UPTEC X 05 023
APR 2005

ISSN 1401-2138

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A novel screening method to identify molecules involved in sensory axon recognition of synaptic target cells in *Drosophila*

Master's degree project

UPPSALA UNIVERSITET

Molecular Biotechnology Programme

Uppsala University School of Engineering

UPTEC X 05 023	**Date of issue 2005-04**

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Title (English)

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Title (Swedish)

Abstract

The formation of a functioning nervous system is a complex task. During embryogenesis peripheral neurons send out axons to connect to the central nervous system. How an axon identifies its target cells remains largely unknown. In an attempt to clarify some steps of the process the fruit fly *Drosophila melanogaster* was used as a model organism. Nerve cords from stage L1 larvae carrying a loss-of-function mutation were screened for defects in the branching pattern of terminal arborisations made by individual sensory neurons. The UAS:GAL4 system was used to visualise the arborisations. Larvae defective in *connectin*, *neurotactin*, *sidestep*, *tenascin major*, *protein tyrosine phosphatase 10D* and *Abl tyrosine kinase* were examined. Loss of fluorescence, increase of background fluorescence and loss of markers during crosses reduced the number of actual studied mutants to one, *connectin*. In the case of *connectin* no mutant phenotype could be observed.

Keywords

Drosophila, PNS, CNS, arborisation, UAS, GAL4, single cell dye injection

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Project name	Sponsors
Language **English**	Security
ISSN 1401-2138	Classification
Supplementary bibliographical information	Pages **28**

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A novel screening method to identify molecules involved in sensory axon recognition of synaptic target cells in *Drosophila*

Henrik Boije

Sammanfattning

Miljarder nervceller bildas då ett humant foster utvecklas. Dessa celler sänder ut utskott, axoner, för att skapa förbindelser med andra celler. På detta sätt skapas kopplingar som gör hjärnan kapabel att lagra information, tolka signaler från omgivningen och tänka. Hur nervcellernas axoner hittar rätt målcell är till stora delar fortfarande oklart. Det här arbetet inriktar sig på hur sensoriska neuroner identifierar sina målceller i det centrala nervsystemet. Eftersom den mänskliga hjärnan är så komplex användes modellorganismen *Drosophila melanogaster* i studien. *Drosophila* tillhör släktet bananflugor och är en av de vanligaste modellorganismerna som används vid forskning. Studier visar att molekyler inblandade i nervsystems uppbyggnad är relativt konserverade emellan olika arter. Upptäckter som görs i en bananfluga speglar väl liknande processer i högre organismer. Många molekyler inblandade in axonguidning i humana nervsystemet är ursprungligen identifierade i experiment nyttjandes just bananflugor. Mer kunskap om hur nervsystem bildas skulle öka vår förståelse och förbättra våra behandlingsmetoder vid nervskador orsakade av olyckor eller sjukdomar.

Projektet gick ut på att med olika metoder visualisera sensoriska nervceller och följa deras axoner in i centrala nervsystemet. Antikroppar, fluorescens och färginjektioner i enstaka nervceller användes för att identifiera axonernas normala mönsterbildning i centrala nervsystemet. Då mönstret är kartlagt kan man generera flugor där en viss gen är ur funktion. Om genen har med axonens förgrening i centrala nervsystemet att göra kan förhoppningsvis ett avvikande mönster observeras. Sex olika kandidatgener undersöktes i studien men inget onormalt mönster kunde observeras.

Examensarbete 20 p, Molekylär Bioteknikprogrammet
Uppsala Universitet, april 2005

Contents

1 Introduction

1.1 Axon guidance

The question at hand is overwhelming; How do 10^{12} neurons make over 10^{15} synaptic connections to form a thinking human brain? During embryonic development, billions of neurons are born; they migrate to their correct positions and extend axons connecting to specific target cells. When the nervous system is established, axon pathfinding is essential for a functional central nervous system (CNS) and peripheral nervous system (PNS). The "wiring up" can be divided into three different phases; axon pathfinding, target recognition and synaptogenesis. The axon guidance is mainly controlled by the tip of the axon, the growth cone (1, 49). The growth cone probes its environment by sending out filopodia, structures largely made up of F-actin bundles (2, 51). When guidance molecules bind receptors on the growth cone it triggers second-messenger cascades. These either stimulate polymerisation or depolymerisation of F-actin in the filopodia steering the axon tip (1). The guidance molecules can be either attractive or repulsive and be either short-range or long-range molecules. The short-range molecules are often physically attached to a cell surface or the extra cellular matrix while the long-range molecules are diffusible and create a gradient (3). How growth cones respond to external signals is dependent on the set of receptors it carries. The same guidance molecule can attract one axon while repelling another (4). Axon pathways seem to be broken down into shorter segments making the process of finding its target cell a stepwise decision (5, 47). Utilising "guide-post" cells and other axons as intermediate targets simplifies the task of locating the final destination (6, 7, 44). When an axon reaches its final destination it forms a specific branching pattern (8). The position and shape of such an arborisation is a delicate transition from axon growth and guidance to branching and synaptogenesis (25, 29, 45). How it takes place and which molecules are involved is mostly unknown.

1.2 Aim

How does an axon "know" when it has found its target, when to stop growing and how to form a specific branching pattern? The aim of this project was to identify molecules that are involved in these events and in the end try to elucidate the cellular mechanisms behind target recognition, growth inhibition and branching activation. Exactly how axons halt is unknown, for example it may encounter a growth-inhibitory zone or pass a border between a promoting and less promoting area caused by axon targeting molecules (26). When it comes to branch formation there appear to exist both positive and negative regulators (7). The specific problem under investigation is how neurons in the PNS recognise their appropriate targets in the CNS. Since the human nervous system is so complex a model organism was used to unravel this problem, the fruit fly *Drosophila melanogaster*. The combination of *Drosophila'*s simple nervous system, quick generation time and well mapped genome makes it a perfect candidate for this type of research. Fruit flies' sensory axons face the same problem in finding its synaptic target as its vertebrate counterpart. Many genes like *semaphorin*, *roundabout* and *slit*, which are known to play a role in vertebrate axon guidance, were all first discovered in mutagenesis screens for guidance genes in *Drosophila* (3). Axon guidance molecules seem to be highly conserved throughout the animal kingdom (3). By finding the genetic program for axon arborisation in a relatively simple organism we hope to be able to draw conclusions relevant for higher animals. This understanding might in the end be used to explain and treat neural damage caused by accidents or disease in humans.

1.3 Drosophila lifecycle

There are four distinct stages in the life of the fruit fly; egg, larvae, pupae and adult. Once a female mates fertilised eggs are laid almost immediately. The embryo is about 0.5 mm long and passes through 17 developmental stages (st1-17) defined by different visual cues (1A, 2A). The embryo has two protective layers, the chorion and the vitelline membrane. During stage 17 a cuticle is also formed around the developing larvae. The larvae hatch after 22-24 hours and immediately commence feeding. The three larval stages, L1, L2 and L3, last for 24, 24 and 48 hours respectively. During larval development its volume is increased 1000 fold. The larva then enters the pupae stage lasting 4-4.5 days. The time past from the egg is laid until the fly hatches varies with temperature but at the optimal temperature of 25°C it takes about 9.5 days (7A). Once the fly emerges the wings will expand within the first hour and full pigmentation occurs within 2-3 hours. The freshly hatched fruit fly will not mate during the first 6-8 hours giving a window where virginity is certain (3A). Its lifespan varies between 45-60 days, with a peak in fertility during the first week (4A). The easiest way to determine the sex of a fly is to examine its genital organs under magnification. The male genitalia have a different shape and are surrounded by dark bristles, which do not occur, on the female (4A). Since female flies have the ability to store sperm and impregnate themselves at a later occasion only virgin females were used in crosses. A virgin fly can either be distinguished by the meconium, a black spot in the gut, which is leftovers in the intestine from their last meal, or by collecting them at 4-6 hour intervals, before the flies are fertile (6A).

1.4 Drosophila nervous system

The *Drosophila* embryo is highly transparent, once the chorion is removed, making visualisation of individual cells under differential interference contrast (DIC) optics relatively easy (fig. 1). The embryo is segmented having a head, three thoracic (T1-3) and eight abdominal (A1-8) segments (2A). The CNS ventral nerve cord (VNC), the fly equivalent to a spinal cord, consists of about 250 motorneurons and interneurons repeated in each segment, most of them individually identified (9). The PNS in the second through seventh abdominal segments is bilaterally symmetrical (10). That means that on each side of the midline in a segment, a hemisegment, one can observe the same pattern of neurons in all segments. In the PNS a set of 42 neurons is repeated in these hemisegments, all individually identified (fig. 2) (11, 12). The sensory neuron cell bodies are located just beneath the epidermis in the body wall greatly simplifying visualisation. The PNS neurons are divided into distinct clusters and classes based on their location, the targets they innervate and their dendritic morphology (50). The neurons are arranged in four clusters; ventral, ventral', lateral and dorsal (10, 11). The classes are; external sensory (es) neurons which has a single dendrite and innervate tactile hairs or chemosensory organs, chordotonal (ch) neurons, also with a single dendrite but innervating chordotonal organs detecting internal stretch and the multiple dendritic (md) neurons innervating skin or trachea (27). The md-neurons are also subdivided into dendritic arborisations (da), bipolar dendrite (bd) or tracheal dendrite (td) neurons (5). Axons from neurons in the ventral and ventral' group enters the CNS via the segmental nerve (SN) while the lateral and dorsal group enters via the intersegmental nerve (ISN) through the posterior fascicle (2A). Each of the 42 neurons form a unique pattern of terminal branches in the CNS, but a certain degree of variability exists (13). Within each class a similar branching pattern can be observed. The ch and md-da neurons generally lie in the longitudinal fascicles within the connective while the es neurons tend to be more branched and not limited to the longitudinal tracts (fig. 3 and 4) (13).

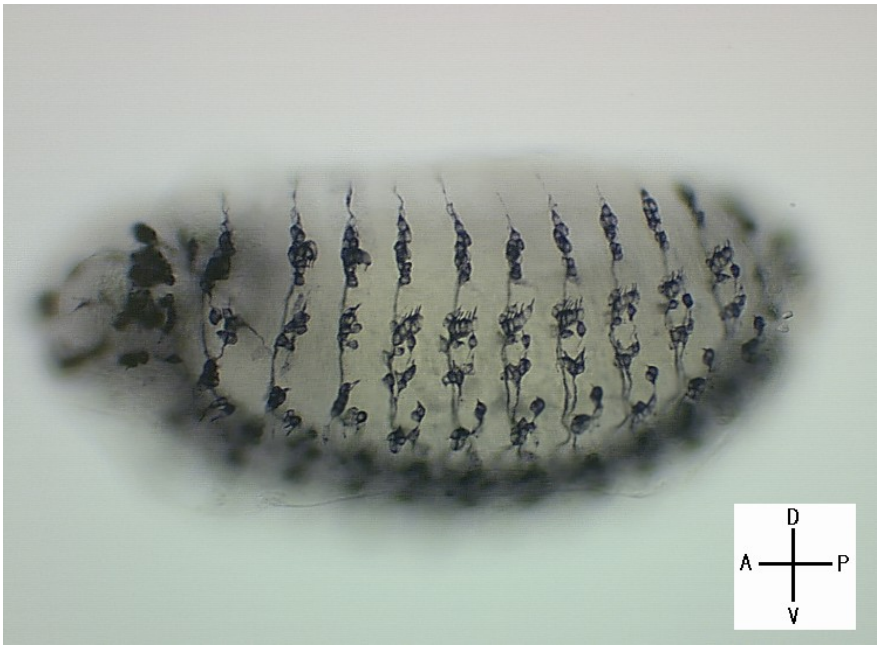

Figure 1: Drosophila embryo stage 16 stained with 22c10 antibody targeting all PNS neurons. Total length of the embryo is 0.5 mm. A-anterior, P-posterior, D-dorsal and V-ventral.

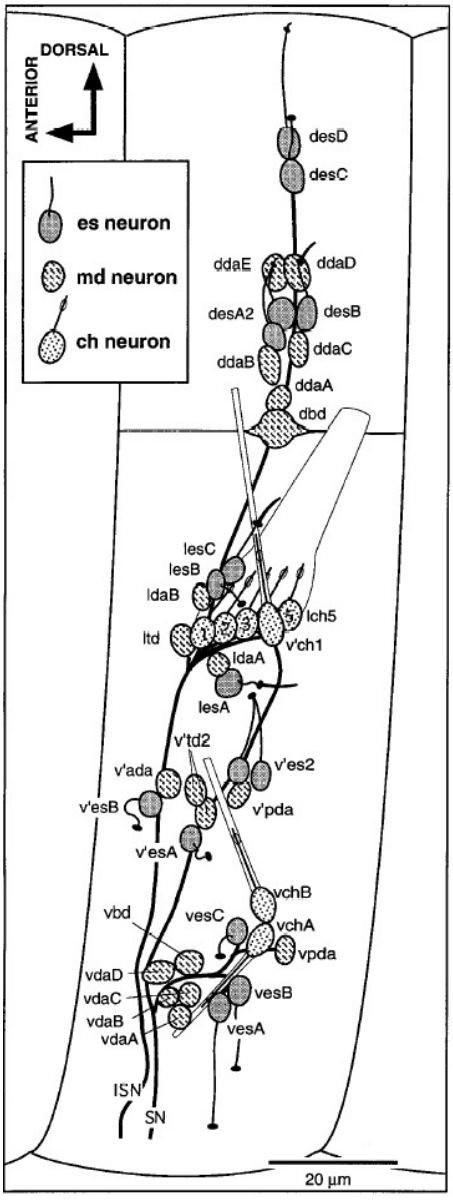

Figure 2: Schematic display of sensory neurons in abdominal hemisegments of the Drosophila embryo (Illustration used with permission from P. Whitington, 13)

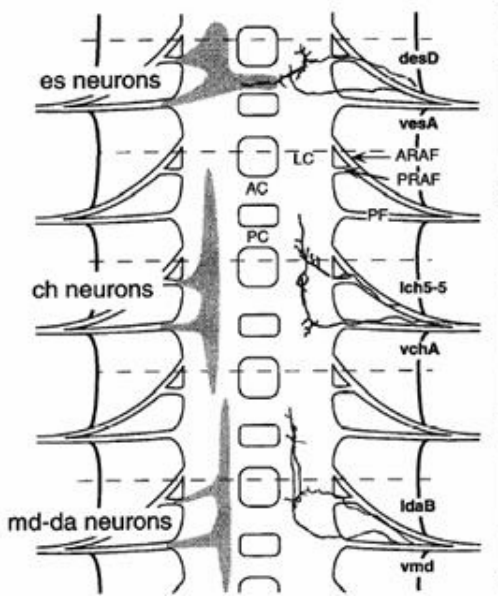

Figure 3: The regions of the VNC where each of the major classes of sensory neurons project is shown on the left hand side. Examples of projections of two neurons from each class are shown on the right hand side. es-external sensory, ch-chordotonal, md-multiple dendritic, da-dendritic arborisation, LC-longitudinal connectives, AC-anterior commissure, PC-posterior commissure, ARAF-anterior root anterior fascicle, PRAF-posterior root anterior fascicle, PF-posterior fascicle. (Illustration used with permission from P. Whitington, 13)

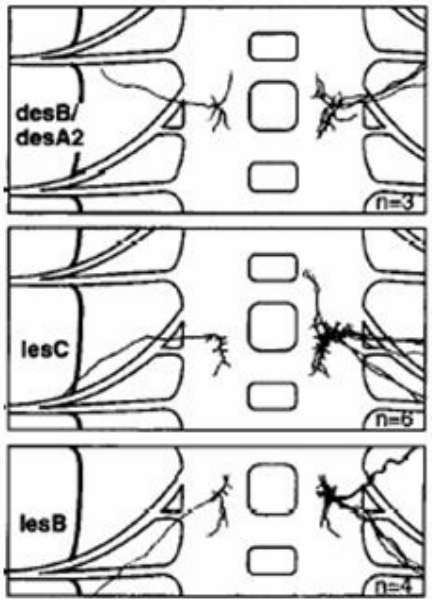

Figure 4: The regions of the VNC where desB/desA2, lesC and lesB project. Single cell injections are superimposed on a standardised nerve cord. desB/desA2 enters through the posterior fascicle, lesC through posterior root anterior fascicle and lesB through anterior root anterior fascicle. (Illustration used with permission from P. Whitington, 13)

1.5 Drosophila genetics

The fruit fly genome consists of four pairs of chromosomes. The genotype is often given for each chromosome separated with a semicolon ($^{X}/_{X}$; $^{2}/_{2}$; $^{3}/_{3}$; $^{4}/_{4}$). Apart from that its genome is sequenced the existence of phenotypic markers and balancer chromosomes greatly simplify the process of manipulating genes. Markers come in all different varieties. The ones used in this project are: Curly-Oster (CyO), Scutoid (Sco), Humeral (H), Serrate (Ser), Stubble (Sb), Tubby (Tb), pAct-GFP and GAL4-KrC (see Table 1 for details) (4A, 5A). A balancer chromosome usually carries a construct containing both phenotypic markers and mutations that are lethal in homozygous form. The balancers prevent recombination by the use of inverted sequences and make sure that altered genes are not lost by the lethal mutations (5A). The presence of inverted sequences minimises the chance of homologous recombination and the lethal mutation prevent viable offspring homozygous for the balancer. Many mutations in developmental genes are homozygous lethal and must be kept in a heterozygous state. If not aided with a balancer chromosome these mutations would be lost in a matter of generations. Most of the markers used in this study are part of a balancer construct.

Table 1: Phenotype of the different markers used	
Marker	Phenotype
CyO	Wings curled up instead of flat
Sco	Missing bristles from posterior thorax
H	Additional humeral bristles
Ser	Notched wing tips
Sb	Short and stubby bristles
Tb	Shorter body length
pAct-GFP	Fluorescent embryonic marker staining actin
GAL4-KrC	Fluorescent embryonic marker staining the bolwig organs

1.6 Methodology

We used classical genetics, i.e. screening for mutant phenotypes, to find genes involved in central arborisation. By using different methods to visualise neurons and their axon trajectories a wild type pattern can be deduced. The presence or absence of misprojections can then be observed in flies with a loss-of-function mutation for a particular gene. The primary step is to generate an understanding of the wild type pattern so that in a later stage multiple mutants can be screened efficiently. There are various ways of visualising neurons and their axons. The ones used in this study are immunohistochemistry, green fluorescent protein (GFP) expression and single cell dye injections (53).

One of the most prominent tools in visualising neurons is the UAS:GAL4 system. The GAL4 protein is originally a transcriptional activator in yeast (14). By p-element mediated transposition the *gal4* gene is randomly inserted into the fly genome (20). By chance, or by including a certain promoter in the p-element, the *gal4* gene can come under the control of wide range of transcriptional enhancers. This generates numerous different GAL4 expression patterns, some unique to only a small subset of cells. GAL4-expressing lines that drive expression in different patterns are collected into databases, such as FlyBase, and many lines can be ordered from stock centres, e.g. Bloomington stock centre (16, 17). The next step is then to cross the fly line expressing GAL4 in a desired subset of cells to a line carrying a reporter gene under the influence of an upstream activated sequence (UAS). When the GAL4 protein and the UAS are located in different transgenic lines nothing will occur. But once they are crossed the GAL4 protein will bind to the UAS and activate transcription of its downstream reporter gene (fig. 5) (15). Reporter genes used in this study were τ-LacZ and mCD8-GFP. The expression can then be visualised either by antibodies or, as in the case of GFP, under fluorescent light. *τ-LacZ* is a fusion gene between *LacZ*, β-galactosidase being its gene product, and *Mapt*, a gene producing the microtubule binding protein Tau (τ) (18). Since the axons are filled with parallel arrays of densely packed microtubules acting as scaffolds they are visualized with this method (28). The GFP gene is originally retrieved from the jelly fish *Aequorea victoria* and produces a relatively small protein, 27 kD (19). The protein is produced as an apoprotein, which is converted to an actively fluorescent form by cyclization and autoxidation (14). The active protein absorbs light at 395 nm and emits green light at 509 nm. mCD8-GFP is a fusion protein between mouse lymphocyte marker CD8 and GFP, labelling the cell surface as a result of mCD8 transmembrane properties (20). The requirement of converting GFP into an active form causes an approximate three hour delay between the onset of expression and visible fluorescence (14).

A confocal microscope was also used to study the GFP expressing nerve cords. The microscope generates optical sections of fluorescent samples. With a laser a small section of the embryo is exposed to the exciting light reducing distortions in the image by background fluorescence. By collecting a stack of optical sections one can walk through the embryo in three dimensions and follow axons. The images can also be superimposed on each other giving an overview of the axon pathways.

Another approach to visualise neurons and axons is single cell dye fill. A dye is backfilled into a sharpened glass electrode. The microelectrode is brought up to the membrane of the cell of choice and the dye is ejected from the electrode by applying current, staining the entire cell, including axonal and dendritic extensions (21). The dye is usually then visualised in a fluorescence microscope. Both living and fixed preparations can be injected.

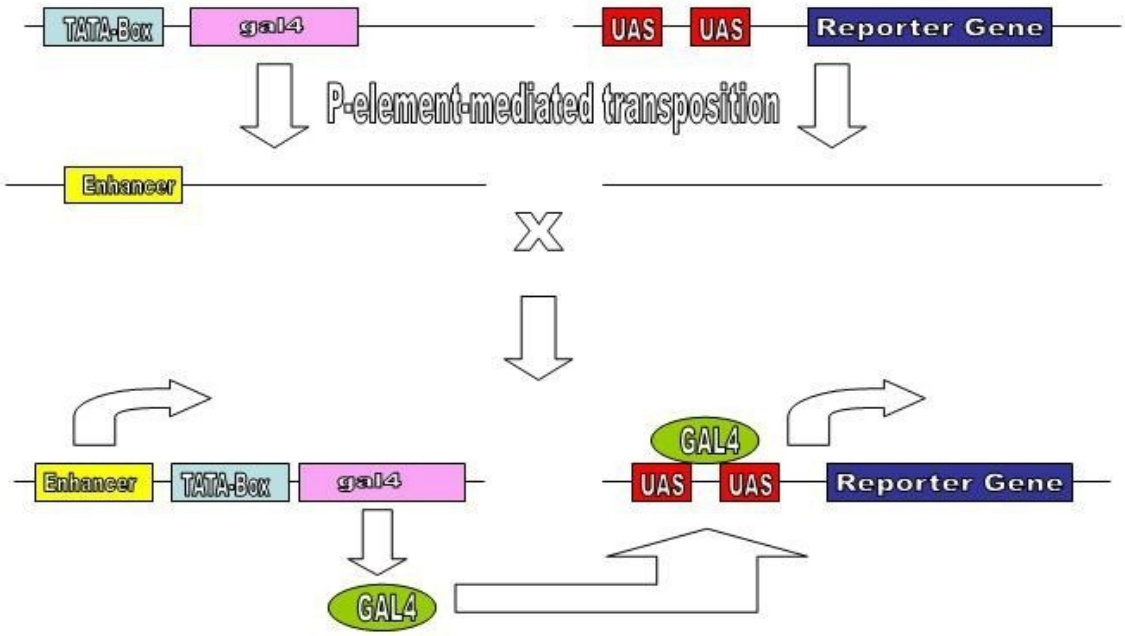

Figure 5: Creating transgenic lines with cell-specific expression of a reporter gene. A vector carrying the gal4 gene is inserted randomly into the genome by P-element-mediated transposition. When inserted close to a genomic enhancer the gal4 expression is activated. When a line carrying a reporter gene controlled by an UAS is crossed to the GAL4 producing line, the reporter gene will be expressed in the same subset of cells as the GAL4-protein.

1.7 The project

The project can be divided into three major parts; characterising GAL4-lines, creating a UAS:GAL4 recombinant line and using the recombinant line to screen loss-of-function mutants. The first step was to find a line of flies driving GAL4 expression in an appropriate subset of neurons so that their axon morphology in the CNS could be visualised. This was done by crossing GAL4 lines to either an UAS-τ-LacZ line or an UAS-mCD8-GFP line to visualise the GAL4 expression pattern. A suitable expression pattern should be a small subset of PNS neurons and minimal expression in CNS and supportive tissue. Once a good candidate line was found, and its wild type pattern thoroughly investigated, a recombinant line was created. This line carried both the GAL4 and the UAS-mCD8-GFP construct on the same chromosome. This allows further crossing without loosing the fluorescent pattern. Once this line was produced it could be used to screen through a set of mutants. One at the time lines, carrying a certain loss-of-function mutation, were crossed to the recombinant line. This created lines carrying a homozygous mutation for a gene and the construct staining a known subset of cells and their axons. The arborisations generated by PNS axons in the CNS were compared to wild type in search of deviations that might suggest that particular genes' involvement in the correct patterning.

1.8 The genes

Molecules involved in mediating recognition between growth cones and their terminal targets are also often involved in other aspects of axon guidance (3). The genes investigated in this study were *sidestep* (*side*), *tenascin major* (*Ten-m*), *neurotactin* (*Nrt*), *protein tyrosine phosphatase 10D* (*Ptp10D*), *connectin* (*Con*) and *abl tyrosine kinase* (*Abl*). *Side* encodes a protein known to be involved in motor axon guidance (30, 33). Neuroatonomy defective *side* mutants have been isolated (32). Among these findings *side* seem essential for motor axons to leave the motor nerves and enter their muscle targets (31). *Ten-m* encodes a structural molecule involved in regulation of cell shape (42). Loss-of-function mutants have been isolated which affect the embryonic CNS (43). *Nrt* encodes a protein involved in cell adhesion (34). Its amino acid sequence contains an esterase/lipase/thioesterase family active site and a carboxylesterase type-B. Mutations in *Nrt* affecting the embryonic CNS have been isolated (35). *Con* also encodes a structural molecule, involved in fasciculation of neurons (5). It interacts with Fas2 and Sema-1a both involved in neurogenesis (36). *Con* has also been shown to both attract and repel motoraxons (22). *Ptp10D* encodes a protein with protein tyrosine phosphatase activity. Its amino acid sequence contains a tyrosine specific protein phosphatase and a cytokine receptor. Members of the *Ptp*-family have been implicated in processes including cell adhesion, cell migration, and development of the immune and nervous systems (41). Recently a receptor *Ptp* was identified as a regulator in axon growth and guidance (23, 40). *Abl* encodes a product with protein tyrosine kinase activity involved in axon guidance which is localized to the axon (37, 38, 39). Its amino acid sequence contains a tyrosine kinase catalytic domain and a src homology 2 domain. It interacts with *Nrt*, *robo* and several other genes (24). Embryos double mutant for *fasciclin I* and *Abl* have major abnormalities in the development of their axon commissures (3, 52). The genes were selected on basis of their previous documented involvement in axon guidance. Since the UAS:GAL4 construct is present on the second chromosome mutations residing on chromosome two can not be used because a homozygous mutant carrying the construct can never be generated.

2 Material and methods

2.1 Drosophila stocks

The following Bloomington (BL) GAL4-stocks were used: 1874, 3042, 6793, 6798, 6980, 6981, 7008, 7010, 7028, and 7148. Stocks were raised on standard cornmeal and sugar medium at 25°C. Virgins were often collected over several days and stored at 18°C to slow down its life cycle until used in a cross. During collection the flies were anaesthetised by CO_2. Regular crosses where embryos or larva was the desired product were set up in egg chambers containing an apple juice agar plate with a dab of yeast paste. About 25 virgin flies were crossed to 5 males at 25°C and plates were changed regularly. Crosses where the next generation of flies were the objective were put up in vials containing cornmeal sugar medium. Three virgin females were crossed to one male and raised at 25°C. After 9 days the adults were removed to facilitate collection of the freshly hatched flies.

2.2 Immunohistochemical staining

2.2.1 Staining of embryos

The embryos were collected from apple juice agar plates from the cross between female UAS-τ-LacZ and male GAL4 lines. Embryo collection was timed to maximize the number of embryos going through stage 15-17 of embryonic development. At this age most axons have reached their targets in the CNS. The chorion was removed by agitation in a 50% bleach solution for 15 minutes. The embryos were thoroughly rinsed in saline (1.9mM KCl, 2.4mM $NaHCO_3$, 1.1mM $CaCl_2*2H_2O$, 0.6mM NaH_2PO4*H_2O and 110mM NaCl) before fixed in a 4% formaldehyde solution for 20 minutes. The formaldehyde was diluted in phosphate buffered saline (PBS; 130mM NaCl, 7mM $NaHPO_4*2H_2O$, 3mM $NaH_2PO_4*2H_2O$; pH 7.0). The vitelline membrane was chemically removed by shaking the embryos for one minute in a 50/50 methanol/heptane solution. The embryos were rinsed in 100% methanol and rehydrated in PBT (PBS +0.2% Triton X-100 and 0.2% bovine serum albumine, BSA). Block solution, PBT-NGS (1:20 dilution of normal goat serum in PBT), was added 30 minutes prior to the primary antibody (Promega, 1:500 dilution of anti-β-gal in PBT-NGS). Embryos were incubated over night at 4°C then rinsed with PT (PBT +0.1% Tween). Secondary antibody (Chemicon Australia, 1:500 dilution of sheep-anti-mouse with conjugated HRP (horseradish peroxidase) in PBT-NGS) was added and allowed to incubate for 2 hours at room temperature. HRP is visualised with DAB as a substrate, which is oxidatively polymerized to a brown insoluble indamine. By addition of $NiCl_2$ the stain obtains a blue colour. The embryos were washed with PBS before preincubated in the dark for 30 minutes in DAB-mix (125 µl DAB, 875 µl PBS and 40 µl $NiCl_2$). Hydrogen peroxide (0.3% H_2O_2) was added to activate the HRP. Once sufficient staining was obtained the embryos were washed with PBS for 30 minutes. The PBS was replaced by 70% glycerol and the embryos mounted on slides. Stage 16 embryos were identified by the presence of three disc-like contractions in the gut (6A). Images were captured using a Zeiss Axioskop, a MTI DAGE 3CCD camera, a Scion CG7 frame-grabber and Scion image 1.62c software.

In a similar way embryos from the cross between female UAS-mCD8-GFP and male GAL4 were collected, stained and images were captured. The primary antibody used was goat-anti-GFP (Jomar Diagnostics,1:500 dilution in PBT). No normal goat serum was added to the PBT during blocking due to the fact that the secondary antibody was a HRP conjugated donkey-anti-goat antibody (Jackson imuno research laboratories,1:500 dilution in PBT).

2.2.2 Staining of VNC

Embryos from a cross between female UAS-τ-LacZ and male GAL4 lines were allowed to develop. Stage L1 larvae were transferred to glass slides precoated with 10% polylysine into a drop of saline. By utilizing a sharp tungsten needle and a pair of forceps the VNC was dissected out and stuck down on the polylysine. The cords were fixed in a 4% formaldehyde solution for 15 minutes. After a couple of washes with PBS the cords were preincubated in block solution (PBT-NGS) for 30 minutes. Cords were then incubated over night at 4°C with the primary antibody (1:500 dilution of anti-β-gal in PBT-NGS). Slides were washed with PT and incubated with the secondary antibody (1:500 dilution of sheep-anti-mouse with conjugated HRP in PBT-NGS) at room temperature for 2 hours. The cords were preincubated in DAB-mix for 30 minutes before 20 µl H_2O_2 (0.3%) was added. The process was stopped after 10 minutes by rinsing the cords in PBS. The PBS was replaced with 70% glycerol and the cords mounted by adding a coverslide with a tab of vaseline in the corners. Images were captured using a Zeiss Axioskop, a MTI DAGE 3CCD camera, a Scion CG7 frame-grabber and Scion image 1.62c software.

In a similar way larval cords from the cross between female UAS-mCD8-GFP and male GAL4 were obtained, stained and images captured. The primary antibody used was goat-anti-GFP (1:500 dilution in PBT). No normal goat serum was added to the PBT during blocking due to the fact that the secondary antibody was a donkey-anti-goat antibody (1:500 dilution in PBT).

2.3 GFP expression

Some Bloomington stocks were crossed directly to UAS-mCD8-GFP; BL 6793, 6798 and 7028, because they were known to have a late onset of expression (unpublished data). Offspring from the cross between female UAS-mCD8-GFP and male GAL4, which express GFP, were directly observed under fluorescent microscopy. Lines showing promising staining pattern with anti-β-gal were also examined. Both embryos and larvae were studied and images were captured with an Olympus AX70 microscope, an I/Pentamax camera and IPlab 3.6 software. A 460-490 nm excitation wavelength filter was used and a 505 nm mirror and a 515-550 nm barrier filter for emission. The embryos were either observed whole or as a fillet, opened along the dorsal midline and the digestive system removed to expose the CNS. The larvae were also studied whole, in a drop of hydrocarbon oil under a coverslip to reduce movement. However, most studies were done on nerve cords dissected out from L1 larvae and stuck down on polylysine coated slides. Confocal microscopy was also used to study stage L1 larvae VNC.

2.4 Dye injections

Embryos and larvae from the cross between female UAS-mCD8-GFP and male GAL4 were used for dye injections. The GFP fluorescence was used to simplify the task of identifying cells. In preparing embryos they were manually dissected from their vitelline membrane. A small cut was done at the anterior end and the embryo was squeezed out onto a polylysine-coated slide. The embryos were either injected whole or filleted, living or fixed for 5 minutes in a 4% formaldehyde solution. Filleting and exposing the CNS prior to injecting instead of afterwards takes the pressure off successfully filleting an already injected embryo. But it forces you to inject the cells from the internal side through the muscle tissue instead of just having to penetrate the epidermis.

A living embryo is easier to penetrate than the rubbery texture of a fixed one but the tissue sticks more easily to the electrode. Nerve cords were dissected out from L1 larvae and stuck down on precoated polylysine slides. Individual cell bodies were visualised under 100X water immersion DIC optics and the position and presence of fluorescence identified the correct cells. A 30-60 Mohm micro electrode (Harvard Apparatus) was filled with 1, 1'-dioctadecyl-3, 3, 3', 3'-tetrametyl indocarbocyanine perchlorat (DiI) 0.1% in 1:9 DMSO:ethanol and the shaft was backfilled with 0.1 M LiCl. DiI has an excitation wavelength of 550 nm and an emission maximum at 565 nm. A 520-550 nm excitation wavelength filter was used and a 565 nm mirror and a 580 nm barrier filter for emission. Under GFP fluorescence the tip of the electrode was brought up to the plasma membrane of the cell. The dye was ejected from the electrode by iontophoresis. About 10 seconds of 0.2 nA dehyperpolarizing current is enough to stain the cell and its axon. Images were captured with the Olympus system, previously mentioned.

2.5 Crosses

2.5.1 Recombination

Two of the Bloomington GAL4-lines, BL1874 and BL7148, were used to create recombinant lines carrying both the GAL4 and the UAS construct on the second chromosome. The first step was to cross females homozygous for the UAS-mCD8-GFP construct to males homozygous for the GAL4 gene. These produce 100% heterozygous $^{UAS}/_{GAL4}$. During the larval stages the vials were moved into 30°C to increase the recombination rate, since it is during this time the oogenesis occurs. A twofold increase in recombination is obtained by the elevation in temperature (3A). Since chromosomes do not recombine during spermatogenesis, only females are of interest. Once pupae started to form the vials were brought back to 25°C to not decrease fertility, which is otherwise the case. From this cross virgin females were collected and crossed to a balancer stock, heterozygous $^{Sco}/_{CyO}$ for the second chromosomes, in egg-chambers. From this cross several different genotypes can emerge. The only fluorescent larvae will be the ones that have recombined resulting in a UAS:GAL4 construct balanced by either CyO or Sco. These larvae were identified by the presence of fluorescence and put into vials. Once hatched the males were selected and crossed individually to females of the same balancer stock as before. In this cross the offspring were selected for CyO and against Sco resulting in a recombinant line with the genotype $^{UAS:GAL4}/_{CyO}$ for the second chromosome. See appendix for more details about the crosses.

2.5.2 Crosses with mutants

When selecting mutants to screen the mutation must lie on another chromosome than the UAS:GAL4 construct to make the crosses possible. Six different mutants were used that could be divided into three categories; homozygous viable mutants, heterozygous mutants over a GFP-balancer and heterozygous mutants not over GFP-balancer. To be able to distinguish between the final genotypes an embryonic marker must be present in the heterozygous mutants. Since the genotypes for these three categories are rather different the crossing scheme for them will differ. For the homozygous mutants the first cross between the recombinant and the mutant will generate $^{UAS:GAL4}/_{+}$; $^{Mut}/_{+}$ by selecting against CyO. By crossing this line to the homozygous mutant once more, selecting only fluorescent larvae, the genotypes $^{UAS:GAL4}/_{+}$; $^{Mut}/_{Mut}$ and $^{UAS:GAL4}/_{+}$; $^{Mut}/_{+}$ are obtained. The heterozygous mutant is assumed to have no phenotype. When the heterozygous mutants already over a GFP marker are crossed to the recombinant line the genotype $^{UAS:GAL4}/_{+}$; $^{Mut}/_{+}$ is obtained by selecting against CyO and visual adult markers included in the GFP marker construct.

This line is crossed once more to the mutant line to get $^{UAS:GAL4}/_{+}$; $^{Mut}/_{Mut}$ and $^{UAS:GAL4}/_{+}$; $^{Mut}/_{+}$. Also here the heterozygous mutant is assumed to have no phenotype. The heterozygous mutants without a GFP marker had to have one introduced. By crossing the recombinant line to a GFP balanced line prior to the mutant crosses the genotypes $^{UAS:GAL4}/_{+}$; $^{Mut}/_{Mut}$ and $^{UAS:GAL4}/_{+}$; $^{Mut}/_{+}$ were acquired much in the same way as the others. See appendix for details about the crosses. The last step of the crosses was put up in egg-chambers. Where necessary the embryos or larvae were selected for embryonic markers. VNC from stage L1 and L2 larvae were dissected out and stuck onto a polylysine coated slide. Images were captured with the Olympus system.

3 Results

3.1 Screening of GAL4-lines

A number of GAL4 lines were used to drive expression of UAS-τ-LacZ and UAS-mCD8-GFP. Embryos and larvae were studied by the aid of immunohistochemistry and fluorescence microscopy in search of a GAL4-line driving expression in a small subset of PNS neurons with minimal expression in CNS neurons. Eight out of the ten investigated lines were dismissed. The discarded lines did not show expression in the PNS or had excessive expression in CNS and other cell types (see table2). Axons in anti-β-gal stains could only be observed for a few cell body lengths. Despite several alterations in the protocol only slight improvement in axon visualisation was accomplished. Cell bodies were nicely stained and the identity of the GAL4-expressing cells could be determined. The pattern of antibody staining allowed us to rule out three of the lines. These either did not show specific neuron staining or were considered too difficult to work with due to excessive staining in other cell types. The seven remaining lines were subdued to a GFP study of larval VNC's which lead us to rule out five more lines. Either because of excessive CNS staining or absence of PNS axons in the CNS. Left were two lines, BL7148 and BL1874, showing promising staining (fig. 6 and 7). BL1874 showed specific staining in a small subset of PNS, probably lesB, lesC and in some segments desB or desA2. Specific staining was seen in the CNS, a clusters of 8-10 cells on each side of the midline. In BL7148 staining was seen in most of the PNS. There was not much staining in the CNS except for a few glial cells.

Embryos stained with anti-GFP showed the same pattern as embryos stained with anti-β-gal. Axons were visualised better but because of the late onset of active GFP and the cuticle forming in stage 17 embryos staining of central terminals of sensory axons was still not satisfactory.

No staining could be observed in anti-β-gal or anti-GFP treated Cords. A few cords showed weak stain in single cells that faded away in a matter of days. VCNs from larvae expressing GFP did not show any sign of staining though they clearly were fluorescent.

Table 2: Observed pattern of staining by immunohistochemistry and GFP in st16 GAL4 driving lines. [1]Stocks known to have late expression were not stained with antibodies, [2]Only stocks with promising antibody staining was tested with GFP expression

Bloomington stock no.	Immunohistochemistry[1]	Fluorescence[2]	Conclusion
7008	Dorsal epidermis. Nothing in CNS. Some PNS in ventral group, not all segments. Some glial cells.		Too much unspecific staining.
7028		Only GFP expression in salivary glands.	Absence of neuron staining.
3042	A band of two cells repeated in each segment of the CNS.		Only CNS staining.
6981	Pattern of glial cells. Some PNS. No CNS staining.	Showed staining in salivary glands but not in CNS or PNS.	Absence of PNS axons in CNS.
6980	Some PNS cells and their support cells.	No staining could be observed in either the PNS or CNS.	Absence of PNS axons in CNS.
1874	Specific staining in a small subset of PNS. Probably lesB, lesC and in some segments desB or desA2. Specific staining in the CNS, clusters of 8-10 cells on each side of the midline.	Strong stain in the salivary glands. PNS and CNS axons visible in CNS. CNS stained neurons are outside axon pathways.	Good candidate.
7010	A few unspecific cells. No neurons.		Absence of PNS staining.
7148	Stains most of the PNS. Not much staining in the CNS except for a few glial cells.	Some staining in CNS. Clear staining in lch5 and some other PNS. Possible to make out weak axons in the CNS. Glial cells in CNS distort visibility. Saliva glands.	Possible candidate.
6793		The lch5 and the dorsal cluster are clearly visible in the embryo. Axons are visible though there is too much staining in the CNS to be able to follow individual axons.	Excessive CNS staining.
6798		Weak staining in the embryo but nice PNS staining in larvae. Visible axons but too much CNS fluorescence to follow individual axons.	Excessive CNS staining.

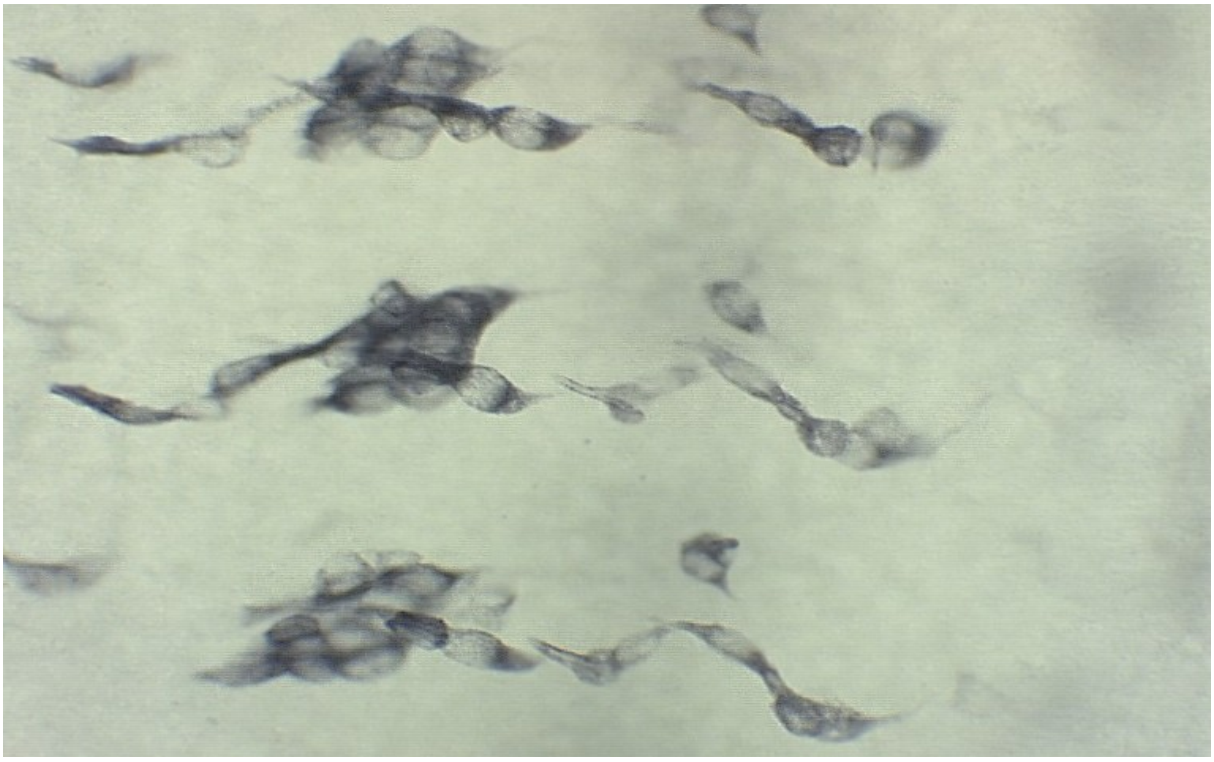

Figure 6: Expression pattern in BL7148. UAS:GAL4 system drives expression of τ-lacZ targeted with anti-β-gal antibodies. Expression in most of PNS. Here we see three hemisegments visualised under DIC optics. Anterior up and ventral left.

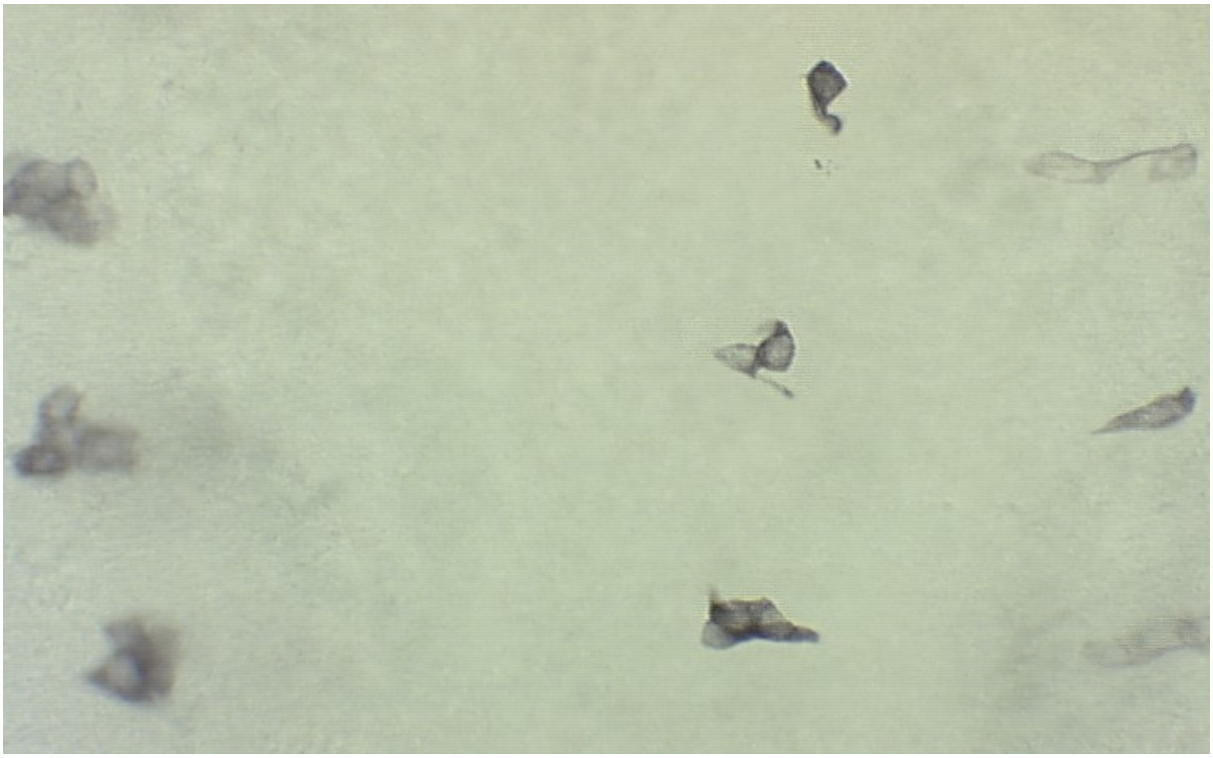

Figure 7: Expression pattern in BL1874. UAS:GAL4 system drives expression of τ-lacZ targeted with anti-β-gal antibodies. Expression in lesB, lesC and desB/desA2. Cluster of cells located ventral are CNS neurons. Here we see three hemisegments visualised under DIC optics. Anterior up and ventral left.

3.2 Recombination

Approximately 1000 larvae were examined for each recombination cross. Of these, about 70 larvae for each cross showed fluorescence and were collected resulting in a recombination rate of 7%. The number of males that hatched from the crosses involving BL1874 and BL7148 were 34 and 19 respectively. Larvae from the cross between the recombinant lines and the balancer were examined. The progeny from five different males from each recombinant were studied. All males examined produced fluorescent larvae but as expected a quarter of the offspring displayed stronger expression, homozygous for the UAS:GAL4 construct, and a quarter had no expression, homozygous for the balancer (data not shown). The technique used to construct the recombinant flies was highly successful and saved a lot of time and work. By identifying the recombinants by their fluorescence made the technique of back crossing, normally used to identify recombinants, unnecessary.

3.3 Characterising the wild type pattern in BL1874

The wild type expression pattern of GAL4 in BL1874 was determined by immunohistochemistry, GFP expression and single cell dye injections. Stage 16 embryos were stained with antibodies to determine which PNS cells drives GAL4 expression. VNCs from L1 larvae expressing GFP were observed to determine the PNS axons central arborisation. Dye injections were performed in stage 16 embryos and L1 larvae to unravel the origin of axons on the CNS. In the characterisation of the BL1874, the goal was to identify the wild type GFP pattern to such extent that defects could be identified. Variation in the intensity of fluorescence as well as variations in which cells express GFP made this a difficult task. The variation in expression existed within lines of flies and even within an embryo.

The antibody staining revealed the identity of the PNS neurons stained. In almost all segment two neurons in the lateral cluster were visible. They were located just anterior to the lch5 cluster, most likely being lesB and lesC (fig. 7). In about half the segments a single cell was observed in the dorsal cluster. This neuron is thought to be either desB or desA2. In the CNS the identities of the cells were more ambiguous. Rather dorsal at the edge of the nerve cord a cluster of 8-10 CNS neurons were driving GAL4 expression. More ventral another cluster of 2-4 cells show expression. No matter which method used, antibody staining, fluorescence observed with the Olympus microscope or confocal microscopy, the origin and pathway of most axons could not be deduced with certainty (fig. 8). Many axons pass through the same point at nearly the same focal plane making it impossible to tell which is which. In larval cords motor axons could be seen leaving the VNC as well as sensory axons entering. Different interneurons could be seen that cross the midline or send axons along the longitudinal connectives. DiI injections were a reliable method to solve this problem. By injecting the fluorescent cells in the larval nerve cord one by one their axons could be observed (fig. 9). In this way some motorneurons sending axons out of the nerve cord and several interneurons sending axons internally in the cord were identified (fig. 10). The PNS neurons that express GFP in the recombinant embryos were also injected and their arborisation in the CNS was examined. All projections of PNS axons seemed to end in the same segment as they entered the cord relatively close to the midline (fig. 11). The axons were seen crossing the longitudinal connective and bending posterior close to the midline. A posterior located interneuron was found sending a branched axon anterior on both sides of the midline. This suggest that the distinctive pattern visible on either side of the midline is an overlap between the PNS arborisations and the axon from a interneuron.

3.4 Mutant studies

The aim was to use the *Drosophila* line BL1874 to determine the pattern of central arborisations for sensory neurons lesB and lesC. The recombinant strain carrying the UAS:GAL4 construct was crossed into a mutant background. Larval cords were observed in search of misprojections in lesB and lesC arborisations. Of the six mutants originally crossed to the BL1874 recombinant stock only *Connectin* produced offspring whose VNC's could be examined for misprojections. The axon projections in *Ptp10D* could not be determined due to the fact that the stock had lost its balancer making identification of the right genotype impossible. Crosses with *Nrt* and *Abl* produced offspring with extremely weak fluorescence making PNS axons invisible. Crosses including *side* and *Ten-m* had profound background staining in the CNS drowning most signals from the regular expression. This is probably because these mutations are embryonic lethal in the homozygous state. As a result no homozygous mutant larvae could be obtained for examination of central sensory projections. All larvae were homozygous or heterozygous for the balancer chromosome carrying the actin-GFP marker. From the one mutant cross that resulted in larvae with the expected level of fluorescence, *Con*, about 30 fluorescent VNC's were examined but no mutant phenotype was observed. The same pattern as discussed before was visible close to the midline. Considering the actual number of segments studied hundreds of terminals were studied without detecting any misprojections.

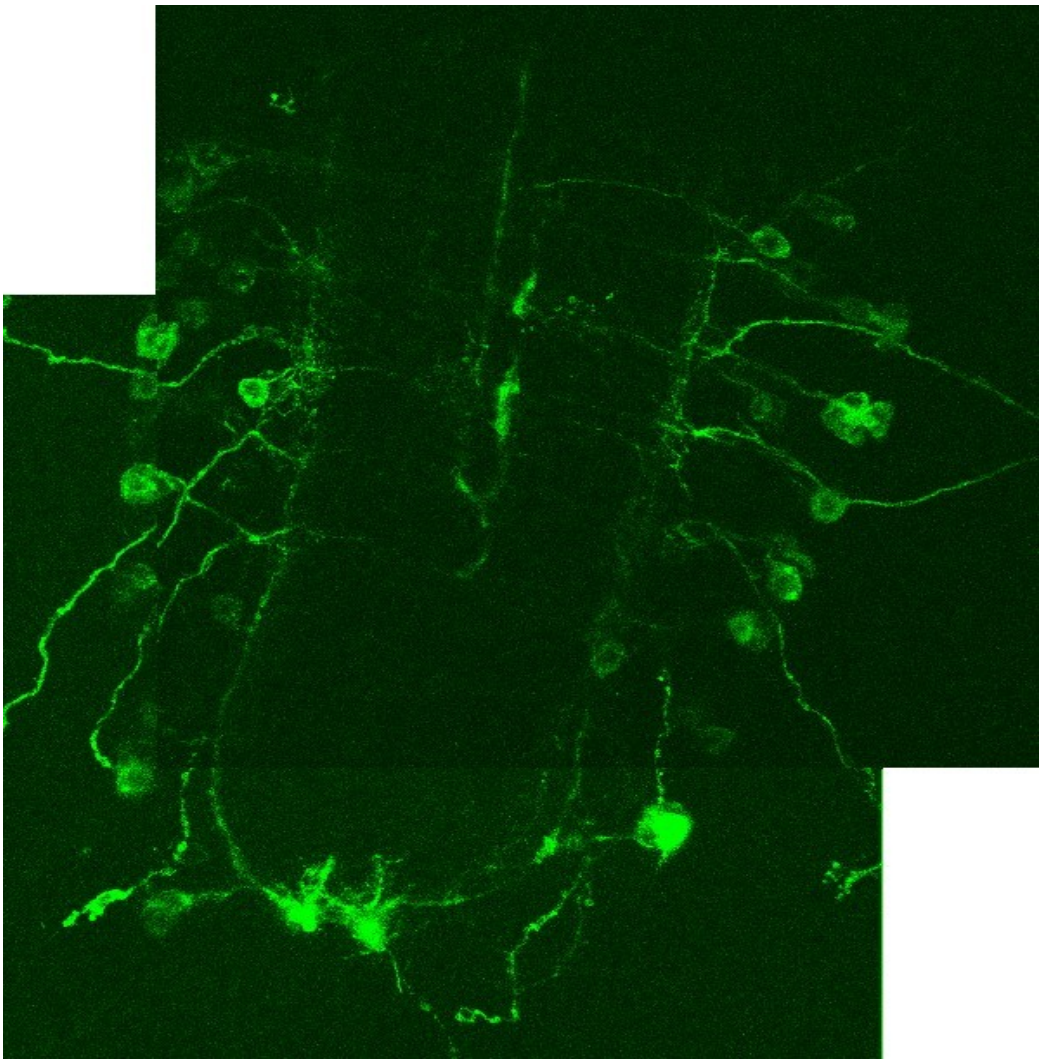

Figure 8: Confocal image of a stage L1 larvae VNC expressing GFP by a UAS:GAL4 system.

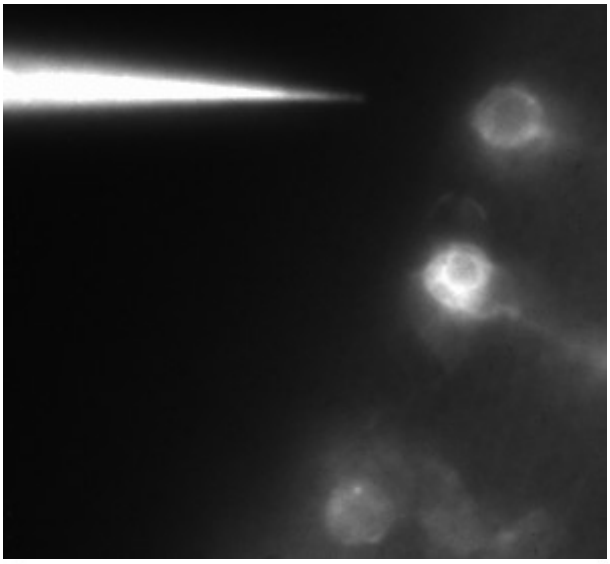

Figure 9: VNC prior to injection under GFP-fluorescence.

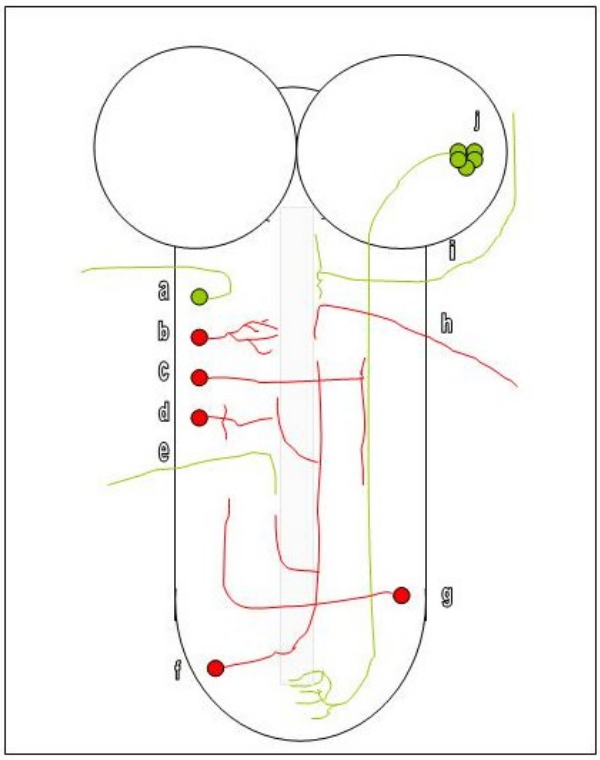

Figure 10: Summary picture of injections, red, and GFP-expression, green, observed in the VNC. a-motorneuron, b, c, d, f, g, j-interneuron, e, h i-sensory axons.

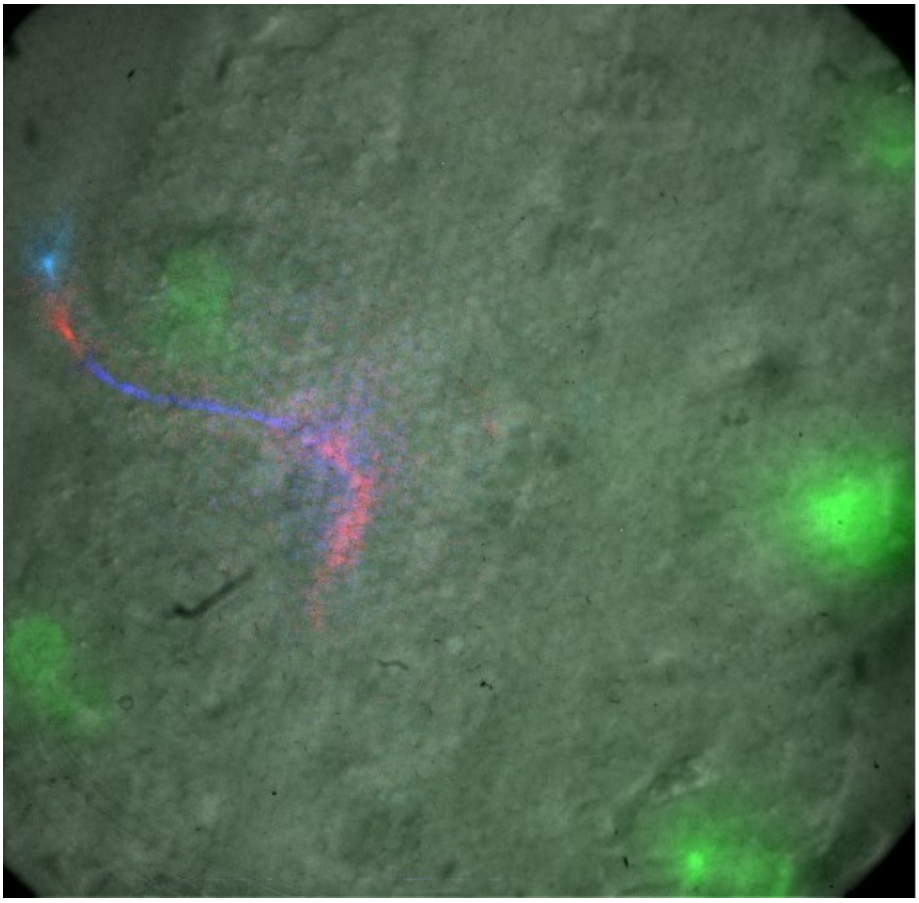

Figure 11: Terminal arborisation of lesB or lesC. Close to the midline the axon bends posterior. The VNC is positioned anterior up. The picture is a merge between a DIC-image, a GFP-image and several different focal planes of DiI-images.

4 Discussion

We set out to create a high throughput method for screening mutants in search of genes involved in PNS axons ability to localize the right target cells in the CNS. By using the UAS:GAL4 system, the central axon morphology of a small subset of sensory neurons was visualized by expression of a membrane targeted GFP. Homozygous mutants for *Con*, *Abl*, *side*, *Nrt* and *Ten-m* carrying a UAS-mCD8-GFP:GAL4 construct were created and central arborisations in their VNCs were examined. This was meant as a faster screening method than the single cell dye injections normally used. The DiI injections is a rather time consuming method resulting in a limitation in the amount of embryos examined. When studying the GFP expression one can study a greater number of nerve cords. This makes it possible to pick up mutants with weaker penetrance and get more reliable statistics.

During the first step, screening of GAL4-lines, a few unexpected problems were encountered. First of all, the fact that some of the GAL4 driving lines in FlyBase did not even show PNS expression revealed the presence of faulty annotations in the database. GAL4-lines were ordered on the basis of previously studied expression patterns. Secondly, the τ-LacZ fusion protein failed to visualise sensory axons in late stage embryos properly. This is peculiar since the Tau-protein is fused to the β-gal protein in order to promote axon staining. Cell bodies were properly stained hinting that transport of lacZ down the axon might be the main issue. It is also noticeable that CNS neurons show staining almost twice the length out the axons compared to the PNS neurons displaying the difference in transport between different cell types. Since the cell bodies could be identified the method still served some of its purpose. A way to solve this problem might be to try with other lacZ-construct to see if transport along the axon is improved.

The reason why no anti-β-gal or anti-GFP staining was obtained in larval nerve cords remains unclear. Many other workers have successfully carried out immunihistochemical staining in larval nerve cords. Compared to these protocols no major differences could be found (7A, 15). In an attempt to clarify this, a fillet stage 16 BL1874 embryo was stained on a slide along with nerve cords from L1 larvae. The embryo was nicely stained while no staining could be seen in the cord (data not shown). Some cords were even torn in half to allow antibodies to pass easily into the cells. This was done in order to rule out the presence of some kind of protective membrane. Despite these alterations in the protocol no staining was observed.

The use of confocal and deconvolution microscopy could not unravel the axon pathways in the CNS in GFP expressing larvae. Overlapping processes makes the origin of an axon difficult to determine. The late onset of GFP expression prevents the use of antibodies to visualise GFP stained axons in the CNS. The cuticle that forms during stage 17 in embryos is impermeable to antibodies and as mentioned above the larval cords did not stain. Single cell injections were more successful in untangling which axons belong to the sensory neurons. The hope was that once this was done only the cords would be required to identify the correct arborisation. If the morphology of the sensory neurons were identified by single cell dye injections the idea was that only GFP preparations would be required to identify them in the VNCs in loss-of-function mutant larvae. The typical pattern seen close to the midline turned out to be made up of both CNS and PNS axons. DiI injections in the PNS neurons, lesB and lesC, place their arborisations close to the midline. Their location and appearance of branching pattern concurs well with previous studies (13). All the sensory neurons have been individually injected and their central arborisation pattern determined. Unfortunately, the posterior located interneuron, sending a branched axon anterior, that overlaps with the PNS arborisations, obstructs the characterisation of the sensory projections.

There are advantages as well as disadvantages with these techniques to visualise the axons. While the single cell dye fill give a strong staining in the whole cell it is a rather time-consuming method. The antibody staining, on the other hand, allows for a processing of a high number of embryos but require that the tissue is fixed thereby stopping development. This can be avoided with GFP expression since it can be followed in living cells allowing time-lapse studies. However, the fluorescence is bleached over time limiting the observation time. Other aspects to take into consideration is that stage 17 embryos and larvae have a cuticle impermeable to antibodies as well as the later onset of GFP due to its activation process. Autofluorescence in the embryo yolk and reflection in the larva cuticle may also distort fluorescent signals. The late onset of GFP prohibits time-lapse studies of PNS axons entering the CNS. No strong GFP fluorescence could be seen until the embryos were late stage16. At this time axons have already arborised in the CNS (51). There exist lines expressing a modified GFP line with earlier onset that might be used for time-lapse. In these studies one would be able to study the PNS axons choice of fascicle pathway into the CNS.

It is difficult to explain why fluorescence was lost in some of the mutant crosses. The same virgin recombinants were used in all crosses, which leave us with random effects and something in the mutant line affecting the expression. The problem of the increased background fluorescence is likely caused by the GFP-balancer. Both *side* and *Ten-m* were balanced with a construct staining Actin with GFP and those were the ones showing elevated background level. The *side* and *Ten-m* mutations are embryonic lethal in the homozygous state so no homozygous mutant larvae could be obtained. All larvae examined were homozygous or heterozygous for the balancer chromosome carrying the actin-GFP marker causing fluorescence in most cells.

The cross including a loss-of-function mutation for *Con* did not display an abnormal phenotype that could be observed. If a loss-of-function does not generate a phenotype it does not mean that the gene is not involved in axon guidance. There might be overlapping gene functions, the gene might disrupt early development resulting in abnormal embryos or the resolution of the screen is to low. These are just a few reasons why a gene might go through the screen unnoticed. The absence of mutant phenotype with *Con* might also be caused by the fact that it is a hypomorph, not a full loss-of-function mutant (54).

This UAS:GAL4 system was developed as a rapid screening method. It looks promising but at this stage it is more useful as a tool for easier and quicker injections. The resolution in the nerve cord centre is almost certainly too low to identify an abnormal phenotype under GFP expression. However, with the presence of fluorescence in the PNS neurons they are easier to find and can be more accurately injected. A mounted embryo can be injected multiple times with the same electrode in different segments. The fluorescence also provides a familiar pattern in the CNS, landmarks to which the PNS arborisations can be compared. One can capture images under both GFP fluorescence and DiI fluorescence and merge the pictures to reveal exact location of the arborisation. By making the injections more efficient a higher number of terminals can be studied resulting in better statistics and the possibility of picking up weaker penetrance.

5 Future prospects

Either one can continue screening new loss-of-function mutants by doing single cell injections in GFP-expressing embryos, or more GAL4-driving lines can be screened in search of a candidate with more suitable expression pattern. If a line driving expression in a few sensory neurons and no VNC cells was found this technique has the potential of being a powerful and rapid screening method.

If a mutant phenotype is identified one could never be sure whether it had been caused by disruptions in the axon or by more general disruptions in the embryo development. To deduce this, a rescue experiment is required. A rescue of the loss-of-function by expressing the gene with a UAS:GAL4 system in the desired subset of cells should eliminate the mutant phenotype. If it does not, the phenotype might be caused by major disturbances in the environment and not by the loss of the gene in the neuron itself. Also, if a phenotype is found, different crossing schemes would be needed to generate offspring that are 100% homozygous for the mutant. This would to further improve statistics and eliminate the assumption of no phenotype in heterozygous mutants.

6 Acknowledgment

Many thanks to Paul Whitington for his never ending commitment to the project, to Martin Steinel, Veronica Martin and Karen Bates for company and assistance both on and off campus and to all the guys in the fly-room for making sorting flies a little bit more fun.

7 References

1. Huber A. B, Cloutier J-F; Signalling at the growth cone: ligand-receptor complexes and the control of axon growth and guidance; Annu. Rev. Neurosci.; 2003; 26: 509-563
2. Lee S, Harris K-L, Whitington P. M, Kolodziej P. A; short stop is allelic to kakapo, and encodes rod-like cytoskeletal-associated proteins required for axon extension; J. neurosci.; 2000; 20: 1096-1108
3. Goodman C. S; Mechanisms and molecules that control growth cone guidance; Annu. Rev. Neurosci.; 1996; 19: 341-377
4. Colamarino S. A, Tessier-Lavigne M; The axonal chemoattractant Netrin-1 is also a chemorepellant for trochlear motor axons; Cell; 1995; 81: 621-629
5. Tessier-Lavigne M, Goodman C. S; The molecular biology of axon guidance; Science; 1996; 274: 1123-1133
6. Whitington P. M, Sink H; Necessity and redundancy of guidepost cells in the embryonic *Drosophila* CNS; Int. J. Devl. Neurosci.; 2004; 22: 157-163
7. Murakami F, Yamamoto N; Wiring of the brain by a range of guidance cues; Progress in neurobiology; 2003; 68: 393-407
8. Zlatic M, Bate M; Genetic specification of axonal arbors: atonal regulates robo3 to position terminal branches in the *Drosophila* nervous system; Neuron; 2003; 37: 41-51
9. Landegraf M, Bate M; The origin, location, and projections of the embryonic abdominal motorneurons of *Drosophila*; J. Neurosci.; 1997; 17: 9642-9655
10. Hoang B, Chiba A; Single-cell analysis of *Drosophila* larval neuromuscular synapses; Developmental biology; 2001; 229: 55-70
11. Bodmer R, Jan Y. N; Morphological differentiation of the embryonic peripheral neurons in *Drosophila*; Roux's Arch Dev Biol; 1987; 196: 69-77
12. Ghysen A, Jan Y. N; Sensory neurons and peripheral pathways in *Drosophila* embryos; Rouxs Arch Dev. Biol; 1986; 195: 281-289
13. Merrit D. J, Whitington P. M; Central projections of sensory neurons in the *Drosophila* embryo correlate with sensory modality, soma position, and proneural gene function; J. neurosci.; 1995; 15: 1755-1767
14. Brand A; GFP in *Drosophila*; TIG; 1995; 11: 324-325
15. www.protocol-online.org/prot/Model_organisms/drosophila/staining (Dec. 2004)
16. http://flybase.bio.indiana.edu/ (Sep. 2004)
17. http://flystocks.bio.indiana.edu/ (Sep. 2004)
18. Callahan C. A, Thomas J. B; Tau-beta-galactosidase, an axon targeted fusion protein; Proc. Natl. Acad. Sci. USA; 1994; 91: 5972-5976
19. Chalfie M, Prasher D. C; Green fluorescent protein as a marker for gene expression; Science; 1994; 263: 802-805
20. Dormand E-L, Brand A. H; The GAL4 system as a tool for unravelling the mysteries of the *Drosophila* nervous system; Current opinion in neurobiology; 1995; 5: 572-578
21. Sink H, Whitington P; Early ablation of target muscles modulates the arborisation pattern of an identified embryonic *Drosophila* motor axon; Development; 1991; 113: 701-707
22. Matthes D. J, Goodman C. S; SemaphorinII can function as a selective inhibitor of specific synaptic arborizations; Cell; 1995; 81: 631-639
23. Desai C. J, Zinn K; Receptor tyrosine phosphatases are required for motor axon guidance in the *Drosophila* embryo; Cell; 1996; 84: 599-609
24. Parsons L, Whitington P. M; roundabout gene family functions during sensory axon guidance in the *Drosophila* embryo are mediated by both Slit-dependent and Slit-independent mechanisms; Developmental biology; 2003; 264: 363-375

25. Garner C. C, Ziv N. E; Molecular mechanisms of CNS synaptogenesis; Trends in neuroscience; 2002; 25: 243-250
26. Jones E. G; Anatomy of cerebral cortex: columnar input-output organization; The organisation of the cerebral cortex; MIT press; Cambridge; 1981; 199-235
27. Hartenstein V; Development of *Drosophila* larval sensory organs: spatiotemporal pattern of sensory neurones, peripheral axonal pathways and sensilla differentiation; Development; 1988; 102: 869-886
28. Butner K. A, Kirshner M. W; Tau protein binds to microtubules through a flexible array of distributed weak sites; J. of Cell biology; 1991; 115: 717-730
29. Keshishian H, Johansen J; Cellular mechanisms governing synaptic development in *Drosophila melanogaster*; J. of Neurobiology; 1993; 24: 757-787
30. Sink H, Goodman C. S; sidestep encodes a target-derived attractant essential for motor axon guidance in *Drosophila*; Cell; 2001; 105: 57-67
31. Thomas; Elucidating the Basis of Sidesteps Function in Motor Axon Guidance; A. Dros. Res. Conf.; 2003; 44: 716B
32. Sink H, Goodman C. S; Sidestep: a mutant with perturbed motor axon pathfinding; A. Dros. Res. Conf; 1996; 37: 279
33. Sink H; Motor axon guidance in the embryonic periphery is mediated by the Sidestep protein; A. Dros. Res. Conf; 2001; 42: 728B
34. Barthalay Y, Piovant M; *Drosophila* neurotactin mediates heterophilic cell adhesion; EMBO J; 1990; 9: 3603-3609
35. Liebl E. C, Seeger M. A; Interactions between the secreted protein Amalgam, its transmembrane receptor Neurotactin and the Abelson tyrosine kinase affect axon pathfinding; Development; 2003; 130: 3217-3226
36. Meadows L. A, White R. A; The cell adhesion molecule, connectin, and the development of the *Drosophila* neuromuscular system; J. Cell Sci; 1994; 107: 321-328
37. Hill K. K, Hoffmann F. M; Genetic interactions between the *Drosophila* Abelson, Abl, tyrosine kinase and failed axon connections (Fax), a novel protein in axon bundles; Genetics; 1995; 141: 595-606
38. Gertler F. B, Hoffmann F. M; *Drosophila* abl tyrosine kinase in embryonic CNS axons: a role in axonogenesis is revealed through dosage-sensitive interactions with disabled; Cell; 1989; Vol. 58; pp. 103-113
39. Wills Z, Van Vactor D; Profilin and the Abl tyrosine kinase are required for motor axon outgrowth in the *Drosophila* embryo; Neuron; 1999; 22: 291-299
40. Sun Q, Zinn K; Receptor tyrosine phosphatases regulate axon guidance across the midline of the *Drosophila* embryo; Development; 2000; 127: 801-812
41. Stoker A. W; Receptor tyrosine phosphatases in axon growth and guidance; Curr. Opin. Neurobiol; 2001; 11: 95-102
42. Dgany O, Wides R; The *Drosophila* odz/ten-m gene encodes a type I, multiply cleaved heterodimeric transmembrane protein; Biochem. J; 2002; 363: 633-643
43. Tucker R. P; The novel extracellular matrix proteins Ten-a and Ten-m are expressed by complementary populations of neurons in the developing nervous systems of *Drosophila* and birds; Molec. Biol. Cell; 1996; 7: 64a
44. Raper J. A, Goodman C. S; Pathfinding neuronal growth cones in grasshopper embryos. II. Selective fasciculation onto specific axonal pathways; J. Neurosci.; 1983; 3: 31-41
45. Halpern M. E, Keshishian H; Growth cone behaviour underlying the development of stereotypic synaptic connections in *Drosophila* embryos; J. neurosci.; 1991; 11: 3227-3238
46. Merrit D. J, Hawken A, Whitington P. M; The role of the cut gene in the specifications of central projections by sensory axons in *Drosophila*; Neuron; 1993; 10: 741-752
47. Harris K-L, Whitington P. M; Pathfinding by sensory axons in *Drosophila*: substrates and choice points in early lch5 axon outgrowth; J. Neurobiol; 2001; 48: 243-255

48. Murray M. J, Whitington P. M; In Vivo dynamics of axon pathfinding in the *Drosophila* CNS: a time-lapse study of an identified Motorneuron; J. Neurobiol; 1998; 37: 607-621
49. Goodman C. S; The likeness of being: phylogenetically conserved molecular mechanisms of growth cone guidance; Cell; 1994; 78: 353-356
50. Bodmer R, Jan Y. N; Neurogenesis of the peripheral nervous system in *Drosophila* embryos: DNA replication patterns and cell lineages; Neuron; 1989; 3: 21-32.
51. Yu T. W, Bargmann C. I; Dynamic regulation of axon guidance; Nat Neurosci; 2001; 1169-76
52. Elkins T, Goodman C. S; Genetic analysis of a *Drosophila* neural cell adhesion molecule: interaction of fasciclin I and Abelson tyrosine kinase mutations; Cell; 1990; 60: 565-75.
53. Bossing T, Technau G. M; The fate of the CNS midline progenitors in *Drosophila* as revealed by a new method for single cell labelling; Development; 1994; 120: 1895-906
54 Nose A, Goodman C. S; Connectin: A homophilic cell adhesion molecule expressed on a subset of muscles and the motorneurons that innervate them in Drosophila; Cell; 1992; 70; 553-567

Additional references

1A. Hartenstein; V; Atlas of Drosophila development; 1993; Cold spring harbor press
2A. Campos-Ortega J. A, Hartenstein V; The embryonic development of *Drosophila melanogaster*; 1997; second edition; Springer
3A. Freenspan R. J; Flypushing, The theory and practise of *Drosophila* genetics; 1997; Cold Spring Harbor Press
4A. Flagg R. O; Carolina *Drosophila* manual; 1988; Carolina biological supply company
5A. Lawerence P. A; The making of a fly: The genetics of animal design; 1992; Blackwell Scientific Publications
6A. Ashburner M; *Drosophila*, a laboratory manual; 1989; Cold Spring Harbor Press
7A. Ashburner M; *Drosophila*, a laboratory handbook; 1989; Cold Spring Harbor Press

Appendix: Crossing schemes

The desired genotype is highlighted in each cross.

Recombination crosses (second chromosome):

First cross:
♀ $^{\text{UAS-mCD8:GFP}}/_{\text{UAS-mCD8:GFP}}$ X $^{\text{GAL4}}/_{\text{GAL4}}$ ♂
⇩
$^{\text{GAL4}}/_{\text{UAS-mCD8:GFP}}$

Second cross:
♀ $^{\text{UAS-mCD8:GFP}}/_{\text{GAL4}}$ X $^{\text{Sco}}/_{\text{CyO}}$[1] ♂
⇩
$^{\text{GAL4}}/_{\text{Sco}}$, $^{\text{GAL4}}/_{\text{CyO}}$, $^{\text{UAS-mCD8:GFP}}/_{\text{Sco}}$, $^{\text{UAS-mCD8:GFP}}/_{\text{CyO}}$ or $^{\text{UAS-mCD8:GFP:GAL4}}/_{\text{CyO or Sco}}$ (Select fluorescent larvae)

Third cross:
♀ $^{\text{Sco}}/_{\text{CyO}}$ X $^{\text{UAS-mCD8:GFP:GAL4}}/_{\text{CyO or Sco}}$ ♂
⇩
II) $^{\text{UAS-mCD8:GFP:GAL4}}/_{\text{CyO}}$, $^{\text{UAS-mCD8:GFP:GAL4}}/_{\text{Sco}}$, $^{\text{Sco}}/_{\text{Sco}}$[8] , $^{\text{Cyo}}/_{\text{Sco}}$[8] or $^{\text{Cyo}}/_{\text{Cyo}}$[8] (Select for Cyo and against Sco)

Final genotype:
$^{\text{UAS-mCD8:GFP:GAL4}}/_{\text{CyO}}$

Crosses with Homozygous mutants:

First cross:
♀ $^{\text{UAS:GAL4}}/_{\text{CyO}}$; $^{+}/_{+}$[2] X $^{+}/_{+}$; $^{\text{Mut}}/_{\text{Mut}}$ ♂
⇩
II)[3] $^{\text{UAS:GAL4}}/_{+}$ or $^{+}/_{\text{CyO}}$ III)[4] $^{\text{Mut}}/_{+}$ (Select against CyO)

Second cross:
♀ $^{\text{UAS:GAL4}}/_{+}$; $^{\text{Mut}}/_{+}$ X $^{+}/_{+}$; $^{\text{Mut}}/_{\text{Mut}}$ ♂
⇩
II) $^{\text{UAS:GAL4}}/_{+}$ or $^{+}/_{+}$ III) $^{\text{Mut}}/_{\text{Mut}}$ or $^{\text{Mut}}/_{+}$ (Select larvae with fluorescent VNC)

Final genotype:
$^{\text{UAS:GAL4}}/_{+}$; $^{\text{Mut}}/_{\text{Mut}}$ or $^{\text{UAS:GAL4}}/_{+}$; $^{\text{Mut}}/_{+}$

Crosses with GFP-balanced mutants:

First cross:
♀ $^{UAS:GAL4}/_{CyO}$; $^{+}/_{+}$ X $^{+}/_{+}$; $^{Mut}/_{TM3:GFP\text{-}pAct}$[5] ♂
⇩
II) $^{UAS:GAL4}/_{+}$ or $^{+}/_{CyO}$ III) $^{Mut}/_{+}$ or $^{+}/_{TM3:GFP\text{-}pAct}$ (Select against CyO and TM3)

Second cross:
♀ $^{UAS:GAL4}/_{+}$; $^{Mut}/_{+}$ X $^{+}/_{+}$; $^{Mut}/_{TM3:GFP\text{-}pAct}$ ♂
⇩
II) $^{UAS:GAL4}/_{+}$ or $^{+}/_{+}$ III) $^{Mut}/_{Mut}$, $^{Mut}/_{+}$, $^{Mut}/_{TM3:GFP\text{-}pAct}$ or $^{+}/_{TM3:GFP\text{-}pAct}$ (Select larvae with fluorescent VNC but without GFP-marker)

Final genotype:
$^{UAS:GAL4}/_{+}$; $^{Mut}/_{Mut}$ or $^{UAS:GAL4}/_{+}$; $^{Mut}/_{+}$

Crosses with Heterozygous mutants without GFP-marker:

First cross:
♀ $^{UAS:GAL4}/_{CyO}$; $^{+}/_{+}$ X $^{+}/_{+}$; $^{Sb}/_{TM3:GFP}$[6] ♂
⇩
II) $^{UAS:GAL4}/_{+}$ or $^{+}/_{CyO}$ III) $^{Sb}/_{+}$ or $^{+}/_{TM3:GFP}$ (Select against CyO and for TM3)

Second cross:
♀ $^{UAS:GAL4}/_{+}$; $^{TM3:GFP}/_{+}$ X $^{+}/_{+}$; $^{Mut}/_{TM6}$[7] ♂
⇩
II) $^{UAS:GAL4}/_{+}$ or $^{+}/_{+}$ III) $^{Mut}/_{TM3:GFP}$, $^{Mut}/_{+}$, $^{TM3:GFP}/_{TM6}$ or $^{+}/_{TM6}$ (Select larvae with fluorescent VNC and adults for TM3 and against TM6)

Third cross:
♀ $^{UAS:GAL4}/_{+}$; $^{TM3:GFP}/_{Mut}$ X $^{+}/_{+}$; $^{Mut}/_{TM6}$ ♂
⇩
II) $^{UAS:GAL4}/_{+}$ or $^{+}/_{+}$ III) $^{TM3:GFP}/_{TM6}$, $^{TM3:GFP}/_{Mut}$, $^{Mut}/_{TM6}$ or $^{Mut}/_{Mut}$ (Select larvae with fluorescent VNC without GFP-marker)

Final genotype:
$^{UAS:GAL4}/_{+}$; $^{Mut}/_{Mut}$ or $^{UAS:GAL4}/_{+}$; $^{Mut}/_{TM6}$

[1]Balancer chromosomes carrying Sco and CyO markers respectively
[2]Wild type chromosome
[3]Genotype for third chromosome
[4]Genotype for second chromosome
[5]Balancer chromosome carrying Ser and pAct-GFP marker
[6]Balancer chromosomes carrying either Sb or Ser and GAL4-KrC
[7]Balancer chromosome carrying Tb and H
[8]Not viable