenesis in the Medulla columns mediated by the

Wnt/PCP pathway [14] and has a repulsion function in the axon guidance in the muscles [16]. The first input about Wnt4 as a candidate gene, came from Johann Markovitsch (PhD student at the Hummel lab). He found that flies with the $Wnt4^{EMS23}$ mutation show a significant change on the axonal innervation pattern of SA_{uni} neurons in the AB. Other flies with gene mutations were tested in the Hummel lab, which are part of the Wnt signaling pathways - including the canonical Wnt pathway and the Wnt/PCP pathway - which showed an inconclusive picture of the bilateral axonal innervation of the SA_{uni} neurons in the AB. Therefore a further analysis is needed to finalize a conclusive picture of the involvement of pathway components to the SA_{uni} axonal innervation in the AB. Additionally, it was discovered by the members in the Hummel lab, that colder temperatures (18 °C instead of 25 °C) provide a sensitized background for bilaterality in the different genotypes. On the one hand, I assess the role of Wnt4 and Wnt signaling pathway components on the axonal innervation of the SA_{uni} neurons in the AB. On the other hand, I want to find out if colder temperature has an effect on this axonal innervation.

2 Zusammenfassung

Asymmetrie ist eine fundamentale, biologische Eigenschaft, welche zu einem breiten Spektrum von strukturellen, funktionellen and verhaltensbiologischen Unterschieden in Tierreich führen [1]. Lateralisierung reflektiert verschiedene Aspekte von Entwicklung, Evolution, Erfahrung, Pathologie und Vererbung [1]. Es ist bekannt, dass die Gehirnhälften in Menschen unterschiedlich verschiedene Informationen verarbeiten, was zu einer Lateralisation in der Informationsverarbeitung führt. Dies führt zu einem Anstieg der Kapazität von Kognition [2], sowie zu mehr "neuronalem Platz" [3].

Im Neurodiversitätskonzept, zum Beispiel im Autismusspektrum [4] und dem Schizophreniespektrum [5] ist bekannt, dass diese Spektra weniger Lateralization im Gehirn als gemeinsames Merkmal auf morphologischem [4, 5] und auf genetischem Level haben [6, 7].

Es ist nicht genau bekannt wie genetische Entwicklungsprogramme zu einer Lateralisierung in neuronalen Schaltkreisen führen. Die Frage, die sich daher stellt, ist welche genetischen Entwicklungsfaktoren zur Lateralisation im Gehirnnetzwerk führen. Eine Vielzahl an Merkmals teilen sich verschiedene Spezies als Resultat der Evolution. Die Etablierung von Modellorganismen resultiert von den Ähnlichkeiten zwisschen Menschen uns anderen Organismus. Wir können diese Fragen beantworten, indem wir anhand der Ergebnisse in Modellorganismen

aufzeigen, welche Moleküle für den Menschen relevant sein könnten. Eine Fülle an genetischen Werkzeugen ist in *Drosophila melanogaster* (*D. melanogaster*) verfügbar, was uns ermöglicht komplexe Fragen zu stellen und Einblicke in die molekularen Ursprünge bestimmter Moleküle und Stoffwechselwege zu gewinnen. Um die Fragen beantworten zu können, habe ich mich entschieden, *D. melanogaster* als Modellorganismus für diese Masterarbeit zu verwenden.
Wenn es um Evolution geht dann sind viele Eigenschaften zwischen den Tierspezies konserviert. Diese gemeinsamen Eigenschaften in Menschen und anderen Organismen führten zur Verwendung von Modellorganismen. Im Falle von *D. melanogaster*, eine Vielzahl von genetischen Werkzeugen ist verfügbar, was erlaubt komplexe Fragen zu stellen und Einblicke in die molekularen Hintergründe von bestimmten Molekülen und Wegen zu bekommen. Diese Erkenntnisse geben die Möglichkeit zu zeigen welche Moleküle in Menschen wichtig sein können um die gestellten Fragen zu beantworten. Daher ist *D. melanogaster* als Modellorganismus hilfreich um Fragen zu beantworten, die eng mit den Fragen in der humanen Neurobiologie verknüpft sind.
Der Zentralkomplex des Gehirns von *D. melanogaster* wird in dieser Masterarbeit verwendet um diese Frage zu beantworten. Im Speziellen wird der asymmetrische Körper (AB) verwendet. Der AB ist ein lateralisiertes Neuropil, welches auf der rechten Seite größer und reicher an Verbindungen ist [8]. Es ist bekannt, dass die Fas2 Expression in SA_{uni} Neuronen lateralisiert in der adulten Fliege ist, während diese Neuronen von einem bilateralen Grundstadium kommen [9].
Wnt Proteine sind bekannt dafür in verschiedenden Signalwegen als segregierte Proteine zu agieren, indem sie Frizzled (Fz) binden und dabei diese Signalwege aktivieren. Im kanonischen Wnt Signalweg eine Bindung von Wnt Proteinen an den Fz Rezeptor führt zu einer Aktivierung von Genen, welche in Zelladhäsion entlang des Zytoskeletts involviert sind [10]. Im Wnt/Planarzellpolaritätsweg (Wnt/PCP) ist die Rolle von Wnt Proteinen kontrovers diskutiert [11], aber die Aktivierung dieses Signalweges führt zu einer Neuordnung des Zytoskeletts, was dann zu einer speziellen Polarität von den Zellen in einer Ebene führt, zum Beispiel in den Flügeln [12], den Ovarien [13] oder den Augen [14]. Wnt4 spielt eine Rolle in der axonalen Wegfindung entlang der ventral-dorsal Achse [15], reguliert die Morphogenese in den Untereinheiten des Auges durch den Wnt/PCP Signalweg [14] und hat eine Repulsionsfunktion in der axonalen Wegfindung in den Muskeln [16].
Der erste Input über eine mögliche Funktion von Wnt4 kam von Johann Markovitsch (PhD Stu-

dent im Labor von Prof. Dr. Thomas Hummel). Er fand heraus, dass ein Fruchtfliegenstamm mit der $Wnt4^{EMS23}$ Mutation eine signifikante Änderung im axonalen Innervationsmuster der SA_{uni} Neuronen im AB führen. Weitere Fliegen mit Genmutationen wurden ebenfalls getestet, wobei auch diese Teil der Wnt Signalwege sind, welche einem nicht schlüssigen Bild der axonalen Inneravationen von SA_{uni} Neuronen im AB führten. Daher ist eine weitere Analyse erforderlich, um ein schlüssiges Bild der Beteiligung von Signalwegkomponenten an der axonalen SA_{uni} Neuroneninnervation im AB zu erhalten. Außerdem wurde von den Mitgliedern des Hummel Labors herausgefunden, dass kälte Temperaturen (18 °C statt 25 °C) einen sensibilisierten Hintergrund für Bilateralität in den verschiedenen Genotypen bieten. Auf der einen Seite schaue ich mir die Rolle von Wnt4 and Wnt Signalwegkomponenten auf die axonale Wegfindung der SA_{uni} Neuronen in den AB an. Auf der anderen Seite, möchte ich herausfinden, ob kühlere Temperaturen einen Effekt auf die Entwicklung der axonalen Wegfindung haben.

3 Acknowledgment

I want to thank Prof. Dr. Thomas Hummel for all the experience I gained in his lab and the scientific growth that I was able to do while feeling welcomed in the lab all day and night. My infinite thank goes to Daniel Mitic, who always gave me the scientific and mental support, which is highly underestimated in the daily science business, and to Johann Markovitsch, who shines with his wealth of knowledge in genetics and neurobiology. Furthermore I want to thank all the lab members for their support during the practical work. I am grateful to Benjamin, my parents, and my friends for their unwavering support and encouragement, which helped me to bring everything together and provide the necessary distractions.

4 Introduction

4.1 Wnt4 in *Drosophila melanogaster*

There are seven known Wnt proteins (Wingless, Wnt2, Wnt4, Wnt5, Wnt6, Wnt10, WntD) in *D. melanogaster*. Wnt4 is a 539 amino acid long protein. It is transcribed from the gene *Wnt4*. Wnt4 is a extracellular signaling protein, which acts in different Wnt signaling pathways (see chatpter "Wnt signaling pathways").

Wnt4 is a secreted protein, thereby functioning in the extracellular space [13]. It is active in

the canonical Wnt signaling pathway [17] as well as the Wnt/PCP pathway [18].
For instance, it is known to function as an attraction molecule in the visual system, enabling axonal development to the dorsoventral specificity of retinal projections in the medulla cortex of *D. melanogaster* [15]. Wnt4 controls the morphogenesis of Medulla Columns by differentiated expression of the protein over the Medulla [14]. It also binds to the Wnt receptors Frizzled1 (Fz^1) and Frizzled2 (Fz^2) [19], both are also active parts in the Wnt signaling pathways[17, 18].

4.2 Wnt signaling pathways and the role of Wnt4

4.2.1 Canonical Wnt pathway

The main function of this pathway is the stabilization of Armadillo (Arm), which is the homolog to human protein β-catenin in *D. melanogaster*. Accordingly, in vertebrates including humans this pathway is commonly known as the Wnt/-catenin signaling pathway. Arm mediates the final activation of transcription of target genes that regulate cell proliferation, survival, differentiation, and migration [20]. Armadillo forms a complex with Pangolin (Pan), the TCF homolog and activates Wnt target genes [21]. The requirement of Arm and Pan for mediating activation and repression of the Wnt target genes was shown by genome-wide studies, which therefore supports the dogma of the canonical Wnt signaling [22].
This pathway is composed of an extracellular signal (Wnt proteins), a membrane receptor (Frizzled (Fz) and lipoprotein receptor (LRP)), a cytoplasmatic cascade (Dishevelled (Dsh), a degradation complex including Axin, APC, CK1, GSK-3β), and a nuclear signal (Arm), which translocates from the cytoplasm to the nucleus, binds to Pan and activated Wnt target genes [23].

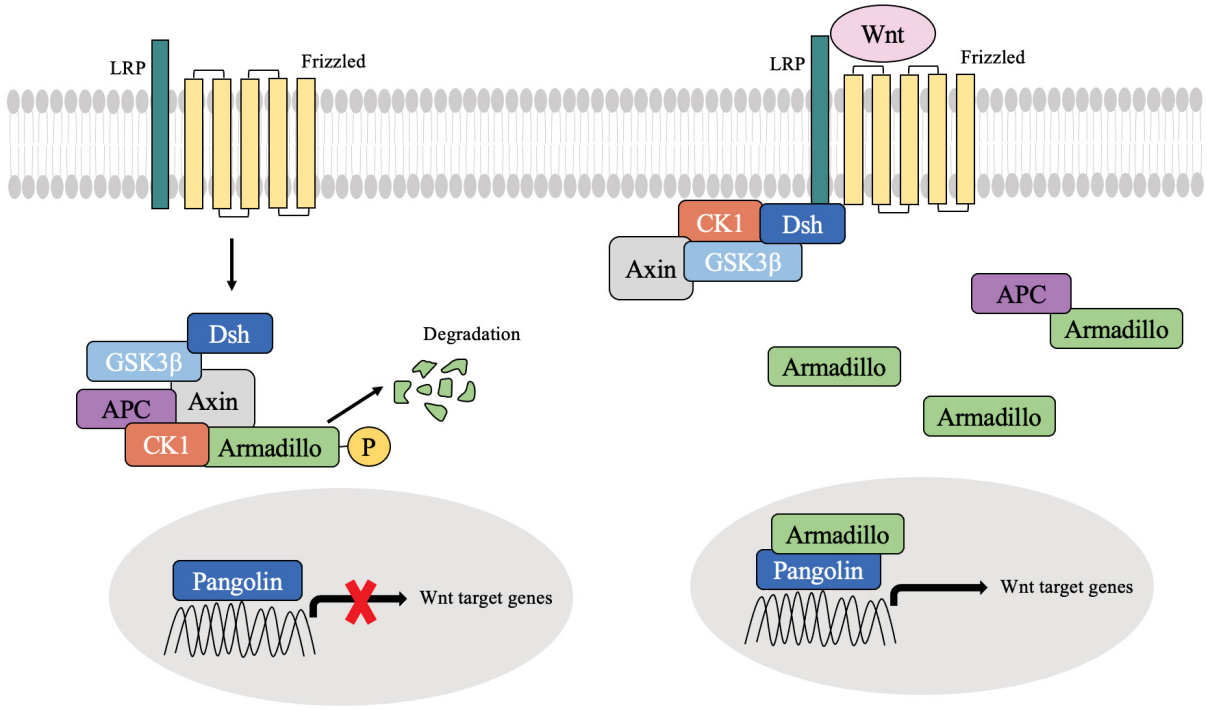

Figure 1: Canonical Wnt pathway. Left: When Wnt proteins is not present and therefore do not bind to the receptors LRP and Frizzled. Arm is degraded in the cytoplasm. Right: When Wnt binds to the receptors, Dsh inhibits the degradation of Arm, whereby Axin, CK1 and GSKβ binds to LRP. The degradation complex is broken, Arm stabilizes and dislocates to the nucleus. Arm binds Pan and activates downstream Wnt target genes.

4.2.2 Wnt/PCP-pathway

The Wnt/PCP-pathway is essential for establishing planar cell polarity (PCP) in different tissues in invertebrates and vertebrates [24]. First descriptions of the PCP were made by Gubb et.al by observations of hair and sensory bristle defects on the wings of *D. melanogaster* [25]. PCP refers to the orientation of cells within epithelial tissue relative along their apical-basal axis.[26]. PCP works by a asymmetrical distribution of the proteins in the cell. The proteins, which are involved in PCP are Fz, Dsh, Flamingo (Fmi), Dgo, Vangogh (Vang) and Prickle (Pk) [27]. Initially PCP proteins are symmetrically distributed on the cell membrane. These proteins relocalize at 26-30 hours after pupal formation (APF) [24]. Fmi, a transmembrane cadherin, gets localized proximal and distal in the cells, while it disappears mostly from the anterior and posterior edges [18], similar to Dgo, which accumulated on proximal and distal sides of the cells [28]. Fz and Dsh localize distal [29] [30], while Vang and Pk are found proximal [31, 32]. The connections between the proximal proteins of one cell and the distal proteins of another cell are thought to stabilize the PCP system along these proximal-distal axis (see Fig. 2) [24].

PCP is found in different tissues - wings, sensory organ precursor and abdomen and eyes [27]. Wnt signaling leads to cytoskeletal rearrangement, which results in the establishment of specific cell polarity in the planar axis. [33]. Specifically Wnt4 is important for the PCP orientation in *D. melanogaster* wings. Therefore Wnt4 modulates the intercellular interaction between Fz and Vang, which is important for the initial polarity - directly affecting PCP [12]. In the wings of *D. melanogaster* Wnt4 is critical for PCP axis definition in early stages [12]. Wnt4 influences projection of the axons to the ventral lamina in the eyes, so that a loss of Wnt4 leads to a misrouting of the axons in the lamina of the eyes [15]. Wnt4 regulates with other Wnt proteins the arrangement and morphogenesis of medulla columns in the brain via the Wnt/PCP-pathway [14]. Wnt4 acts as repulsive cue in muscle cells, and inhibits the formation of synapsis via Fz^2, Dsh and other proteins in the motor neurons there [16].

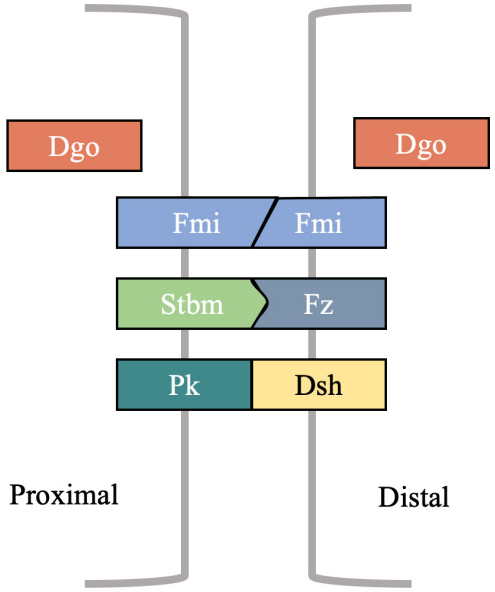

Figure 2: Protein distribution on proximal and distal side of cells, including connections of different proteins to stabilize the PCP. Dgo and Fmi are found on both sides of the cells similar distributed, while Vang and Pk are more on proximal side and Fz and Dsh are more on distal side.

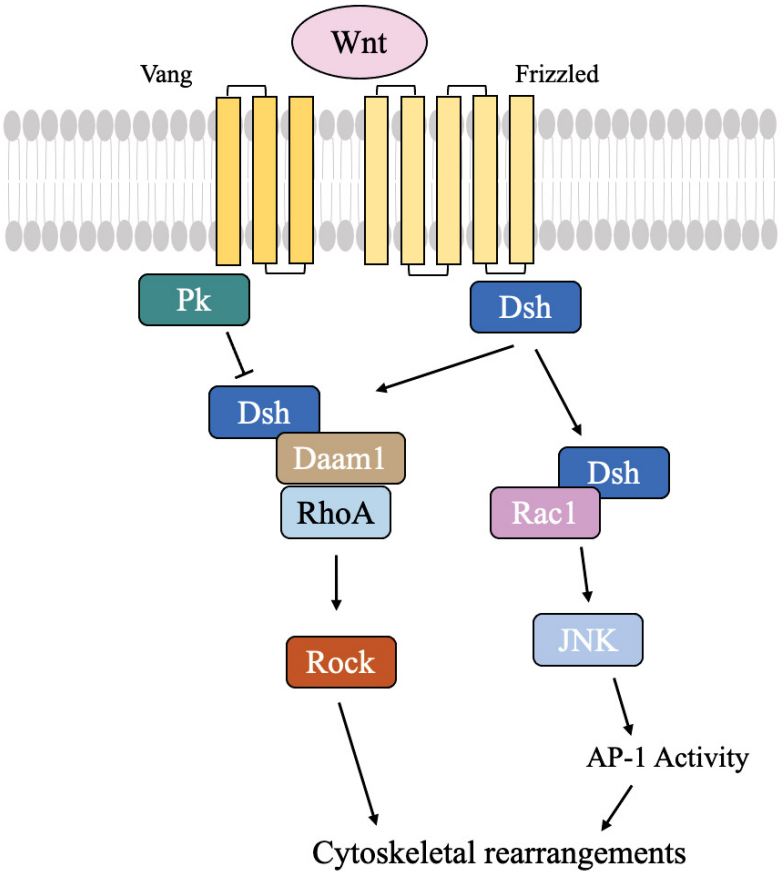

Figure 3: Wnt/PCP pathway. The binding of Wnt leads to Fz/Dsh and Vang/Pk complexes, which lead to cytoskeletal rearrangement via a complex signaling cascade.

4.3 Neural asymmetry in the central nerve system - a biological function?

"Nous parlons avec l'hémisphère gauche" ("We speak with the left hemisphere") [34] - the famous statement of Pierre Paul Broca together with the presentation of his summarized work is considered as the starting point of cerebral asymmetry research [35].

Structural and behavioral differences are based on asymmetry in the brain [1]. Lateralization happens on different levels - from development [36], evolution [1], neurochemical asymmetries [37] and genetic factors [38]. Processing information in the brain hemispheres happens lateralized, which leads to an increase of capacity of cognition [2] and therefore provides more "neural space" [3]. There is a relative, not absolute, relationship between several neurodevelopmental and psychiatric disorder and atypcial functional and structural asymmetries in the structure of the brain hemispheres of humans. Some patients show more atypical asymmetries on average, but there are also patients, which show typical asymmetries. There is a relation

between a decrease in asymmetry with many disorders, rarely an increase of those asymmetries [39]. Additional in autism spectrum it was found that there is reduced asymmetry in cortical thickness in most part of the cortex as well as obsessive-compulsive disorder in children is connected with changes in asymmetries in subcortical areas [40]. However, brain asymmetries are not unique to humans; they are an evolutionary conserved trait shared by vertebrates [1, 3] and invertebrates [38, 41]. Neural asymmetry shapes the cognition and behavior also in *D. melanogaster* [42]. Findings indicate that neural asymmetry is associated with cognition and behavior in *D. melanogaster*, affecting how the organism responds to environmental stimuli. These asymmetries reflect underlying gene interactions, as neurogenic genes exhibit functional hierarchies and asymmetric relationships [43]. *D. melanogaster´s* asymmetric neurotransmitter release from olfactory receptor neurons allows flies to lateralize odor detection, enabling them to turn toward the direction of the intensely stimulated antennae, which enhances their olfactory behavior [42]. Individual locomotor handedness, a manifestation of behavioral asymmetry, is genetically encoded, driving a complex interaction between neural architecture and behavioral outcomes. Specifically, columnar neurons in the central complex of the brain are involved in modulating decision-making and exploratory behavior. [44].

4.3.1 Evolutionary conservation of neuronal asymmetry

Neuronal asymmetry is evolutionarily conserved across species, from invertebrates to vertebrates. Asymmetry is not only unique to humans - it is observed in different animal species. A study in *C. elegans* suggests a hereditary foundation for neuronal asymmetry, providing evidence for its evolutionary conservation [38]. Lateralization in vertebrates is suggested to be influenced by multiple genes, focusing on the complexity of neuronal asymmetries and their adaptive function in ecological contexts [45]. The emergence of asymmetrical circuits indicates a more efficient processing of information and thereby reflects a dynamic interplay of environmental and genetic factors - leading to cognitive evolution across diverse taxa [46].

4.3.2 The Central complex in *D. melanogaster*

The Central complex in *D. melanogaster* is composed of the ellipsoid body, the fan-shaped body, the protocerebral bridge, the nodulus and the asymmetrical body (AB) [8]. The central complex regulates a diverse spectrum of motor and behavioral responses, as well as using inputs for migration, navigation and orientation [8]. A variety of functions of the central complex are

locomotor behaviors like handedness [44], turning directions [47], walking behaviors [48], motor control during walking [49] and spatial memory during locomotion [50] and flight [51] as well as more special behaviors like courtship [52], sleep [53, 54], hunger [55] or gravitaxis [56]. The central brain is suggested to adapt dynamically to estimate orientation in a visual surrounding [57]. Furthermore, the central complex contains a ring attractor network, which encodes for head direction, which brings sensory information into the central complex - connecting between visual cues and head direction - for navigation and orientation in visual conditions [58]. Not only visual input is processed in the central brain, also olfactory input [59] and gustatory inputs [60] are processed there. The formation and recall of short- and long term memories are also a part of the central complex [50, 61].

4.3.3 The Asymmetrical body (AB) and the SA_{uni} neurons

The AB is located at the midline, directly next the ventral Fan-shaped body [8]. In 2004, the AB was first described in flies only on the right hemisphere by immunolabeling with with Fasciclin II (FasII) protein, which is expressed in this structure, getting to the conclusion that the AB is only in the right hemisphere of the brain. Based on this fact the name "asymmetrical body" was chosen for this structure [62]. Flies with symmetrical FasII expression in the ABs have reduced long-term memory [62]. Nowadays it is known that SA_{uni} neurons only innervating the right AB in most cases ($\sim$95%), therefore FasII is only found in the right AB [63, 36]. Three neuron classes are part of the AB: SA_{uni} (SLP-AB) neurons, SA_{bi} (AB-FB) neurons and SAF (SLP-AB-FB) neurons [8, 64]. Neuronal asymmetry is linked to functional and structural differences between the left and right sides of the brain, as exemplified by AB neurons. Whereby these AB neurons play an essential role in interval timing behaviors [65] and in olfactory learning [64], which show that a distinct activity pattern of these neurons influences the retrieval and memory formation [65, 64]. The NetrinB (NetB) is essential for the establishment of the right-sided projections into the AB and is one of the molecules which leads to asymmetries of neuronal projections. NetB is required for the asymmetrical projection of bilateral neurons that innervate the right AB [36].

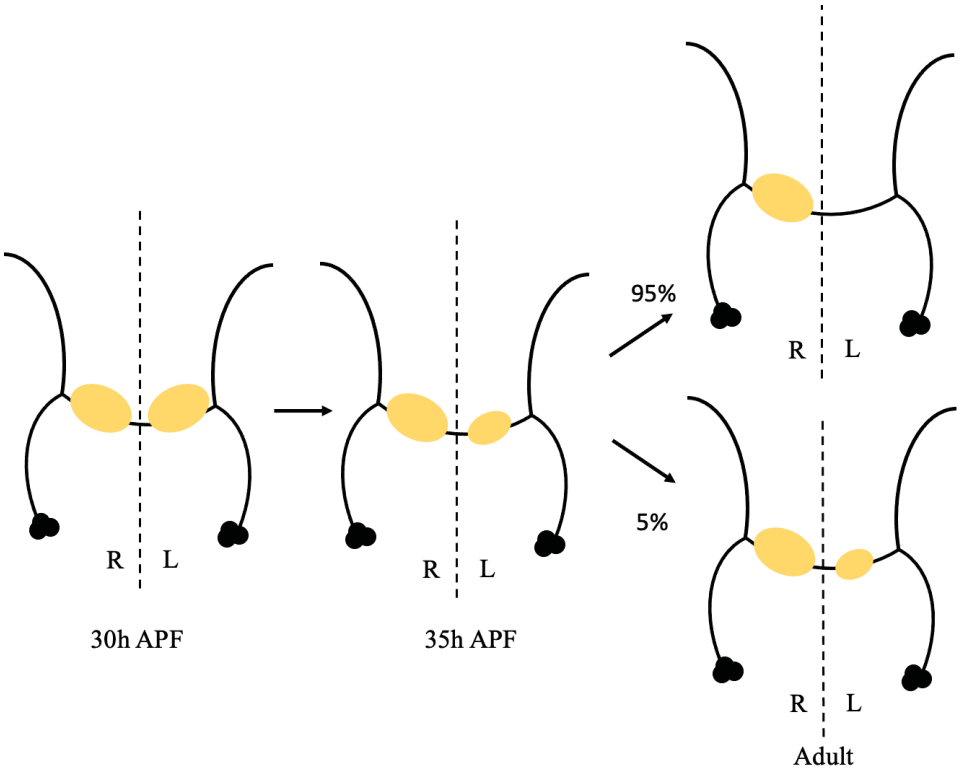

Figure 4: Development of axonal innervation of the SA_{uni} neuron into the AB. At 30h APF the SA_{uni} neurons exhibit a bilateral state of innervation (yellow) of the AB. By 35h APF, this bilateral projection begins to diminish. In the adult flies in 95% the flies have an unilateral innervation of the AB, while only 5% retrain bilateral innervation. Modified on the basis of [62, 63]

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4.4 Temperature

Temperature is an easily controllable parameter, when growing fruit flies. Temperature has an effect on protein folding and localization [66]. The process of the development is influenced by temperature [67]. It is known that high temperature has an effect on the canonical Wnt signaling pathways [68]. Exposure to high and low temperatures has negative effects on cognition and mental health in children [69]. In *D. melanogaster*, sub-adult heat stress leads to reduced learning performance (odor) [70] and reduced long-term memory performance [71].

4.5 Aims of this thesis

First, the goal was to determine the localization of the Wnt4 protein in the central complex in *D. melanogaster*. The information of the Wnt4 localization answers the question if Wnt4 could have an influence on the SA_{uni} neurons in the central complex. Therefore, it was essential to employ fly strains that either directly or indirectly tag the expression of Wnt4 for precise localization. I tested two fly strains, one tags the Wnt4 directly via Wnt4-nls-T2A-GFP and

the other one tags Wnt4 indirectly via a binary expression system (Gal4-UAS) and Flag as tag. These tests were done in pupa and adult flies.
Second, I tested if Wnt4 has an influence on the SA_{uni} development in the AB. Therefore different fly strains with loss of function mutations of Wnt4 were tested.
Third, the objective was to determine whether temperature affects the axonal innervation pattern of the SA_{uni} neurons. Specifically, the investigation focused on the components of the Wnt signaling pathway, as well as the Wnt4 protein. Two distinct Wnt4 mutations, along with various Wnt signaling pathway components, were investigated. Wnt4 downregulation was induced via RNA interference (RNAi), and interactions between Wnt4 mutations and other pathway components were analyzed. A comparative analysis of the two Wnt4 mutation alleles was also conducted.

5 Methods

5.1 Model organism

The model organism *Drosophila melanogaster* was used for all experiments. Different genetic modified genotypes (see Table 1 and 2) were used for the analyses of the given questions.

5.2 Experimental setup

The same setup for all experiments is used.

- Food: Standard fly food was used for all fly strains.
- Temperature: The temperature was controlled during the whole developmental phase. Flies, which were crossed for specific phenotypes were incubated 1-3 days on 25 °C, afterwards put at 18 °C or stay at 25 °C for rest of development.
- Dissections: Flies, which grew on 25 °C are dissected, which hedged on days 1-7 starting from the first fly hedges, while flies growing on 18 °C were dissected after 1-10 days after the first fly hedged.

5.3 Preparation of brains

The preparation of the brains happened in seven steps.

1. The brains are dissected in 1X Phosphate Buffer Saline (PBS).

2. After dissection they are fixed in PFA solution, either in 2% PFA for 1 hour or in 4% PFA for 20 minutes.

3. After the fixation the brains are washed four times in a PBT solution (0,3% TritonX-100 in PBS), for at least 10 minutes each time.

4. The brains are now blocked in 10% goat serum for 1 hour.

5. After removal of the goat Serum, the brains are then incubated with the first antibodies (1° ab) at 4 °C on the shaker overnight.

6. The brains are washed on the next day, as described in step (3). Afterwards the second antibodies (2° ab) are incubated at 4°C on the shaker overnight.

7. The brains are washed as described in step (3) and then mounted in Vectashield® on a slide. Now the brains are ready for the confocal microscopy.

5.4 Antibodies

Typ	Antibody	Origin
Primary	α-Fascilin2	Mouse
Primary	α-Ncad	Rat
Primary	α-GFP	Rabbit
Primary	α-Flag	Rat
Secondary	α-Rabbit 488	Goat
Secondary	α-Mouse High 488	Goat
Secondary	α-Mouse High 568	Goat
Secondary	α-Rat 647	Goat

Table 1: List of antibodies combinations used for staining

5.5 *D. melanogaster* Lines and Genetic Crosses

5.5.1 *D. melanogaster* Lines

Genotype	Source
Wnt4 protein localization	
;Wnt4$^{\text{nlsGFP-T2A}}$;	Bloomington Drosophila Stock Center (BL)#94901
Wnt4 mutations	
w^{1118};Wnt4$^{\text{C1}}$/II;	BL#6651
w^{1118};Wnt4$^{\text{C1}}$/CyO;	BL#6651
;Wnt4$^{\text{EMS23}}$,bw^{1}/II;	BL#6650
Wnt pathways components	
;dgo^{380}/II;	BL#41786
;Vang$^{\text{stbm6}}$/II;	BL#6918
;Vang$^{\text{stbm6}}$/II;fz^{23},Df(3l)fz^{2}/TM6B	Stock collection (Hummel lab)
dsh^{3}/Fm7c;;	Stock collection (Hummel lab): 738
w-,dsh^{1}/II;;	BL#5298

Table 2: *D. melanogaster* Lines utilized in the experiments.

5.5.2 *D. melanogaster* Crosses

Genotype (F1-generation)	Genotype (F0-generation), female	Genotype (F0-generation), male
Wnt4 protein localization		
;Wnt4-Gal4;UAS-Flag	;Wnt4-Gal4;	;;UAS-Flag
Wnt4 mutations		
;Wnt4$^{\text{EMS23}}$,bw^{1}/+;	CantonS	;Wnt4$^{\text{EMS23}}$,bw^{1}/II;
;Wnt4$^{\text{C1}}$/Wnt4$^{\text{EMS23}}$;	CantonS	w^{1118};Wnt4$^{\text{C1}}$/CyO;
Wnt4$^{\text{RNAi}}$		
;;UAS-Wnt4$^{\text{RNAi}}$/+	CantonS	;;UAS-Wnt4$^{\text{RNAi}}$
;Wnt4-Gal4/+;	CantonS	;Wnt4-Gal4/CyO;
;Wnt4-Gal4/+;UAS-Wnt4$^{\text{RNAi}}$/+	;;UAS-Wnt4$^{\text{RNAi}}$	;Wnt4-Gal4/CyO;
Wnt pathways components		
;;fz^{15}/fz^{23},Df(3l)fz^{2}	;;fz^{15}	;;fz^{23},Df(3l)fz^{2}
dsh^{3}/w-,dsh^{1};;	dsh^{3}/Fm7c;;	w-,dsh^{1}/II;;
Wnt4 mutations and Vang mutation		
;Wnt4$^{\text{C1}}$/Vang$^{\text{stbm6}}$;	w1118;Wnt4$^{\text{C1}}$/CyO;	;Vang$^{\text{stbm6}}$;
;Wnt4$^{\text{EMS23}}$/Vang$^{\text{stbm6}}$;	;Wnt4$^{\text{EMS23}}$,bw^{1}/II;	;Vang$^{\text{stbm6}}$;

Table 3: Genetic Crosses between Female and Male Individuals resulting in the required F1 Generation. Only the Genotypes used in the experiments are presented.

5.6 Genetic methods

5.6.1 Wnt4 localization

;Wnt4$^{\text{nlsGFP-T2A}}$; At the N-terminal end of the open reading frame, a sequence encoding GFP tagged with a single loxP site, a T2A sequence, and the nuclear localization signal (nls) has been inserted into the endogenous Wnt4 locus. The nls-GFP and Wnt4 proteins can be translated independently thanks to the T2A sequence, with the help of endogenous regulatory sequences. (Wnt4-nlsGFP-T2A).

;Wnt4-Gal4;UAS-Flag The binary expression system Gal4/UAS is used here. The expression of the Wnt4 protein leads to expression of the Flag protein. Flag is then easily stained via immunohistochemistry.

5.6.2 Wnt4 mutants

For this thesis two Wnt4 mutations were used: Wnt4$^{\text{EMS23}}$ and Wnt4$^{\text{C1}}$. Both Wnt4 mutations are located on the second chromosome of the *Drosophila melanogaster*. Different combinations were tested.

5.6.3 Wnt4$^{\text{RNAi}}$

The binary expression system Gal4/UAS-system is used in combination with RNA interference (RNAi). The expression of the Wnt4 gene is inhibited by the Wnt4$^{\text{RNAi}}$, when the Wnt4 protein is expressed via the Wnt4-Gal4 therefore binding of the Wnt4-Gal4 to the UAS-Wnt4$^{\text{RNAi}}$ leads to production of the Wnt4$^{\text{RNAi}}$. Wnt4$^{\text{RNAi}}$ inhibits the expression of the Wnt4 protein by binding to the Wnt4 gene sequence. This method was used to knock down the expression of the Wnt4 protein. As a control the lines, which were used for the binary expression system were crossed with CantonS flies.

5.6.4 Wnt signaling pathways mutants

Different mutants of the Wnt signaling pathway were used.

5.7 Imaging and processing

The brains were imaged with a Leica Confocal Microscope TCS SP5II. The scans were performed with a resolution of 512x512 pixels at 400 Hz, with a space of 1.5 μm between the single optical sections. The images were processed with a ImageJ/Fiji®. Statistical analysis and graph preparation was done with Rstudio.

6 Results

For simplification reason, the term "bilateral AB" and "unilateral AB" is used, which always means that the SA_{uni} neurons innervate the AB bilateral or unilateral with their axons.

6.1 Localization of Wnt4 protein

The localization of the Wnt4 protein provides insight into the region where Wnt4 may influence the morphological development of surrounding neurons. The Wnt4 protein is secreted on both sides of the central complex of the *D. melanogaster* brain (in pupa and adults, see Figures 5-7). While Wnt4 protein distributed on both sides in the central complex of the brain in the pupal brain 30-31 hours APF (see Figure 5), Wnt4 protein is localized in the FB columnar-neurons (PFN neurons) in the adult fly (see Figures 6-7).

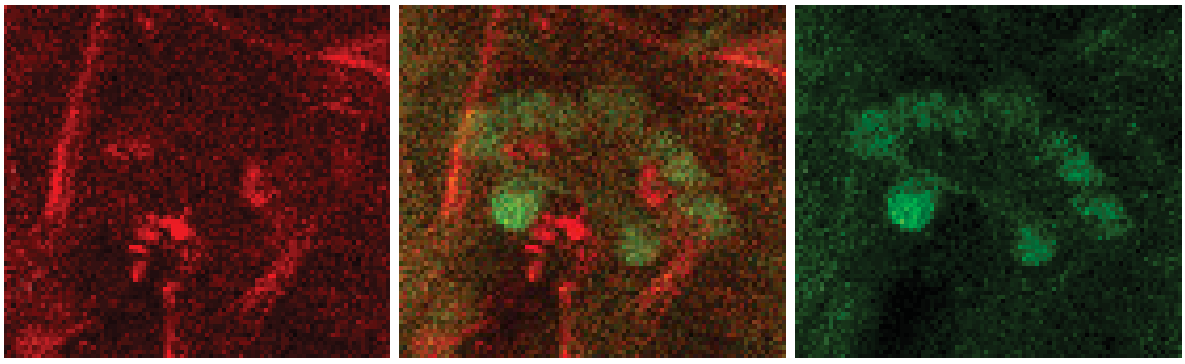

Figure 5: Localization of Wnt4 protein in ;Wnt4-nls-T2A-GFP; genotype in pupa 30-31 APF (n=4). Antibody staining: Red=Fas2, Green=Wnt4, Grey=Ncad. **All.** Background straining by Ncad protein **Left.** Fas2 protein staining shows a similar distribution on both sides of the central complex. **Middle.** Overlaying the staining signals reveals the distinct distribution patterns of Fas2 protein and Wnt4 protein. **Right.** Wnt4 protein is localized in precursor structures that later differentiate into structures of the central complex.

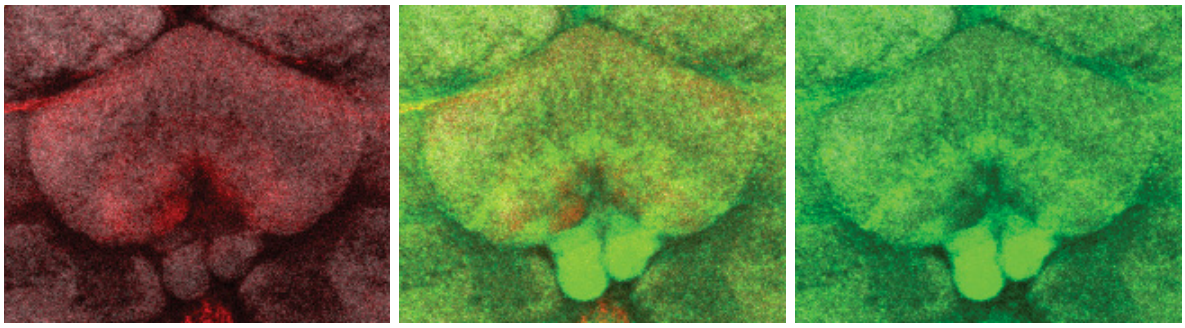

Figure 6: Localization of Wnt4 protein in ;Wnt4-nls-T2A-GFP; genotype in adults (n=41). Antibody staining: Red=Fas2, Green=Wnt4, Grey=Ncad. **All.** Background staining by Ncad protein. **Left.** Here Fas2 protein shows the AB, which is innervated by SA_{uni} neurons on the right side of AB. **Middle.** By overlaying the staining signals, no Wnt4 expression was detected in the SA_{uni} neurons. **Right.** Wnt4 protein is primarily localized in PFN neurons in the Noduli of the central complex.

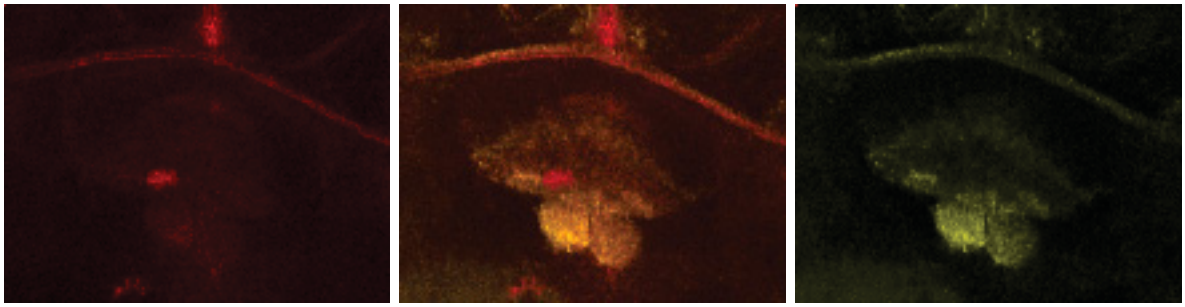

Figure 7: Localization of Wnt4 protein in ;Wnt4-Gal4;UAS-Flag genotype in adults (n=22). Antibody staining: Yellow=Flag, Red=Fas2. **Left.** Here Fas2 protein shows the AB, which is innervated by SA_{uni} neurons on the right side of AB. **Middle.** By overlaying staining signals, it becomes evident that Wnt4 is not in the SA_{uni} neurons,and thus not in the AB. **Right.** Wnt4 protein is mainly localized in Noduli of the central complex.

6.2 Wnt4

6.2.1 Wnt4 mutants

Different mutants of Wnt4 were used to analyze the possible effects of Wnt4 on the SA_{uni} neurons into the AB. First, the genotype *;$Wnt4^{EMS23}$/II;* show 50% bilateral ABs at 25 °C (n=72) while the other tested genotypes *;$Wnt4^{EMS23}$/+;* (n=27), *;$Wnt4^{EMS23}$/$Wnt4^{C1}$;* (n=36), *;$Wnt4^{C1}$/II;* (n=14) and *;$Wnt4^{C1}$/CyO;* (n=61) show ≤10% or no bilateral ABs at 25°C. Second, the same genotypes were tested at 18°C. Four out of five genotypes exhibited a higher number of bilateral ABs at 18°C compared to 25°C. *;$Wnt4^{EMS23}$/II;* (n=24), *;$Wnt4^{EMS23}$/+;* (n=6), *;$Wnt4^{EMS23}$/$Wnt4^{C1}$;* (n=16) and *;$Wnt4^{C1}$/CyO;* (n=41) show significantly higher effects at 18°C compared to those at 25°C. Only *;$Wnt4^{C1}$/II;* (n=10) showed a smaller difference with 10% bilateral brains and no significant difference. Nonetheless, it can be concluded that the flies raised at 18°C have more bilateral ABs than those raised at 25°C.

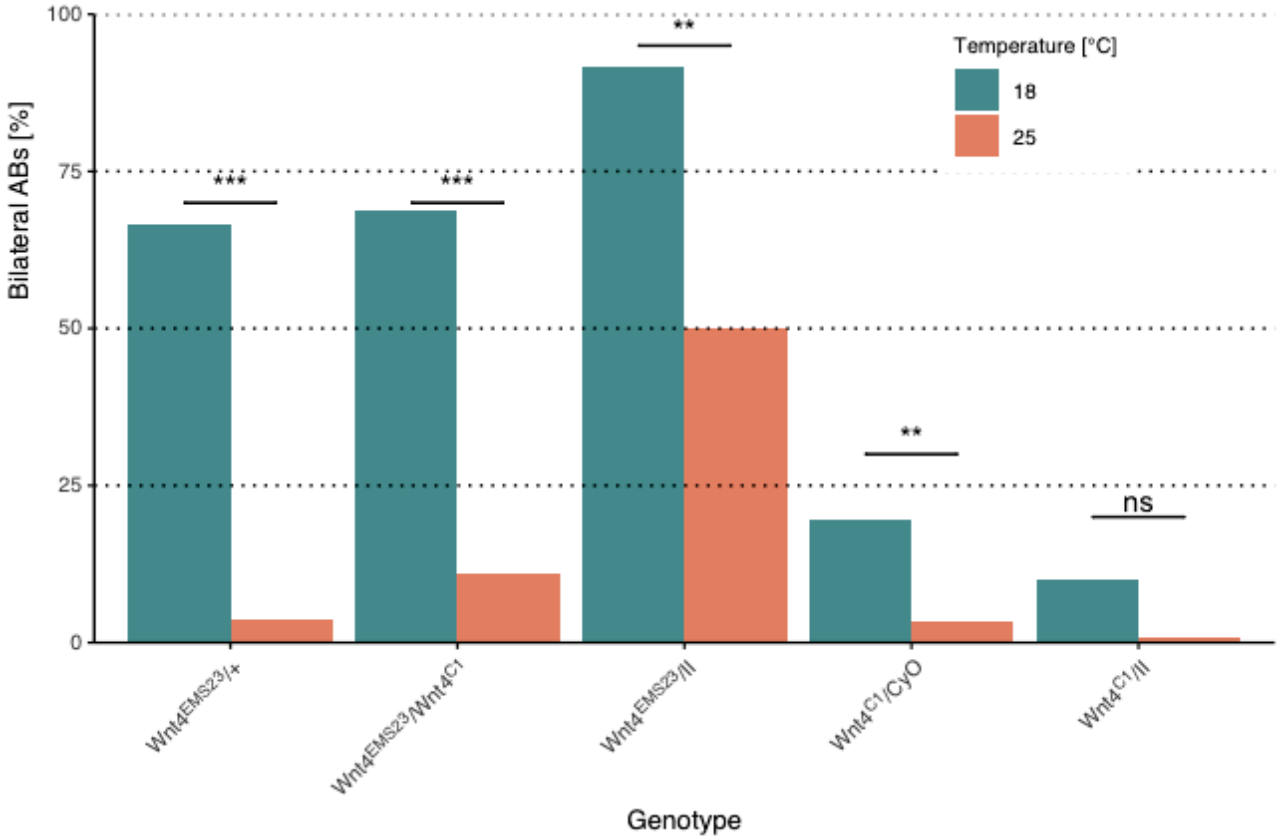

Figure 8: Wnt4 mutation lines and crosses. Temperature and genetic factors influence the innervation of SA_{uni} neurons in the AB. % of ABs with bilateral innervation of SA_{uni} neurons in different Wnt4 mutation combinations. χ^2-test, p-value: <0.001="***", <0.01)="**",<0.05="*", not significant="ns".

6.2.2 $\mathbf{Wnt4^{RNAi}}$

At 25 °C, the *Wnt-Gal4/+;UAS-Wnt4^{RNAi}/+* (n=28) genotype exhibits no significant effect on the bilateral innervation of SA_{uni} neurons, while at 18°C, *Wnt-Gal4/+;UAS-Wnt4^{RNAi}/+* (n=49) shows ∼45% bilateral ABs. Control crosses, *;UAS-Wnt4^{RNAi}/+;* (n=16) and *;Wnt4-Gal4/+;* (n=17) show no significant effects at 25°C. *;UAS-Wnt4^{RNAi}/+;* (n=14) shows no effect at 18°C, while *;Wnt4-Gal4/+;* (n=23) displays ∼25% bilateral ABs at 18°C.

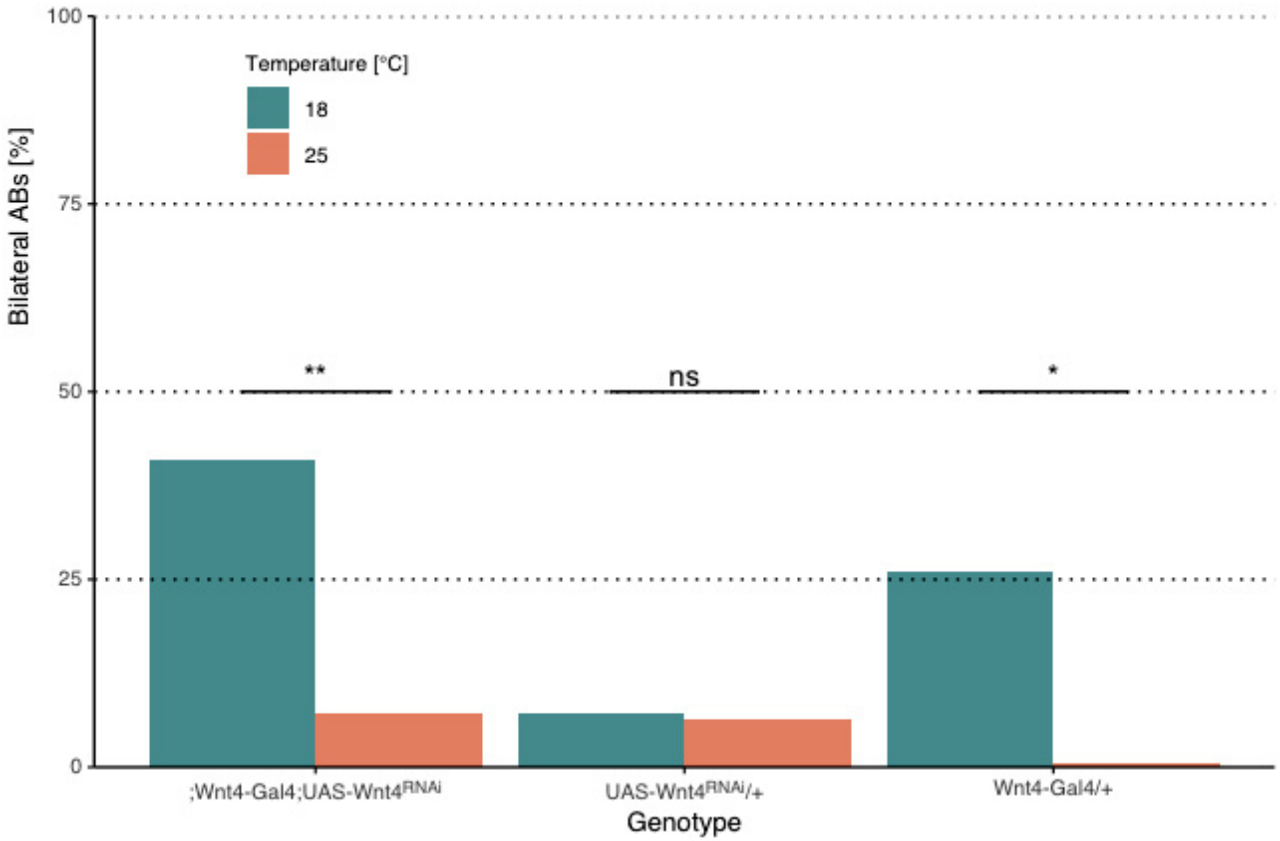

Figure 9: $Wnt4^{RNAi}$. Temperature and genetics effect innervation of SA_{uni} neurons in the AB. % of ABs with bilateral innervation of SA_{uni} neurons in different Wnt4 mutation combinations. χ^2-test, p-value: <0.001="***", <0.01)="**",<0.05="*", not significant="ns".

6.3 Wnt signaling pathways

The genotypes dsh^3/dsh^1;; (n=16) and dsh^1;; (n=11) show an increase of bilateral ABs, while dsh^3;; (n=23) has no effect at 25°C. At 18°C dsh^3/dsh^1;; (n=10), dsh^1;; (n=21) and dsh^3;; (n=18) exhibit a high frequency of bilateral ABs (>20%). $Vang^{stbm6}$ (n=23) leads to a small increase of bilateral innervation of SA_{uni} neurons at 25°C, while the increase at 18°C (n=28) is >45%. The $;Vang^{stbm6}/CyO;fz^{23},Df(3l)\text{-}fz^2/TM6B$; (n=21) which leads to a non-functional Vang receptor as well as non functional Fz^1 and Fz^2 receptors lead to a small increase of bilateral ABs at 25 °C, while flies with those phenotype (n=44) lead to no increase at 18°C. At 25°C $;;fz^{15}/fz^{23},Df(3l)fz^2$ (n=21), which has heterozygous non-functional fz^1 and fz^2 receptors lead to ∼12% bilateral ABs and dgo^{380}/II; (n=30), which leads to non-functional dgo show ∼12.5% bilateral ABs. $;;fz^{15}/fz^{23},Df(3l)fz^2$ (n=17) and $;dgo^{380}$; (n=30) show no effect on 18°C.

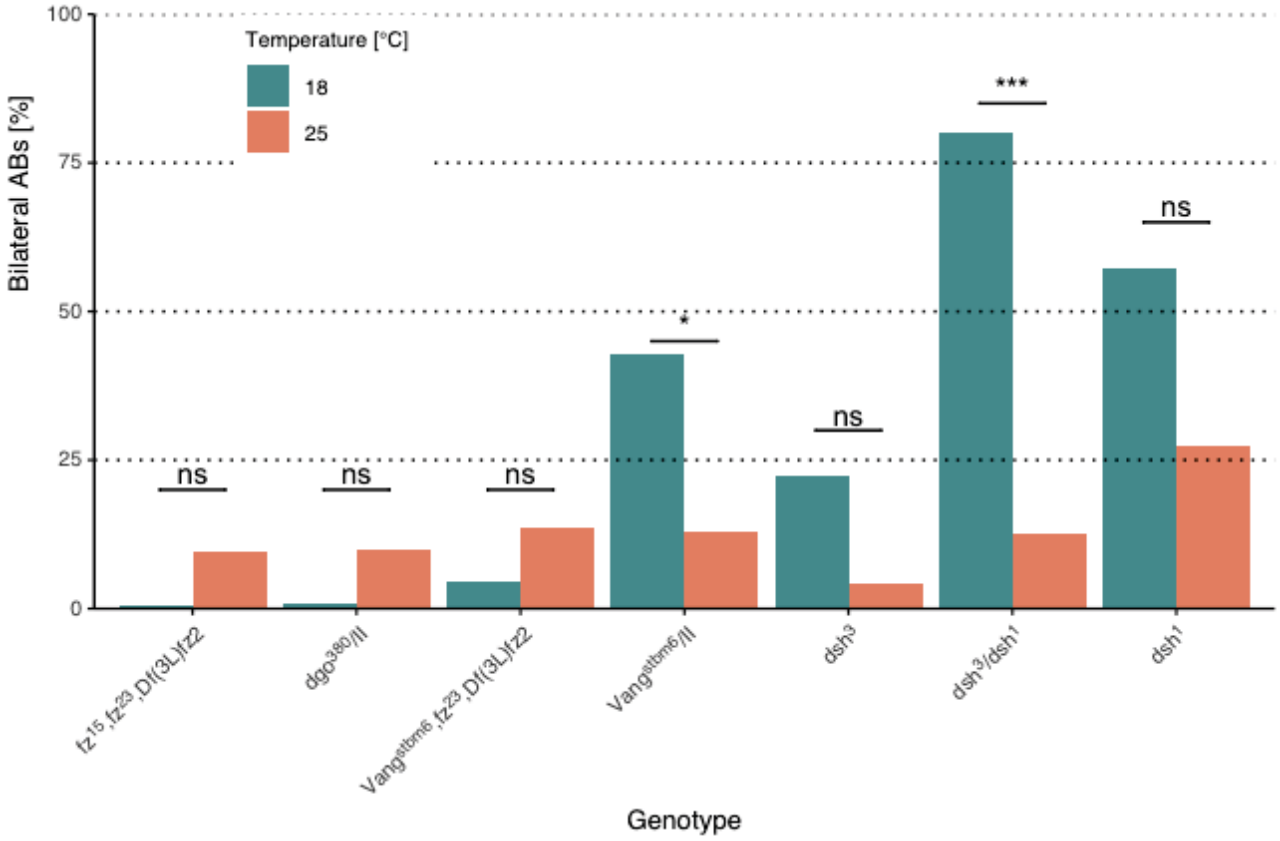

Figure 10: Mutations in Wnt pathway genes. Temperature and genetics effect innervation of SA_{uni} neurons in the AB. % of ABs with bilateral innervation of SA_{uni} neurons in different Wnt4 mutation combinations. χ^2-test, p-value: <0.001="***", <0.01)="**",<0.05="*", not significant="ns".

6.4 Wnt4 mutations and Vang mutation

There are no effects in *;$Wnt4^{EMS23}$/$Vang^{stbm6}$;* (n=3) and *;$Wnt4^{C1}$/$Vang^{stbm6}$;* (n=25) at 25°C as well as in *;$Wnt4^{EMS23}$/$Vang^{stbm6}$;* (n=24) and *;$Wnt4^{C1}$/$Vang^{stbm6}$;* (n=23) at 18°C.

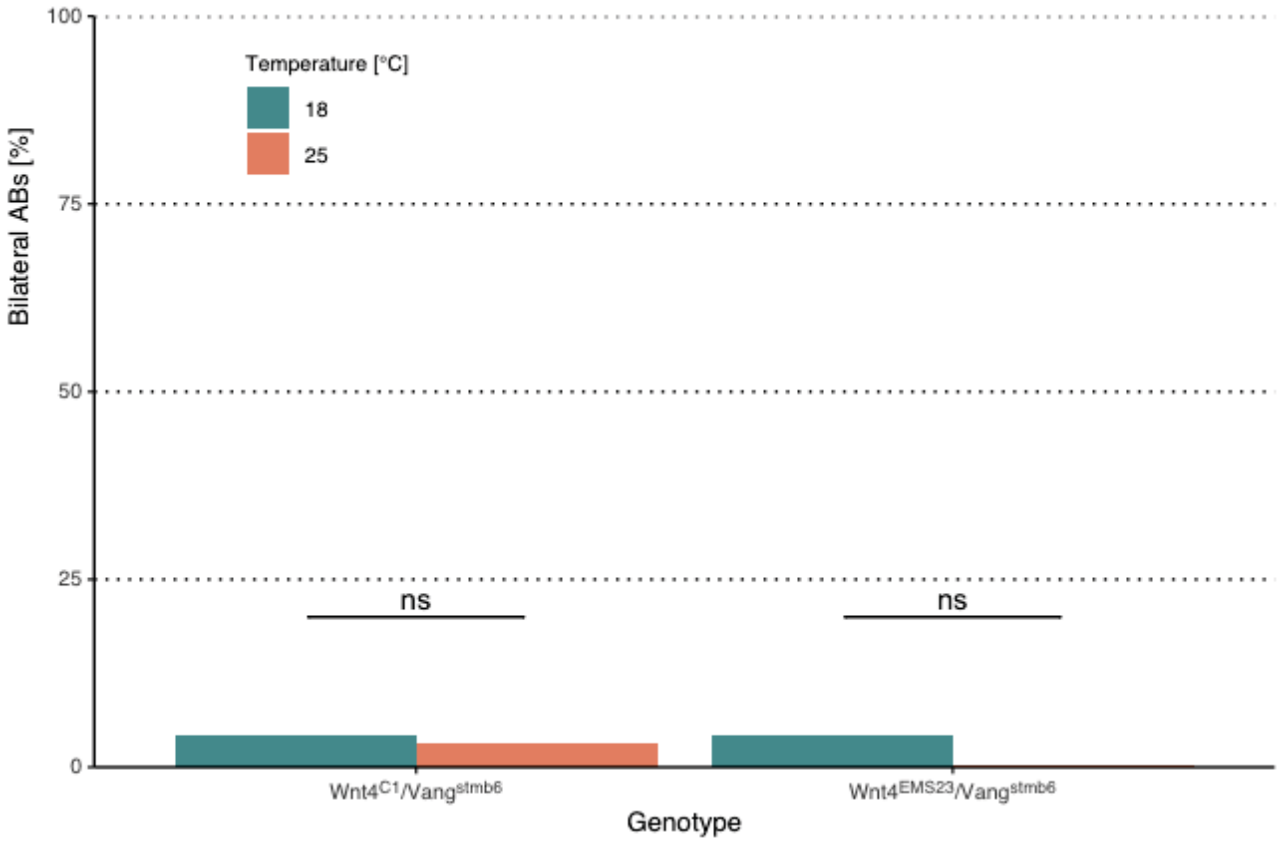

Figure 11: Mutated Wnt4 and mutant $Vang^{stbm6}$. Temperature and genetics effect innervation of SA_{uni} neurons in the AB. % of ABs with bilateral innervation of SA_{uni} neurons in different Wnt4 mutation combinations. χ^2-test, p-value: <0.001="***", <0.01)="**",<0.05="*", not significant="ns".

7 Discussion

While the concept of asymmetry is an widely established concept, it is not finally known how neurons grow asymmetrically in specific structures, while others stay symmetric. The SA_{uni} neurons are the main interest in this thesis. Wnt4 protein and Wnt signaling pathway components are considered potential pieces of the still unsolved puzzle. Overall, the findings suggest that lower temperature (18°C) disrupt the developmental process of SA_{uni} neuron innervation into the AB.

7.1 Localization of Wnt4 protein

The localization data of the Wnt4 protein show that Wnt4 protein is localized in the precursor structures of the central complex in pupae at 30–31h APF. Thereby it can be concluded that Wnt4 protein could influence the development of the neurons in this early stage. In adult flies, Wnt4 protein is found in the PFN neurons, which innervate the noduli in the central complex.

There is an indication over all samples, that Wnt4 stays in the central complex during the development. Based on observations in pupae at 30–31 hours APF, Wnt4 appears to play a potential role in the neuronal development of the central complex. In all genotypes, which were used for localization of Wnt4 protein, Wnt4 protein exhibits a symmetrical distribution in the brain, regardless of the unilateral or bilateral nature of SA^{uni} innervation in the AB in individual adult flies.

7.2 Wnt4

7.2.1 Wnt4 mutants

Wnt4 is actively involved in the axon guidance in *D. melanogaster*, for example as attractive cue in the eyes [15] as well as a repulsive cue in muscles[16]. Under comfortable temperature (25 °C) for flies, only the flies with the *;Wnt4^{EMS23};* genotype show more bilateral ABs compared to all other tested lines and crosses. The controversially results of those *;Wnt4^{EMS23};* flies could be explained by the unknown genetic background beside the Wnt4 mutation of those flies. It is not known, if there are also other mutations, which inhibit the SA_{uni} neurons from retraction from the left AB.
In summary, the development of the SA_{uni} neurons and thereby the innervation of those into the AB does not appear to be regulated by Wnt4 protein.

7.2.2 $Wnt4^{RNAi}$

To test, if a downregulation of Wnt4 leads to any effect, RNAi method was used. The downregulation of Wnt4 protein by *;Wnt4-Gal4;UAS-Wnt4^{RNAi}* leads to no phenotype at 25 °C. In this experiment the lower temperature (18 °C) plays a role, leading to ∼40% bilateral phenotypes. This findings underline the findings of the experiment with the Wnt4 mutants, which lead to the conclusion that Wnt4 itself does not play a role in this pathway.

7.3 Wnt signaling pathways

The observed effects of the *dsh* mutations indicate that the dsh protein is important for SA_{uni} neuron innervation, though not in an all-or-nothing manner; instead, its role appears to be relative, influencing but not solely determining the developmental outcome.
Vang^{stmb6}, which is a loss-of-function mutation of Vang, likely results in a non-functional Wnt

receptor, potentially impairing the Wnt/PCP signaling pathway. As a result, Wnt proteins may fail to bind appropriately, and the downstream signaling cascade is not activated. Conversely, loss of the Vang receptor and loss of fz^1 and fz^2 receptors leads to only a minor effect on the bilateral innervation of SA_{uni} neurons. These findings suggest that, in the absence of functional Wnt/PCP signaling, an alternative signaling pathway may be engaged to compensate for the loss, leading to the observed pattern of SA_{uni} neuron retraction from the left side of the AB.
The loss of dgo lead to more bilateral brains at 25 °C, the effect completely disappears at 18°C. Therefore dgo mutants seems to stabilize the innervation process under temperature stress.

7.4 Temperature

Both bilateral ABs [62] and sub-adult heat stress [71] are known to impair long-term memory performance. This raises the question of whether the reduced performance under heat stress is also linked to the presence of bilateral ABs.
So it seems that leaving the comfort zone of the flies lead to a stress response and therefore reduced ability for the retraction of SA_{uni} neurons in the AB. The lower temperature plays a role in all tested parts of this thesis. It leads to more bilateral ABs in most genotypes at 18 °C, which indicates for reduction in long-term memory performance. While there were no behavioral tests done in this thesis, this part stays speculative. In the $Wnt4^{EMS23}$ mutants the effects are extremely high, which indicates a different binding motif of this mutant Wnt4. This could be to an effect on protein folding or localization, which can be induced by temperature [66]. A similar effect as in $Wnt4^{EMS23}$ was seen in the $Wnt4^{RNAi}$ experiment, where lower temperature leads to significant more bilateral ABs, indicating some kind of stress response. Mutations in *dsh* indicate that the Dsh protein has a modulatory effect on the development of SA_{uni} neurons, influencing the process without fully determining the outcome.

7.5 Wnt4 mutations and Vang mutation

As an additional experiment, a combination of $Vang^{stbm6}$ and Wnt4 mutants was tested. While $Vang^{stbm}$ as well as the Wnt4 mutants ($Wnt4^{EMS23}$, $Wnt4^{C1}$) individually showed some bilateral ABs, the combination did not result in any significant difference across all variants. This suggests that the interaction between these genes is not critical for the development of SA_{uni} neurons, which aligns with previous findings indicating that *Wnt4* is not involved in SA_{uni} developmental process.

7.6 Next steps

Within the scope of this thesis, the factors regulating the developmental innervation of SA_{uni} neurons into the AB remain unresolved; however, it can be definitively concluded that Wnt4 is not a regulator of this process. As a next step the remaining Wnt proteins could be tested, in view of the Wnt signaling pathway, were some components already seem the have an relative impact on the developmental process of the SA_{uni} neurons. The fact that lower temperature leads to a higher bilateral AB rate in most genotypes leaves the question, which effect temperature has on the development of the central complex and more specific on the neurons innervating the AB. It would be highly interesting to get further insides into the complexity of this system, which seems to be easily disturbed by lower temperature. Based on this thesis it would be highly interesting if lower temperature and bilateral AB are directly connected and show an effect on the long-term memory in *D. melanogaster*. Furthermore it seems that temperature itself induce less asymmetry. Temperature, asymmetry, cognition and mental health form a composite in which all of them are connected to each other.

7.7 Conclusion

Asymmetries cover a broad research area that spans multiple levels of biological organization, ranging from molecular and cellular asymmetries to morphological and behavioral asymmetries. This thesis aimed to determine whether Wnt4 plays a role in the development of nervous system asymmetries, specifically whether SA_{uni} neurons are influenced by Wnt4. The results clearly indicate that Wnt4 does not have a direct role; however, the involvement of the broader Wnt signaling pathway cannot be excluded. Numerous questions remain open and should be addressed in future studies to elucidate the influence of these pathways and other Wnt proteins. The most striking finding is the significant impact of temperature on the majority of tested genotypes, which was previously unforeseen. This observation opens substantial potential for future research aimed at elucidating how temperature variations affect the development of neurons associated with the AB.

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