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Introduction

In order for an organism to develop, grow, mature and function properly the cells within that organism need to communicate with each other and their environment. *wnt* genes encode highly conserved secreted proteins that are essential for cell-to-cell communication during the development of a variety of organisms. They play diverse roles in nervous system development, adult homeostasis, regeneration and stem cell specification. When misregulated they can cause various diseases such as cancer, tetra-amelia, bone density defects and vascular defects. Unraveling the signaling cascades mediated by these extracellular signals provides a greater comprehension of how organisms develop and function, and moreover offers opportunities to manipulate these signals for therapeutic benefit.

1.1. WNT SIGNALING IN THE CNS

The first member of the Wnt family (WNT1) was discovered in a retroviral insertion mutagenesis screen by Nusse and Varmus in 1982 and cloned as a candidate proto-oncogene (1,2). Over the years many more *wnt* genes were identified in a number of species: there are 19 mammalian Wnt family homologues (3,4). This family of secreted lipid-modified glycoproteins (reviewed by (5,6)) is highly conserved amongst vertebrates; all Wnts contain a signal sequence for secretion, a number of highly charged amino-acid residues and many potential glycosylation sites. They also have 22 cysteine residues that are invariably spaced within Wnt proteins, suggesting that protein folding requires the formation of multiple intramolecular disulfide bonds (7). Their hydrophobic character came to light when trying to purify active Wnt protein. Analyses of the Wnt proteins by use of mass spectroscopy unveiled that the first absolutely conserved cysteine residue within the Wnt protein family is modified by the attachment of a palmitate moiety (8). Accordingly, most Wnts were found to be associated to cell membranes and the extracellular matrix (ECM) (9). Up till now there have been two papers on the crystal structure of the interaction between Wnt and a receptor (10,11) that will certainly aid in elucidating binding specificities and provide insights for designing specific inhibitors to fight cancer.

Despite these similarities Wnts are very divergent in the functions they can perform, due to specific sequence variations between family members, but also due to their highly divergent temporal and spatial expression patterns. Remarkably we find a diverse Wnt protein collection already present in Cnidarians, an ancient metazoan phylum containing over 10,000 species amongst which are fresh water polyps and sea anemones (12). Besides the fact that this indicates the early time point for *wnt* gene diversity in evolution, more importantly it denotes that it has remained essential for the proper development of multicellular organisms (13). The intricacy and complexity of Wnt-signaling is significantly enhanced by the plethora of potential Wnt-receptors, enabling the Wnt-family to activate a number of different signaling

pathways. Emerging data suggests there is considerable crosstalk between the different Wnt pathways, implying that these pathways are not autonomous and therefore not to be thought of as separate, linear pathways. In this chapter I will very briefly discuss the different Wnt-pathways known to date and introduce our model system; *Drosophila melanogaster*. Both the nervous system and musculature of *Drosophila*/fruit fly were employed to study Wnt-signaling through RYK which is the subject of the work presented in the remainder of this thesis.

1.1.1 The Canonical WNT Pathway

The canonical Wnt pathway is the most intensely studied Wnt pathway (reviewed by (14). A number of the components downstream of the canonical Wnt ligand have been identified and their roles in development and their involvement in a variety of cancers elucidated. In the absence of Wnt-signaling the complex of Axin, Adenomatous polyposis coli (APC),

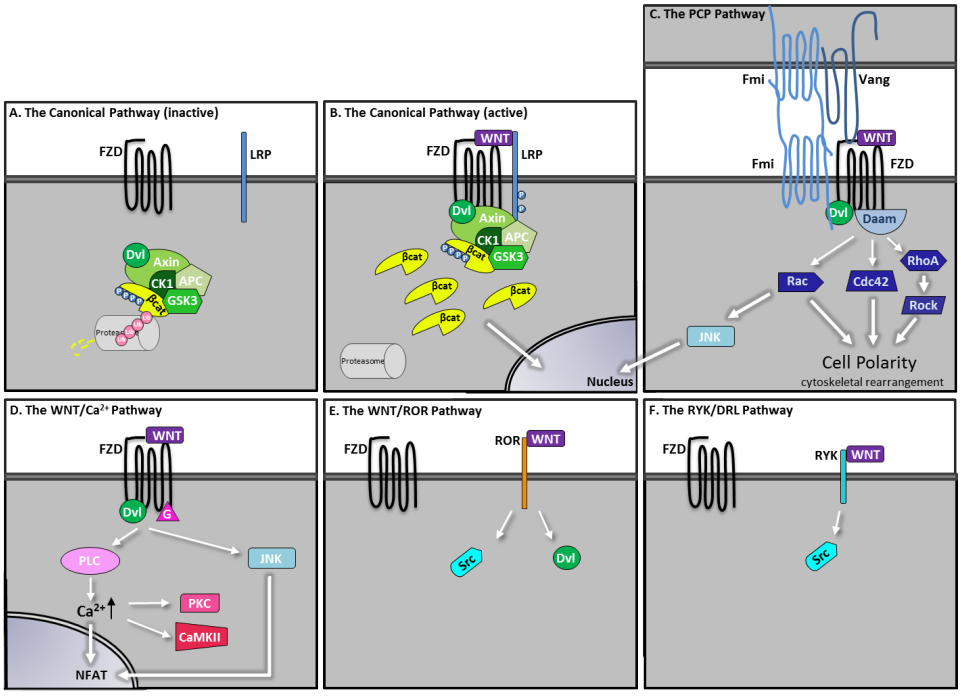

Fig 1. Schematic representation of the known Wnt-pathways. The inactive (A) canonical pathway is portrayed and ends with the degradation of β-catenin (βcat). When Wnt binds the complex of Frizzled (FZD) and Low Density Lipoprotein (LDL) receptor related protein (LRP), β-catenin accumulates and translocates to the nucleus where it regulates targeted gene expression. In the Planar Cell Polarity pathway (C) Wnt binds to another complex of FZD with Flamingo (Fmi) and Van Gogh (Vang) to ultimately guide cell polarity and cytoskeletal rearrangement. The Calcium pathway (D) activates transcription factors through another cascade of proteins. In (E) and (F) two other receptors are shown that are receptors for Wnt and provide a downstream signal upon ligand-binding. All of the above pathways will be addressed in more detail below.

Casein Kinase 1α (CK1α) and glycogen synthase kinase 3 (GSK3) is responsible for the degradation of cytosolic β-catenin, keeping cytoplasmic levels low (15–17). In the presence of the canonical Wnts (Wnt1, Wnt3, Wnt3a, Wnt8 or Wnt8a) β-catenin accumulates in the cytosol and subsequently translocates to the nucleus, where it affects transcription. Many studies have focused on the interactions of Wnt ligands with their canonical receptors, the Frizzled proteins (18,19). Once Wnts are secreted they can bind to the extracellular Cysteine Rich Domain (CRD) of the Frizzled (FZD) protein family (20,21) that is in a complex with the low density lipoprotein (LDL) receptor related protein (LRP5/6) (22–25). FZDs are sevenpass-transmembrane receptors of which 10 family members are found in mice and humans and 4 family members are present in *C. Elegans* and *Drosophila*. When Wnt binds the receptor complex of FZD and LRP this induces phosphorylation of LRP that allows for the subsequent recruitment of Axin (26–28). Another protein called Dishevelled (Dsh) is also phosphorylated in a FZD dependent fashion (29,30) and inhibits the complex formed by Axin, APC and GSK3. This inhibition leads to the prevention of GSK3-dependent phosphorylation of β-catenin (31), resulting in the accumulation of β-catenin in the cytosol and translocation into the nucleus. Nuclear β-catenin regulates target gene expression by displacing Groucho and binding to a family of transcription factors named T cell-specific transcription factor/lymphoid enhancer-binding factor 1 (TCF/LEF) (32–35) and induces the transcription of Wnt-target genes. A comprehensive overview of Wnt target genes known up to date have been listed by Roel Nusse on his website; http://www.stanford.edu/group/nusselab/cgi-bin/Wnt/target_genes.

1.1.2. The WNT/Planar Cell Polarity Pathway

The polarity of cells is essential for developing embryos, allowing cells to align correctly with respect to each other and orient specifically with respect to tissue axes within the plane of a sheet. The Wnt/Planar Cell Polarity (PCP) pathway regulates different cellular behaviors such as cell motility and polarity that are mediated by cytoskeletal remodeling (reviewed in (36,37). A number of the components of the PCP pathway were first identified in *Drosophila* using genetic screens, molecular cloning and functional analyses of the patterning of the adult wing, abdomen, notum and eye (38–41). FZD was found to be an essential component of PCP signaling (42–44). The extracellular domain (ECD) of FZD is able to interact with the transmembrane protein Van Gogh (Vang) that resides at the plasma membrane (PM) of a neighboring cell (45). This intercellular contact between FZD and Vang is facilitated by homophilic interactions of two Flamingo (Fmi) proteins that bridge the two neighboring cells and interact with both FZD and Vang on either side (46–49). However, the ligand interacting with FZD to activate the *Drosophila* Wnt/PCP pathway remains to be identified. It has been shown, that Wingless (Wg) and DWNT4 can modulate the interactions between FZD and Vang (50). Furthermore, there is evidence that the vertebrate PCP signaling is established with the help of Wnt5a and Wnt11 (51–55). Nonetheless, it is thought that downstream of FZD

through Dsh, two parallel pathways lead to the activation of the small GTPases Rho and Rac (41,56–59). The small GTPase Rac can activate the c-Jun N-terminal kinase (JNK) (60,61), the other small GTPase Rho recruits Dishevelled associated activator of morphogenesis (DAAM1) (62), leading to the activation of Rho associated kinase (ROK) (63–66). Both parallel pathways mediate the reorganization of the actin cytoskeleton that is necessary to assume the correct orientation and position of the individual cells within a tissue.

1.1.3. The WNT/Ca^{2+} Pathway

Activation of the Wnt/Ca^{2+} pathway via the FZD receptor leads to an increase in the concentration of Ca^{2+} that subsequently leads to a modulation of the expression of target genes, resulting in cellular responses, such as changes in cell movement and cell adhesion. There are reports that show an increase in the release of intracellular calcium ions for WNT4, WNT5A and WNT11 (67–71). It is thought that after FZD activation, signaling is transduced via heterotrimeric G proteins that can activate phosphodiesterase 6 (PDE6) and Phospholipase C (PLC), of which PLC gives rise to Inositol triphosphate (IP_3) and Diacylglycerol (DAG). IP_3 induces Ca^{2+} release from the ER which together with the abundantly present calmodulin activates CamKII (72). Whereas DAG activates protein kinase C (PKC) (73), both CamKII (67–71) and PKC can induce the nuclear transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-KB). PKC is also able to induce another transcription factor; cAMP response element-binding protein (CREB), and the released Ca^{2+} can activate the widely expressed protein phosphatase calcineurin (Cn) that induces Nuclear Factor of Activated T-cells (NFAT) (74)], a third transcription factor. These transcription factors can modulate the expression of several target genes thereby altering cellular behavior.

1.1.4. The WNT/ROR pathway

In contrast to the above described Wnt pathways the WNT/ROR pathway does not act via the FZD receptor, but signals through the receptor tyrosine kinase-like orphan receptor (ROR) (75–78). Interestingly, a number of examples have been described in which this pathway feeds back into the canonical Wnt pathway and the PCP pathway (79–82). There is also a study that shows signaling of WNT5a through ROR that is independent of FZD as a co-receptor (83).

ROR is a member of the receptor tyrosine kinase family of proteins. A number of organisms express two structurally related members, ROR1 and ROR2. *Drosophila* has two Ror orthologues named ROR and NRK. They are conserved amongst species and their extracellular domains (ECDs) contain an immunoglobin (Ig) domain, a Frizzled-like cysteine-rich domain (CRD) and a Kringle (Kr) domain (84). The CRD is crucial for the interaction with Wnt ligands (75–77,85–88). The functions for the Kringle domain have not yet been resolved,

but are presumed to bind other Wnt regulatory proteins (89,90). The Ig-like domains, found in the N-terminal extracellular regions, are present in most species, except for *Drosophila* ROR and NRK. The Ig-like domains can be found in many more proteins and seem to be involved in protein-protein and protein-ligand interactions and are implicated in cell adhesion.

The intracellular domain possesses a tyrosine kinase domain; a proline-rich domain (PRD) that is surrounded by two Ser/Thr-domains; Ser/Thr1 and Ser/Thr2 (84). The combination of a PRD and two Ser/Thr-domains have not been found in any other protein, but are thought to interact with signaling mediators. The PRD domain of ROR2 was found to associate with the actin binding protein filamin A (FLNa) and this interaction is essential in filopodia formation and Wnt5a-induced cell migration (91). The PRD domain was also found to be responsible for the recruitment of SRC leading to the phosphorylation of mROR2 causing activation of the receptor (92). Furthermore, the ROR cytoplasmic domain contains potential phosphorylation sites and several consensus motifs for protein-protein interactions, such as the WW domain, the SH3 motif, and the SH2 motif. These domains are likely required for the association of Ror-family members with adaptors and signaling molecules in the signaling transduction cascade.

In vertebrates, the data so far suggests that Ror signaling is mediated via the non-canonical Wnt, WNT5a. WNT5a/ROR2 signaling has, amongst others, been shown to play a crucial role in inhibiting WNT3a mediated canonical Wnt signaling at the level of TCF/LEF-mediated transcription (76,93,94). The downstream signaling partners in the Ror pathway that have been elucidated so far are Dsh, that is phosphorylated in a WNT5a/ROR dependent fashion (83) and SRC that has been shown to phosphorylate mROR2 (92)(reviewed in Petrova et al, 2014 (78)).

The expression of Rors during development has been addressed in several papers (see review (78,95). *ror2* mutant mice die neonatally likely due to respiratory dysfunction and cyanosis, just as observed in *wnt5a* mutants and *ror1* mutants. The phenotypes of *ror2* and *wnt5* mutant mice are similar and exhibit dwarfism, facial anomalies, short limbs and tails (76,96–98). Although *ror1* mutant mice die like their *wnt5a* and *ror2* mutant counterparts they do not display any of the apparent morphological phenotypes and their size is comparable to wild-type mice (99).

In humans, mutations within *ror2* gene cause autosomal dominant brachydactyly type B (BDB) (100,101), and an autosomal recessive form of Robinow syndrome (RRS), (102,103) reviewed in (104,105). Increased expression of ROR2 has been observed in squamous cell carcinoma of the oral cavity in which expression levels correlate to the degree of the

malignancy (106). Activation of non-canonical WNT5a/ROR2 signaling causes osteosarcoma cell lines to demonstrate invasive properties in vitro (107). ROR1 overexpression is associated with hematopoietic malignancies of B-cell lineage in humans (108–112). More research is needed to elucidate the signaling cascade of Rors.

1.1.5 The WNT/RYK/DRL Pathway

One of the more recently identified Wnt driven pathways is employing RYK as a receptor (reviewed in (113)). The first report published that RYK is a receptor for WNT5 was in 2003

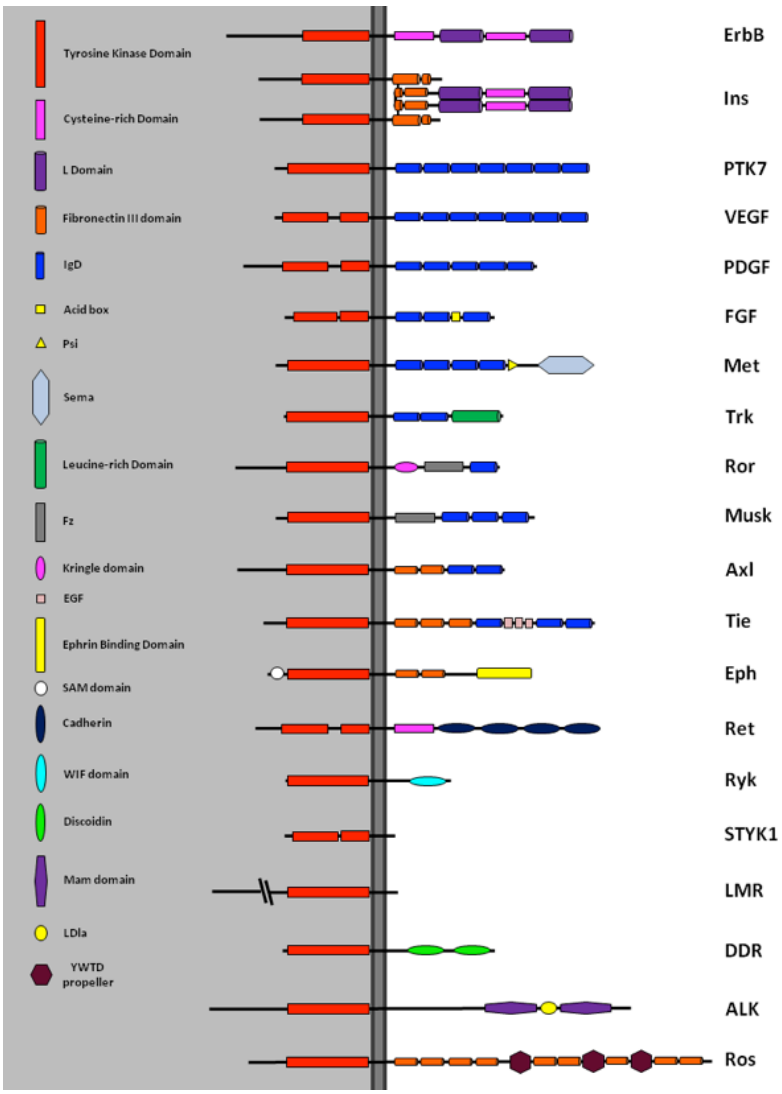

Fig 2 A schematic representation of the 20 subfamilies that make up the receptor tyrosine kinase family. The red rectangles illustrate the intracellular tyrosine kinase domain(s).

in *Drosophila* (114) that showed that WNT5 repels RYK expressing axons, playing a role in axon guidance in the ventral nerve cord during embryogenesis. In mice it was also shown that RYK expressing axons could be repelled by WNT5a as well as WNT1 (115,116). In zebrafish the interaction between WNT5b and RYK was found to regulate the directional cell movement during gastrulation (117) which was also affected in the African clawed frog (118) yet here RYK employed besides WNT11 also co-receptor Frizzled 7. RYKs are members of the receptor tyrosine kinase (RTK) family. This family is composed of 58 receptors that span the membrane once, have an extracellular ligand binding domain that can bind growth factors, cytokines and hormones with high affinity and transduce signals via their cytoplasmic tyrosine kinase domain (reviewed in (119,120)). These signals regulate a range of important cellular responses but can also play a critical role in the development and progression of cancer.

The RTK family can be subdivided in different classes according to function, of which class XVI describes receptor related to tyrosine kinase (RYK) that was uncovered by a PCR-based screen for novel RTKs in 1992 (121,122). The conservation of RYK between species is relatively high: comparison between human and mouse RYK indicates that the homology at the amino acid level is 97%. Single RYK orthologues have also been identified in other organisms, like *Danio rerio*, *Xenopus laevis*, *Caenorhabditis elegans*, however *Drosophila melanogaster* has three orthologues; Derailed (DRL), Derailed-2 (DRL-2) and Doughnut on 2 (DNT) (exhibiting 60% amino acid (aa) identity to DRL) (113,123). Downstream of DRL it was shown that DRL can interact with kinase SRC in the *Drosophila* nervous system (124). Before the WNT5/RYK/DRL pathway is examined in more detail, the model system *Drosophila melanogaster* is introduced in the next paragraphs.

1.2. *DROSOPHILA MELANOGASTER* AS A MODEL ORGANISM

For over a century *Drosophila* has been used in scientific research. Initially, it was the ease of forward genetics that made the fruit fly an attractive model and enabled the discovery of genes that are involved in body patterning, organogenesis, nervous system development, muscle development, but also genes required for learning/memory, pain, audition, olfactory, vision and courtship (125). The *Drosophila* generation time is about ten days when kept at 25°C: the *Drosophila* life-cycle starts with the embryonic stage followed by three larval instar stages (molds) and a pupa stage giving ultimately rise to an adult fly. Genetic manipulation and the generation of transgenic flies became technically possible with the development of the P-element mediated germ line transformation technique established by Spradling and Rubin in 1982 (126). A further important advance for the study of gene function was made by Andrea Brand and Norbert Perrimon in 1993 (127) who developed a method which

makes use of a bipartite yeast Gal4/UAS system to allow stable differential spatial-temporal transgenic expression of any gene of interest in the fly genome. The Gal4 gene encodes the yeast transcription protein that specifically binds the UAS (upstream activating sequence), a short section of the promoter region. Furthermore there are many tissue specific Gal4 drivers publicly available, that allows expression of your gene of interest in a temporal/spatial pattern in a specific genetic background. The *Drosophila* genome was fully sequenced and published in 2000 which greatly added the implementation of novel genome wide reverse genetic approaches to uncover gene function. In 2001 Rong and Golic (128) introduced homologous recombination in the fruit fly, and more recently, a publicly available genome-comprehensive transgenic RNAi library has been developed for knock down expression in specific tissues. This is especially useful for mutations that cause lethality, since dsRNA against your gene of interest can be expressed with the Gal4/UAS system in a restricted pattern. The genetic knock down in a desired spatial/temporal pattern allows the study of its consequences where that is impossible in a complete knock out that could be potentially lethal. As of 2012 the CRISPR/Cas system (clustered regularly interspaced short palindromic repeat/CRISPR-associated) is the newest genetic editing tool. In origin it is a bacterial defense system that protects bacteria against invading viruses and plasmids. It has been adapted to create targeted double strand break(s) (DSB) in the genome. A single DSB will lead to small deletions and insertions by non-homologous end joining at the target site, whereas a double cut can lead to bigger deletions of the target area. In addition, homologous recombination with a desired template allows even more possibilities to edit the genome. Interestingly, of all known genes that cause human disease about 75% of them have a conserved orthologue in the genome of *Drosophila* (129).

1.2.1 The *Drosophila* Central Nervous System

The brain and spinal cord, which make up the Central Nervous System (CNS), receive and interpret signals from the peripheral nervous system. Neurons in the brain extend their axons to their targets throughout development. During this growth they interact with specific guidance cues through cell adhesion molecules (CAMs) to direct them to their target region and to establish appropriate connections called synapses (130). In order to facilitate correct navigation and migration the milieu for growing axons is requiring temporal and spatial control of many and often dynamic cues. Some of these axons can traverse large distances (many centimeters or even meters), creating an elaborate and precise network throughout the nervous system. The known proteins that are involved in axon guidance during development are in many cases conserved amongst a large variety of vertebrate and invertebrate species (reviewed in (131,132)).

The *Drosophila* brain develops from the neuroectoderm that gives rise to either neuroblasts or progenitor cells of the epidermis (133). Approximately 500 neuroblasts progress deeper into the embryo to produce a central neural primordium. The progenitors of the epidermis give rise to the epidermal sheet as well as to the peripheral nervous system (PNS). An overview of the embryonal nervous system situated within the embryo around stage 16 can be seen in Fig 3A. The BP102 antibody highlights all CNS axons present within the embryo. The top of the is the anterior part of the embryo containing the developing brain hemispheres and the ventral nerve cord (VNC) can be seen running down from the brain hemispheres to the posterior

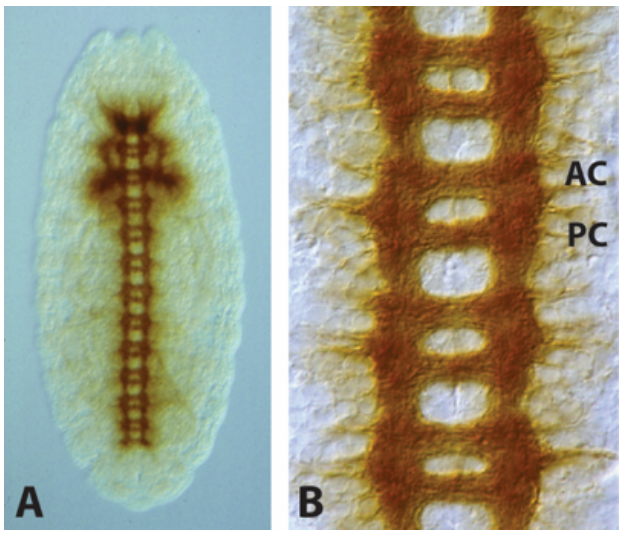

Fig 3 The nervous system of a *Drosophila* embryo at stage 16 (3A) with two bilateral brain lobes and the ventral nerve cord in situ, with two longitudinal tracts that run along either side of the midline. The zoom in (B) on four embryonal segments provides more detail for the anterior commissures (AC) and the posterior commissures that create an environment in each segment where axons traverse the midline. Anterior is up.

part of the embryo. The neurons send their axons down the VNC in the embryo along two longitudinal tracts that run alongside the midline. In each embryonal segment axons can cross the midline through either the anterior commissure (AC) or the posterior commissure (PC) (Fig 3B) and continue to their respective target site and establish a synaptic contact.

During embryogenesis the neuroblasts form three neuromeres/neural segments: the protocerebrum, deutocerebrum and the tritocerebrum which make up the brain hemisphere at the larval stage. This brain hemisphere of the larvae becomes the supraesophageal ganglion of the adult (133), whereas the neuromeres of the mandible, maxilla and labium (three gnathal segments) give rise to the subesophageal ganglion. The eye-antennal disc

does not develop till the third instar larvae stadium, generating the antennal and optic lobes. The major brain centers that ultimately make up the adult *Drosophila* brain are depicted in the figure 4. Two brain structures, the antennal lobes and the mushroom bodies have been the subject of intense study and have been shown to be required for proper behavior and

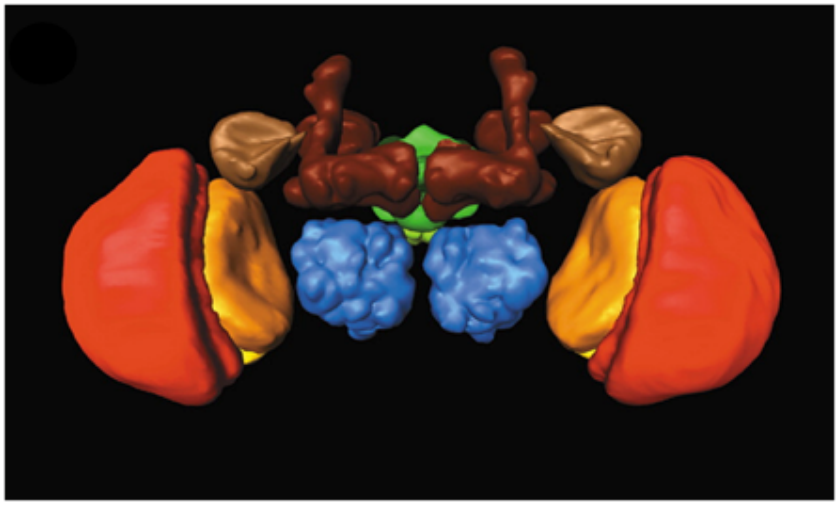

Fig 4 A polygonal brain model of an adult brain generated from an individual labeled data set. Colour code: Red: medulla; orange: lobula; yellow lobula plate; chocolate brown: mushroom body; beige; lateral horn; blue: antennal lobe; olive green: noduli; dark green: ellipsoid body; light green: fan-shaped body; grayish green: protocerebral ridge (not visible, behind fan-shaped body). Taken from: Rein, K; Zöckler, M et al (2002) *Current biology: **CB*** vol. 12 (3) p. 227-31 with permission from last author.

learning and memory of the adult fly (reviewed in (134,135)). These structures are discussed in greater detail below.

1.2.2 The antennal lobes and mushroom bodies of *Drosophila*

In 1850 the French biologist Félix Dujardin first discovered the Mushroom Body (MB) and it is thought to be common to all arthropods (136). The Mushroom Bodies (MB) are some of the most clearly distinguishable neuropile structures in the insect brain consisting of a dense network of glia, dendrites and axons. It receives information amongst others from the olfactory system. Odor is first detected by olfactory receptor neurons (ORNs) in the antennae and the maxillary palp (137,138) and conducted to the antennal lobe (Fig. 5). The antennal lobes - are the primary olfactory centers of the brain (139) consisting of about 50 glomeruli of which each glomerulus receives input from specific ORNs. Subsequently the antennal lobe launches projection neurons through three antennal cerebral tracts that in turn project to the lateral horn in the *Drosophila* brain however the inner antennal cerebral tracts sends collaterals to the MB

There are three compartments that make up the MB; the calyx, the pedunculus and the lobes (140–143) and can be seen as two bilateral symmetrical clusters (Fig. 5). The cell bodies of the MB intrinsic neurons (also known as Kenyon cells reside in the dorsal posterior brain and form a cluster, sending their dendritic branches in the anterior direction (the calyx), which converge to form the pedunculus. From this pedunculus the axons bifurcate at its anterior end producing vertical lobes (α' and α) and medial lobes (β', β and γ).

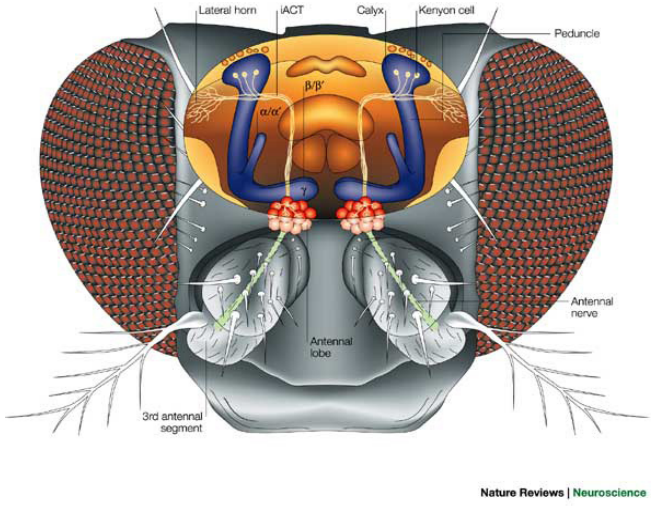

Fig 5 The position of the Antennal lobes in red, the Mushroom Body (in blue) and the central complex is outlined in the middle and back of the *Drosophila* head. Taken from: Heisenberg, Martin (2003) *Nature reviews. Neuroscience* vol. 4 (4) p. 266-75 with permission from author.

The MB is regarded as an integrative center for a multitude of sensory information and is shown to be specifically important for olfactory learning and memory (144–146). A detailed description of other tasks the MB could be involved in, like sleep, place, memory and others can be found in Tanaka et al; 2008 (147). The MB have also been shown to be dispensable for certain experience-independent olfactory related behaviors (148–151). After critical evaluation of the literature, Ito et al 1998 (152) and Tanaka et al 2008 (147) postulate that the mushroom body itself might not be the center for learning and memory, it can equally be considered as a preprocessor of sensory signals on their way to "higher" protocerebral regions. Still little is known about the exact function and identity of neural circuits that are responsible for specific behaviors.

Furthermore, still much needs to be learned about how other sensory information is relayed to the MB and to which brain regions output neurons project to. Assigning functions to defined brain regions is the place to start since it has proven to be exceedingly difficult to integrate the

individual contributions of brain regions into a behavioral model of the brain. The insect brain is relatively small (about 200.000 neurons) and there are some straight-forward behavioral paradigms available that allow the dissection of circuits underlying complex behavior. It is of interest to extrapolate the information gained from the *Drosophila* MB to circuit function in a vertebrate brain. So far, it is found that the genes that are expressed early in the development of the MB share considerable homology with genes involved in the early development of specific regions in the mammalian prosencephalon (142).

1.2.3 The embryonic musculature of *Drosophila*

The *Drosophila* musculature has been used as a model to identify genes that are needed for the different stages of muscle formation, such as mesoderm specification, myogenesis, myoblast fusion, muscle fiber specification and guidance of muscle fibers to their attachment sites. Many genes that are required for muscle formation during embryogenesis are highly conserved in sequence and function from insects to vertebrates. The process of myogenesis in *Drosophila* takes about 5.5 hours to complete. Muscles stem from a germ layer named the mesoderm, which is first specified at the blastoderm stage as the most ventral cells. These cells are formed under the control of the gene dorsal that is maternally contributed and establishes the dorsoventral axis in the embryo (153). During gastrulation, invagination

Fig 6 Displays the muscles of a living *Drosophila* embryo, which nicely presents the reiterated pattern of all segments. Downloaded from: http://www.biochem.mpg.de/schnorrer/research/index.html, with permission of Frank Schnorrer.

of these specified cells takes place along this dorsoventral axis and they continue to migrate dorsally, eventually lining the ectoderm of the embryo (154). Meanwhile, transcription factors twist and snail are upregulated under control of dorsal in all mesodermal cells. Since the mesoderm gives rise to several different tissues, i.e. the visceral mesoderm, somatic mesoderm, heart and fat body (155), the uniform mesoderm is regionally and temporally specified during embryonic development. An important developmental regulator of dorsal versus ventral structures in the embryo is the morphogen Decapentaplegic (Dpp), while the Twist protein is an important determinator of muscle fate. Twist expression is first detected uniformly at stage 5, low twist expression is maintained in cells that express the segmentation gene evenskipped (156). High twist expression is maintained, till stage 11, by cells that express another transcriptionfactor named; sloppy paired (slp), which is under control of Wg (157). These cells eventually produce the somatic mesoderm (158), among them are the body wall muscle fibers.

The embryonic somatic musculature forms an intricate network of about 600 individual muscle fibers that are arranged beneath the epidermis in a stereotypical segmentally reiterated pattern (159), that is visualized in figure 6. This network is sustained by the fact that all muscles attach at both ends to a tendon-like cell that is part of the exoskeleton, thereby providing the integrity needed to withstand the contractile forces exerted by the muscles. Each hemisegment consists of 30 muscles and each muscle is initiated by a single muscle founder cell. All 30 fibers are defined by size, position, orientation, attachment sites and innervation (reviewed in (160)). The muscle founder cell undergoes a series of processes leading to the fusion of the muscle founder cell with a number of surrounding fusion competent myoblasts (FCMs). This fusion process creates multinucleated myofibers whose two leading edges subsequently migrate towards clusters of tendon cell progenitors in the epidermis (161–163). The initial determination of the tendon cell progenitors in *Drosophila* is provided by segment polarity genes such as *wingless* and *hedgehog* (156). They are responsible for activating the early growth response (Egr)-like transcription factor Stripe in segmentally-reiterated clusters of epidermal cells (164). Stripe expression is both necessary and sufficient to promote muscle migration towards these clusters of tendon cell progenitors (165–167). The approaching myofiber itself, however, induces final differentiation of the single selected tendon cell by secreting the protein vein (reviewed in Schweitzer, et al 2010 (162)). The neuregulin-like ligand Vein accumulates at the muscle tendon junction to activate the Epidermal Growth Factor (EGF) pathway solely in the tendon cell progenitor that is contacted by the muscle fiber (168) (Fig. 7).

This pathway upregulates Stripe expression, which further activates other proteins, like guidance cues and terminal differentiation factors for the tendon cell. The tendon precursor

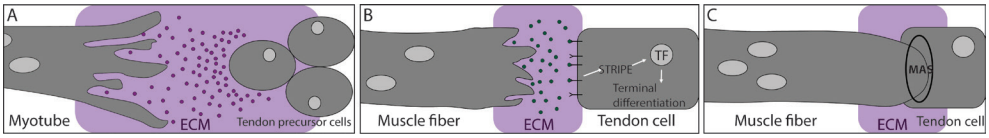

Fig 7 Schematical representation of myotube guidance. In (A) the approaching myotube is attracted by unknown factors to the tendon precursor cells. In close proximity of the tendon cells (B) the muscle fiber starts expressing Vein that induces tendon cell specification and ends in the establishment of a myotendinous junctions.

cells that are not contacted by a muscle fiber cease to express Stripe and do not differentiate into a tendon cell. The final stage of tendon cell determination is defined by the association of muscle specific αPS2βPS integrin with tendon-secreted ECM-component Thrombospondin (Tsp). The tendon specific αPS1βPS integrin binds to the extracellular Laminin (Lam). Both muscle and tendon cell secrete several factors to the ECM, like Tiggrin (169) and Slowdown (Slow; a regulator of Tsp) respectively. Many proteins mediate the formation of a myotendinous junction. Both muscle and tendon specific Integrins are associated with the actin cytoskeleton in each cell, enabling the myotendinous junctions (MTJs) to withstand the mechanical forces that occur during larval locomotion.

The basic morphology of the body wall musculature of the embryo is carried over into the three larval molds however muscle size increases about a hundred fold (170). After the third larval stage, *Drosophila* undergoes pupal metamorphosis during which the larval somatic musculature degenerates and the presumptive adult musculature, where multiple fibers are bundled into single muscles, will be formed.

One can consider three distinct phases in *Drosophila* myotube guidance; unidirectional migration, bidirectional migration and attachment, which are also employed in vertebrate myotube progression. There are some significant differences in the mechanisms of muscle formation in vertebrates and invertebrates, especially during the earlier phases. Whereas *Drosophila* muscle founder cells are programmed from the start, the vertebrate myoblasts receive their specification when they contact their respective tendons. This suggests that the more complex vertebrate musculature has acquired more flexibility during myoblast specification to increase the plasticity of fiber formation in a complex muscle field. But the final stages of *Drosophila* muscle guidance and attachment show many similarities to these operating in vertebrates on a cell biological level. The molecules that are involved in establishing the musculature are only just emerging (171); work done in *Drosophila* can provide a steady stepping stone for understanding this process in vertebrates. In addition, there is accumulating evidence that a number of conserved molecules, such as Slit and DRL,

are important players not only in the guidance of muscles to their tendon cells but also in guidance of axons to their target cells.

1.3 BIOLOGICAL ROLES OF WNT/RYK SIGNALING

The generation of the *ryk* knockout mice in 1992 gave important insights into the function of RYK in mammals (172). Without RYK present mice die perinatally, likely as a result of cardiovascular impairments (172,173). During embryogenesis the *ryk*$^{-/-}$ embryos present with craniofacial and skeletal defects, which include cleft palate and significant shortening of the long bones of the fore- and hindlimbs respectively (172). Besides its roles for correct development of a number of tissues during development, RYKs are also of importance in the adult, since misregulation of RYK is associated with human disease in later life (174), predominantly cancer (175–180).

ryk expression is observed in many different mammalian tissues during development as well as in adults (see review Serfas and Tyner, 1998 (181)), but in the fruit fly DRL, DRL-2 and DNT seem to be restricted to distinct subsets of cells in the nervous system and the musculature. *drl* was initially identified in a screen for genes that are required for axon pathfinding in the *Drosophila* embryo (182) and for learning and memory in the adult fly (183,184). Recently, reports have been published on describing roles for DRL as an axonal guidance cue operating in the mushroom bodies (185), the antennal lobes (186) and as a protein important for synapse formation at the neuromuscular junction (NMJ) (187). Outside the CNS DRL has been involved in correct muscle guidance of the lateral transverse muscles (188) and in the formation of the salivary glands (189). A role for RYK in tissues other than the nervous system has also been uncovered in *C. elegans*, in which embryonic cell fate patterning in the vulval cell lineage is regulated by Wnt induction of RYK (190).

In the embryonal ventral nerve cord of *Drosophila* there are two major routes available to cross the ventral midline; the anterior commissure (AC) and the posterior commissure (PC) as aforementioned in section 1.2.1. In the wildtype embryo the axons that express DRL traverse the midline via the AC where the PC neurons that do not express DRL cross the midline through the PC (191,192). However when DRL is ectopically expressed in the PC neurons, the PC neurons switch to project through the AC (114,193). This misrouting can be suppressed by heterozygosity for *wnt5*. This result can be explained to mean that WNT5 which is normally expressed at the PC acts as a repellent for DRL expressing neurons (114,193). And indeed, when WNT5 is misexpressed in the midline glia (Sim-Gal4) the DRL expressing neurons do no longer cross the ventral nerve cord (VNC), hence the AC is not formed and the neurons are stalled at the intersections. The penetrance of this midline overexpression dominant gain of function phenotype is dependent on the level of WNT5 expression. The assay can be used

to screen for genes that are required for WNT5 signaling, i.e. candidate genes are evaluated for their ability to suppress this induced phenotype (114,124,193).

In Chapter 2 we use an *in vivo* **axon guidance assay** developed by the Thomas lab (114,192) that is named; the *drl*-mediated commissure switching assay (Fig 8). This assay is based on the ability of WNT5 to repel DRL$^+$ axons. When *drl* is ectopically expressed in all *eg*$^+$ neurons using the *eg-Gal4* driver and a *UAS-drl* transcript the neurons that normally cross the midline through the PC are forced to cross the midline through the AC (Fig 8, right panel). The penetrance of this phenotype depends on the *drl* expression levels, allowing amongst others the assessment of different *drl* mutants in a biological context. Both the commissure

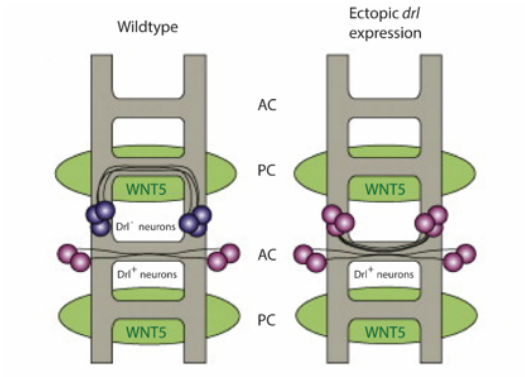

Fig 8 Illustration of the *drl*-mediated commissure switching assay. The crossing of *drl*$^-$ neurons via the PC in the wildtype is depicticted in the left panel. When *drl* is expressed ectopically (right panel) these neurons are repelled by WNT5 and proceed to cross the midline through the AC.

switching after ectopic overexpression of DRL and the midline WNT5 overexpression assay have been successfully used by our lab and others to identify novel members of the WNT5/DRL pathways such as the non-receptor kinase SRC64B (124).

In contrast to the roles of DRL as a signaling receptor in the embryonic VNC, there is also evidence to show that DRL can sequester its ligand; WNT5, acting as a sink to titrate out the amount of DRL that can interact with an alternative, signaling receptor. This was shown in the mushroom body (MB) where the *drl* mutant was also found to display a phenotype: the αα' vertical lobes are reduced and the β lobes can cross the midline in the *drl* mutant, see fig 9 (185,194). However the specific expression of DRL in the MB in an otherwise *drl null* background does not rescue the phenotype implying that DRL is not intrinsically present in the MB (194). Furthermore it was shown that this *drl* mutant phenotype was rescued by expressing DRL without its intracellular domain (ICD). This dual mechanism of DRL action; acting as a sink vs a signaling receptor, is probably determined by co-receptor context.

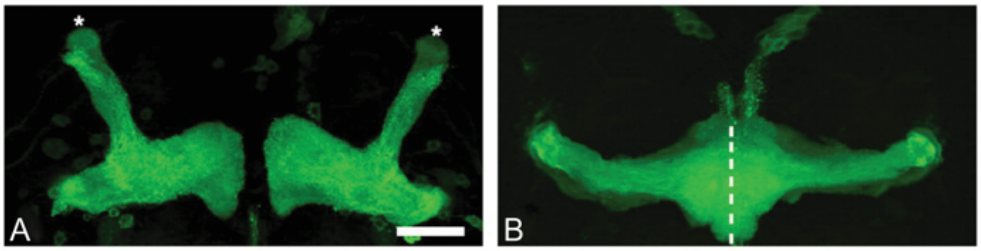

Fig 9 The wildtype mushroom body is displayed in (A) where the asterisks indicate the αα′ vertical lobes that are present in the wild-type situation or reduced in the mutant situation (B). The midline is shown by a dashed line and midline crossing can also observed in the *drl* mutant. Scale bars: 50 μm in A. Adapted from *Grillenzoni N, et al Development 2007* (194) with permission.

DRL can be in a complex where it is able to transduce a signal through Src kinase (see section 1.3.3) or it can be in a different setting where its only role could be to down regulate the amount of WNT5 by its sequestration. The role of DRL during the MB development will be addressed in Chapter 3 (this thesis). Despite the many questions remaining on the biochemical mechanisms by which DRL functions, there are important roles for RYK/DRL during the development and in the adult *Drosophila* nervous system and in its musculature (114,182–188,193,195,196).

The phenotype of the musculature in the *drl* mutant was first described by Callahan et al 1995 (188). Normally the lateral transverse muscles 21-23 (LTMs 21-23) present in the embryonal muscle segment (schematically represented in Fig 10A) can be seen to attach ventrally at a tendon cell at the level of muscle 12 whereas in the *drl* mutant we find that muscle 21 or occasionally 23 overshoots its normal attachment site and establishes an ectopic attachment site beyond muscle 13 (Fig10B). This phenotype is not fully penetrant and therefore we used this **muscle guidance assay** in Chapter 4 to assess genetic interactions that are either

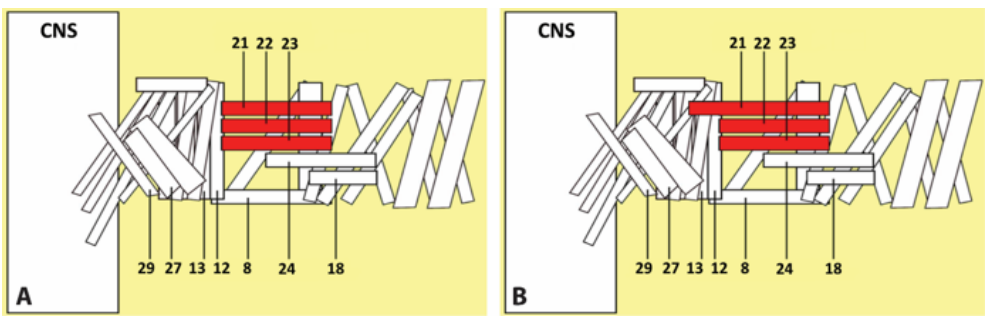

Fig 10 This representation of a single muscle segment demonstrates most of the 30 muscles that make up a muscle segment. The LTMs 21-23 are portrayed in red in the wildtype (A) and in the *drl* mutant (B). The bypassing muscle phenotype in the *drl* mutant is illustrated by LTM 21 that bypasses its normal muscle attachment site and attaches further ventral beyond muscle 13.

capable of phenotypic rescue or increasing the penetrance of the phenotype.

The Wnt-receptor RYK was also found to be expressed in many tumor cell types in cancer; neuroblastomas, primitive neuroectodermal tumors (PNETs), Wilm's tumors and melanoma (197). H-RYK is also overexpressed in malignant ovarian tumors (177), and overexpression of H-RYK causes transformation *in vitro* and tumor formation *in vivo* (178). And there are implications for RYK, through the interaction with for instance EphB2 and EphB3, to possibly mediate tumour migration, invasion and metastasis (123). Recently, an article was published indicating a role for WNT5a-RYK signaling in hematopoietic stem cell proliferation and repopulation (198).

1.3.1 RYK/DRL Protein Structure

The protein encoded by the *ryk/drl* gene is an atypical member of the RTK family because of the lack of conservation of certain amino acid residues (see section 1.3.2). The ECD of RYK/DRL contains about 250 amino acids, which is considerable less than the average of 400 amino acids present in other ECD of RTKs. These 250 amino acids have five potential

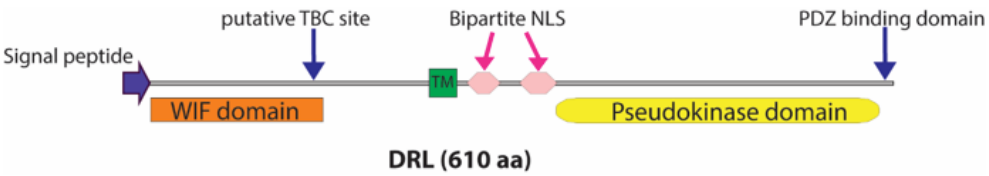

Fig 11 Illustration of the domains present within DRL that starts at the N-terminus with the signal peptide. The ECD contains the WIF domain that also holds the putative TBC site. In green the transmembrane region (TM) is portrayed, followed by the bipartite nuclear localization signal (NLS) that resides in the ICD of DRL. The ICD also includes the pseudokinase domain as well as the PDZ binding domain.

N-glycosylation sites, a putative tetra-basic protease cleavage (TBC) site and importantly there are two leucine-rich motifs near the N-terminus that bear homology to the secreted Wnt inhibitory factor (WIF), illustrated in Fig 11. It has been established that RYK can bind Wnts

(WNT1, 3, 3a and 5a) via its WIF domain (114,173,199–201). The TBC site (KRRK) can be recognized by the furin family of proteases and therefore allows posttranslational modification. After cleavage an α- and β-subunit of the RYK receptor can theoretically be formed which can be linked via a disulfide bond on the cell surface, a mechanism that is experimentally shown for some integrin and insulin receptors (123). Within the ECD of RYK/DRL there is a cysteine available on either side of the TBC motif that could potentially cause the disulfide bond formation linking the two subunits (202). The transmembrane domain (TMD) of RYK also contains two cysteines that allow the formation of disulfide bonds. However, *Drosophila* DRL and DRL-2 do not contain cysteines in the TMD, whereas DNT has a single cysteine residue

residing within the TMD. This could be functionally significant since cysteines as well as other amino acid motifs can play a role in dimerization and activation of transmembrane proteins. It was established that the disulfide-linked insulin receptor is able to form homodimers without being bound by its ligand (203–205). The preformed homodimer is believed to undergo a conformational change upon ligand binding that causes transphosphorylation of both ICDs and activates a signaling cascade. This mechanism could also be operating for RYK and DRL activation as they too belong to the RTK family and are disulfide linked.

The ICD amongst others contains a PDZ-binding domain (TRYV at the very end of the DRL-ICD sequence) (Fig 11 and 12) and could therefore potentially interact with proteins containing a PDZ domain (206). The most likely candidate would be Dsh (207), since this PDZ containing protein has been implicated to play a role in most pathways that are activated by Wnts. In addition, it has been shown that the noncanonical WNT11/ FZD7-mediated endocytosis of Dsh requires RYK, both RYK and FZD7 are needed to bind WNT11 ultimately regulating the convergent extension (CE) movements in *Xenopus leavis* (118).

Furthermore, it has been reported that when cells are stimulated with WNT3 this results in proteolytic cleavage of RYK. The first cleavage event takes place at the TBC motif of RYK. A second cleavage event within RYK's TMD is mediated by γ-secretase and results in a cytosolic fragment that is stabilized and transported to the nucleus by interacting with Cdc37 (208,209). This nuclear localized RYK ICD most likely functions as a transcriptional regulator as published for ErbB4 (210,211), but it does not seem to activate the TCF-luciferase reporter (208). This RYK processing is required for neural progenitor cell differentiation (208).

The examination of the homology for DRL and its siblings DRL-2 and DNT reveals high homology especially for the WIF- and pseudokinase domain that are present in all three proteins. The homology is also maintained for the putative TBC site, KRKK in DRL, and that resides within the WIF domain. However, the bipartite nuclear localization signal (NLS), highlighted in purple in the DRL sequence only (see Fig 12), shows relatively low homology compared to DRL-2 and DNT and even between DRL-2 and DNT. Suggesting, that both DRL-2 and DNT have lost their NLS in the genomic duplication event. The final four amino acids, TRYV in DRL, represent the PDZ binding domain which is identical in DNT yet altered in DRL-2. Although the preservation of the second to last residue, a tyrosine, could be sufficient to maintain its PDZ binding capacity.

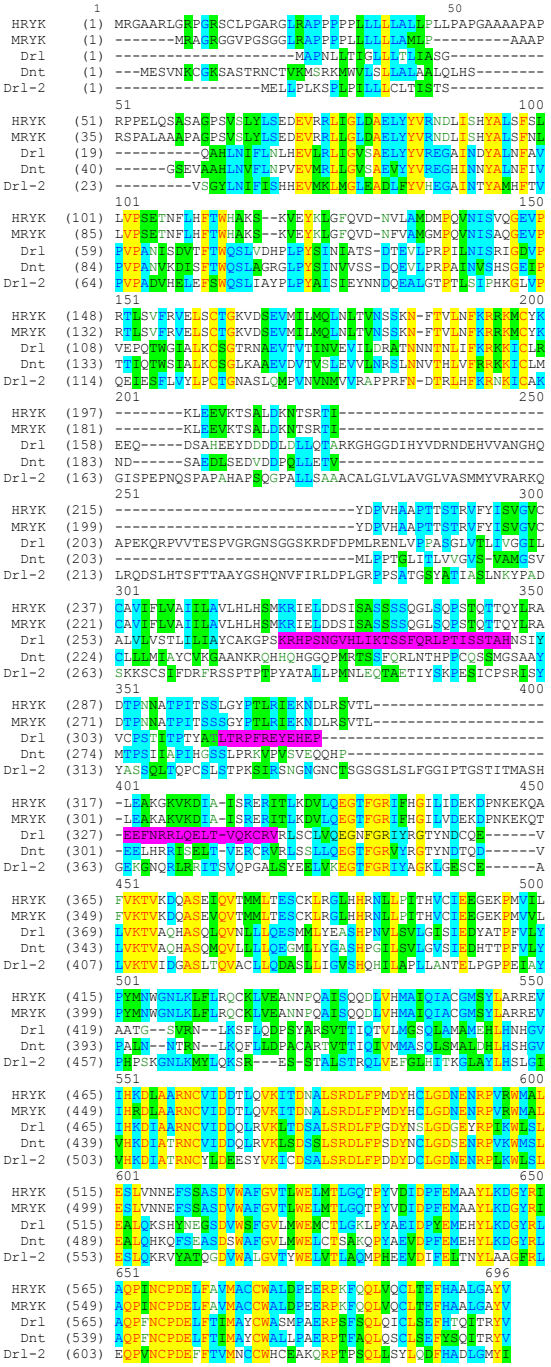

Fig 12 Alignment of the amino acid sequence of human RYK (HRYK), murine RYK (MRYK) and their *Drosophila* counterparts Derailed (DRL), Doughnut (DNT) and Derailed-2 (DRL-2). The yellow highlights represent the amino acids (aa) that are conserved amongst these species, whereas blue displays the aa that are most conserved yet not within all proteins. The green color highlights aa that are either conserved or similar and could perform the same function within a protein structure. The purple highlights in the DRL sequence only represents the bipartite nuclear localization signal.

1.3.2 Are RYKs Pseudokinases?

The intracellular domain (ICD) of RYK contains a number of subdomains that are hallmarks of the larger family of protein kinases (212). Yet a number of conserved phosphotyrosine kinase (PTK) domains seem to harbor amino acid substitutions, rendering them to be likely catalytically inactive as a kinase (see Table 1).

Table 1 The left column contains the species and the three *Drosophila* family members. The other columns display the different conserved PTK domains that are established for RTKs and that are not conserved in RYK/DRL.

Conserved PTK domain	Subdomain I GXGXXG	Subdomain II VAVK	Subdomain VIb IHRDLAARN	Subdomain VII DFG	Subdomain VIII KWMAPE
HRYK	**Q**EGTFG	**AF**VK	IH**K**DLAARN	D**NALS**	**R**WMA**L**E
MRYK	**Q**EGTFG	**TF**VK	IHRDLAARN	D**NALS**	**R**WMA**L**E
DRL	**Q**EGNFG	V**L**VK	IH**K**D**I**AARN	D**SALS**	KW**LSL**E
DRL-2	**K**EGTFG	**AL**VK	**T**H**K**D**I**A**T**RN	D**SALS**	KW**LSL**E
DNT	**Q**EGTFG	V**L**VK	**T**H**K**D**I**A**T**RN	D**SSLS**	KWM**SL**E

Within the kinase domain the glycine-rich loop with consensus sequence GxGxxG together with subdomain II constitutes the putative ATP binding site in conjunction with subdomain VII and is one of the most conserved sequence motifs in protein kinases (212,213). In RYK as well as DRL, the first nonpolar glycine of subdomain I is replaced by a polar glutamine, which could interfere with the binding of ATP. Furthermore, subdomain II and VII contain significant amino acid substitutions. Although the aspartate residue (D) for subdomain VII is present, it is anticipated that the subsequent residue alterations could disrupt the interaction with ATP. Thus the putative ATP binding site of RYK/DRL is therefore structurally different from those found in other PTKs and these aa sequence changes could prevent an interaction with ATP.

Furthermore, an aspartate (D) in another catalytic loop (IHRDLAARN) is in both RYK and DRL not preceded by an arginine residue (R); they can therefore be included in an expanding subfamily of proteins that have recently been considered non-RD kinases (214). The distinguishing feature of these assumed non RD-kinases or pseudokinases is that their potential activation domain does not appear to be phosphorylated, in direct contrast to the RD kinases (i.e., MAPK, IRK, and FGFR kinase) (215). However it has to be noted that, in murine RYK, subdomain VIb does match the conserved kinase domain, whereas in human and *Drosophila* RYK it does not. In mice, RYK could therefore theoretically function as a RD kinase, which one needs to take into account when extrapolating RYK data from mice to human. The other motifs presented in Table 1 also contain amino acid substitutions believed to interfere with the kinase activity of RYK/DRL. Nonetheless, there are other PTK domains that are conserved in RYK/DRL, leaving the possibility open for kinase activity within the cytoplasmic domain.

Several studies have been done to address the kinase activity of RYKs PTK domain experimentally. For that purpose a RYK antibody was produced and phosphorylation of RYK was detected in an *in vitro* kinase assay of immunoprecipitated RYK (121). In contrast, a FLAG:RYK fusion protein in bacteria was used and no phosphorylated tyrosines could be detected. These conflicting results might be explained by the possibility that the RYK phosphorylation, that is detected, is performed by another kinase that is associated with RYK. It has to be further taken into account that both experiments were done in the absence of a ligand which could be necessary to stimulate the intrinsic kinase activity of RYK. In another study, a chimeric receptor approach using the nerve growth factor (NGF) receptor TrkA was used to analyze the function of H-RYK (216). This chimeric TrkA:RYK receptor was made by fusing the ECD of TrkA to the transmembrane and cytoplasmic domains of the H-RYK receptor. The authors were unable to detect autophosphorylation of the chimeric receptor after stimulation with NGF, suggesting that H-RYK is catalytically inactive under these conditions. Alternatively, one could interpret this result to mean that the interaction between NGF and the ECD of TrkA does not mimick the interaction between RYK and a specific WNT. If a conformational switch is needed to bring the ICDs in proximity of each other, one could argue that the chimeric receptor is unable to induce a conformational change and therefore no autophosphorylation will be detected. It was observed, however, that NGF stimulation of the chimeric receptor was able to activate the mitogen-activated protein kinase (MAPK) pathway. More recently, it has been found that RYK binds another kinase; SRC and therefore, the above mentioned results might be explained by the recruitment of this other kinase.

In order to address the catalytic capacity of RYK/DRL more directly, a *drl* mutant was made that is regarded as catalytically inactive/kinase dead. Since the lysine in subdomain II at amino acid position 371 is thought to be involved in the phosphotransfer reaction and has been shown to be essential for catalytic activity of tyrosine kinases (217), this lysine was mutated to an alanine. This DRL K371A mutant was expressed in *Drosophila* embryos and was able to rescue the muscle bypass phenotype in a DRL mutant background. In addition, when DRL K371A was misexpressed in PC neurons, the PC neurons switched their axonal projections to the AC, just as is observed when DRL wildtype protein is misexpressed in the PC neurons. We can conclude that the substitution of K371 to an alanine does not interfere with the downstream signaling cascade of DRL. However, it does not exclude phosphorylation of DRL via an alternate route, such as the recruitment of another kinase.

1.3.3 SRC as a functional binding partner for RYK/DRL

An indication for such an interaction with another kinase came from the observation that the RYK ICD has been found to interact with SRC (124). Src family kinases (SFKs) that belong to the nonreceptor tyrosine kinases and are highly conserved amongst species. These allosteric

enzymes can exist in at least two conformational states and both states can be stabilized. What state is stabilized depends on the interactions with other proteins of two different domains (218–220). The first domain through which the SFKs can bind other proteins is the phosphorylation-dependent SH2 domain and the second is the phosphorylation-independent SH3 domain. Figure 13 demonstrates that in the inactive state SRC is phosphorylated on residue Y527 (221) and interacts with SH2 while the SH3 domain interacts with the kinase domain rendering it inactive. Upon dephosphorylation of Y527 the conformation of both SH2 and SH3 open up (Fig. 13) and autophosphorylation of Y416 causes Src-activation(222–224).

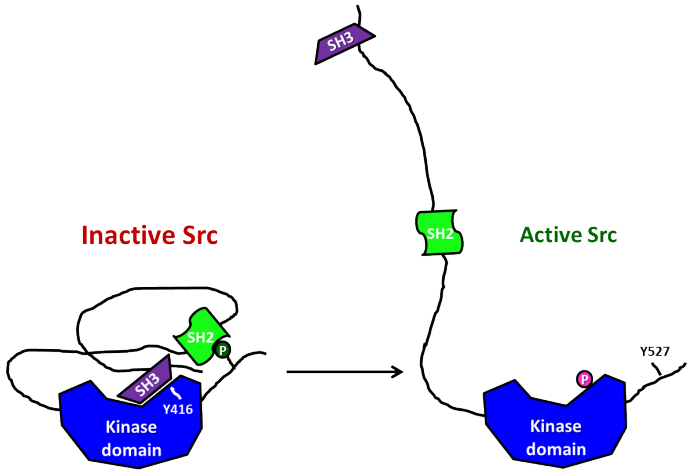

Fig 13 Schematic of the activational opening of SH2 and SH3 generating an active Src molecule.

The *Drosphila* genome has two SFKs; SRC64B (225) and SRC42A, both of which are able to interact with DRL (124,226). It has also been shown that SRC42A has at least partial redundancy to SRC64B (124,189,227,228). The same complex can be formed in mammalian cells between RYK and c-SRC, therefore the interaction between RYK and SRC seems to be evolutionary conserved (124).

1.4 GENERAL OUTLINE

In **Chapter 1** a general overview of Wnt signaling is presented which is followed by a more in-depth review of what is known about a specific non-canonical Wnt-receptor, RYK/DRL. DRL acts as a transmembrane receptor for the *Drosophila* Wnt family member, WNT5. The implications of this conserved interaction during development of vertebrates, invertebrates and in human disease will be addressed. Finally, our model organism *Drosophila melanogaster* is introduced with which most of the research presented in this thesis has been executed, with special attention for the CNS and musculature. This will provide the essential

background for the work presented in the following thesis chapters.

Chapter 2 focuses on the biochemical properties of RYK/DRL. We show that DRL can form homodimers and can also from heterodimers with two DRL protein orthologues, DRL-2 and DNT. We proceed to show that dimerization is dependent on its transmembrane domain and increased upon stimulation with WNT5. The downstream kinase that is associated with DRL, i.e. SRC64B, and the DRL/SRC64B interaction is discussed into detail and the specific domains required for their interaction presented. Furthermore, we show the functional relevance of these domains *in vivo*, specifically, we show their requirements for axonal guidance in the *Drosophila* ventral nerve cord during embryogenesis.

Chapter 3 describes an important function for DRL in the patterning of the mushroom body, a learning and memory center within the brain of *Drosophila*. DRL-2 is one of two family members of DRL that can also bind WNT5, providing a repulsive cue for the growing axon. Previously, DRL, DRL-2 and WNT5 have been shown to play a role in antennal lobe patterning. The authors showed that DRL in the antennal lobe is able to sequester WNT5 from DRL-2, thus preventing signaling through DRL-2. In contrast, we show here that extrinsic DRL binds its ligand WNT5, this extracellular complex is subsequently cleaved and the WNT5/DRL cleaved extracellular complex is presented as a repulsive ligand for DRL-2 expressing mushroom body axonal growth cones. The DRL, DRL-2, WNT5 ternary complex described here in the developing adult brain is, to our knowledge, novel to the Wnt-field.

In **Chapter 4** we show that not only axon guidance is affected in the WNT5 and DRL mutants, but also a subset of muscles fibers is misguided and overshoots their normal targets. Specifically, the lateral transverse muscles 21-23 are affected in this phenotype, one out of the three muscles does not attach at the appropriate muscle attachment site (MAS). We also show that DNT, another family member of DRL displays the same phenotype. Furthermore the new ectopic MASs are fully functional throughout embryonic and larval stages of development. These data reveal a remarkable evolutionary conservation and re-employment of the molecular machinery for both early initial guidance of axons in the embryonic CNS and for guidance of individual muscle fibers to their final attachment targets in the embryonic epidermis.

The results presented in this thesis and the future plans that emanate from this work are discussed in **Chapter 5**.

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