UNIVERSITY OF CRETE

M.Sc. program: “Molecular biology and biomedicine”

Departments of Biology and Medical school

M.Sc. Thesis

“Investigation of spatiotemporal expression of proneural bound enhancers in embryo, larva and adult Drosophila melanogaster “

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Abstract

The central nervous system in *Drosophila melanogaster* originates from a single cell type, the Neuroblasts (NBs). It is known that some proneural genes (*ac, sc, l'sc, ase*) form a complex, the Achaete-Scute Complex (AS-C) which seems to be necessary for the differentiation of the nervous system, both central and peripheral. In order to elucidate the chromatin alterations in NBs during differentiation, chip-seq (Chromatin Immunoprecipitation followed by deep sequencing) was performed for proneural transcription factors in embryonic neuroectoderm (st. 8-11 – the stages where NBs are normally formed) and 11 regions related with genes that promote neural fate, were found to act as enhancers. In order to investigate the dependence of these enhancers' activation with the AS-C, transgenic flies with these enhancers expressed under a reporter gene were made and scanned in embryos mutated for these genes (*ac, sc, l'sc, ase*), named *Def(1)sc*^{B57} (loss of AS-C). No expression of the enhancers in question was observed (submitted data of V. Theodorou et al). In this study, we investigated whether their expression could be rescued in the presence of *ase*, a proneural gene that seems to have a different functional role than the rest. We saw that the expression of these same enhancers is partially saved in embryos mutated for *ac, sc, l'sc* but with spared *ase* (named *Def(1)sc*^{19} embryos), which indicates that *ase* might be sufficient for their activation. The second question that we raised was whether the same enhancers are active in different tissues and developmental stages where the AS-C has been documented to be active. We checked their expression in larval brain, larval imaginal discs and adult gut and we saw that most of them are widely expressed in more than one tissue. These data indicate that the differentiation of the nervous system in *D. melanogaster* could use the same set of genes and enhancers throughout development and adult life, and that it is closely related with the AS-C.

CONTENTS

1. Introduction

1.1: *Drosophila melanogaster* as an animal model.

The fruit flies *Drosophila*, are small flies that belong to the order of Diptera and the family Drosophilidae. *Drosophila melanogaster* is in the subgenus Sophophora and it has been used as animal model for over a century; its debut in the laboratories began in Harvard, by the William Castel's team, but the fame came with Morgan at the Columbia University, who defined the genes and established that they were found in the chromosomes, greatly enriching Mendel's theory of inheritance. Thereafter, D. melanogaster had been one of the most useful and common animal models (1,2).

Nowadays, *D. melanogaster* is used by thousands of laboratories worldwide to answer scientific questions related to regulation of gene expression, development, neurobiology, aging, cell biology, pathogenic mechanisms of disease, adaptation to new environments, behavior and many more. Among the reasons that make it such a useful animal model, are the facts that it is an organism that can be very easily and inexpensively cultured in laboratory conditions, it has a short life cycle while producing large number of externally laid embryos, and it is suitable for genetic modifications. The lack of chromosomal recombination in males and the vast genome data available make it an easy to use model for genetic analysis. Moreover, a key reason to its extended usage in the research community is the presence of numerous orthologous genes associated with human disease (1–5).

Figure 1: Drosophila melanogaster wild type. A female (left) and a male (right) D. Melanogaster, WT ("normal" – not genetically modified).

1.2: The life cycle of *D. melanogaster*.

One of the main reasons that *Drosophila* is widely used as a model is its short life cycle. In the laboratory conditions, it is cultured at 25 or 18°C (see material and methods for more). The time required for each developmental stage is affected by the temperature. The life expectancy varies from 60 to 80 days, depending on the conditions in which it is raised. The process of developing from a fertilized egg to an adult takes approximately 9-10 days at 25°C, but at 18°C it is prolonged to ~19 days (1). *Drosophila* is a holometabolous insect, and its life can be divided into four stages: embryo, larva, pupa and adult (**fig. 2a**). From now on, the time that is required for each developmental stage will be referring to flies that are cultured in 25°C, unless indicated otherwise.

Embryogenesis starts after the fertilization and formation of the zygote and happens within the egg membrane. It lasts only 24h and it is followed by three larva stages (termed first, second and third instar) with a molting event at each transition. Each of the first two larva stages lasts one day and the larvae are feeding within the medium. The 3rd instar larvae (this stage lasts 2 days) leave the medium and start wondering at the walls of the tube in order to find a spot and start the metamorphosis (**fig. 2b**). A hard, protective chitin-based pupal case is formed from the outer larval cuticle and the pupa stage begins. It lasts for 4-5 days and ecdysone, a steroid hormone, plays crucial role in this metamorphosis (1). Most of the larva tissues are degraded and the adult structures develop from 19 imaginal disks which are undifferentiated sacs of cells, present in the larvae. After the adult flies emerge, it takes 8-10 hours for them to become sexually mature and for the life cycle to repeat (6).

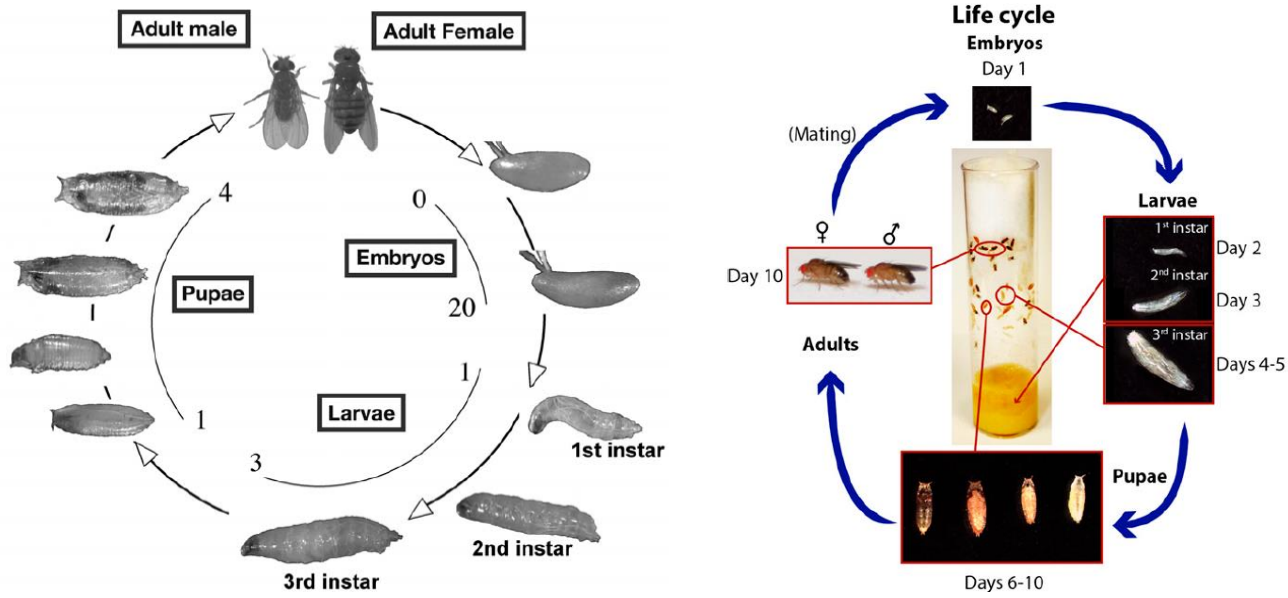


Figure 2: life cycle of *Drosophila melanogaster* at 25°C. a) The time indicated for embryos is in hours whereas for larvae it is in days b) the different larvae stages and their place in the vial where they are growing and feeding.

1.3: The genome of *Drosophila melanogaster*

The genome of *D. melanogaster* was fully sequenced for the first time in 2000, making it possible to perform sophisticated functional studies (7). The size of the genome is calculated in 180Mb (8) whereas the number of genes it is thought to reach the 14.000 (9). The genome is organized in four chromosomal pairs, three of which are autosomes (II, III &IV) and the remaining one is sex chromosomes (I). The Y chromosome is mostly composed by heterochromatin. It has very few genes, regulating spermatogenesis but it does not participate in sex determination directly. The X chromosome is large in size and contains many genes. Sex determination in fruit flies, in contrast with mammals, is not based in the presence of the Y chromosome but in a mechanism that "senses" the dosage of the X chromosome. Diploid flies with two X chromosomes are female and those with one X are males (10). Chromosomes 2 and 3 are large in size, whereas chromosome 4 (referring as the "dote chromosome") is a very small autosome (1).

1.4: Embryogenesis in *D. melanogaster*.

Embryogenesis begins with the fertilization of the oocyte in the uterus of a female *D. melanogaster* from a sperm (1st embryonic stage). Females can store sperm for up to 2 weeks in "specialized organs, named seminal receptacles and spermathecae" (11). After mating, females refuse to mate again for several days although they can store sperm from more than one male. Fertilization itself does not occur until the egg is ready to be laid. Before fertilization, the meiotic divisions of the oocyte had stopped temporarily in the metaphase of meiosis I. These meiotic divisions are activated again after the merge of the two gametes, which happens when a mature egg leaves the ovaries and travels through the oviduct, during which time some of the stored sperm is released (1). The diploid nucleus that is formed performs 13, extremely fast, nuclear divisions which are not followed by cytoplasmic divisions. Therefore, the embryo, in early stages, is a syncytium with 128 nuclei. During the first 8 divisions, the nuclei are located as an ellipsoid centrally in the yolk. During the 9th division (4th embryonic stage), most of them migrate towards the surface of the egg to form the somatic buds of the syncytial blastoderm. Up to this point, the divisions are synchronized. Some of the nuclei, called vitellophages, remain in the yolky egg center. In the 10th division that follows, the nuclei are starting to be surrounded by membranes which are the extension of membrane furrows between the syncytial blastoderm nuclei. Hence, the first embryonic cells are starting to be formed and give rise to a single layered cellular blastoderm. Cellularization marks the beginning of asynchronous cell divisions. The blastoderm is not completely formed until 3.5h after

fertilization, in the end of the 13th nuclear divisions. At this point, approximately 6000 cells are lining in the surface of the egg with yolk in the interior of the zygote (12). Before the cellular blastoderm is formed, when most of the nuclei migrate to the surface of the egg, 5-10 nuclei move to the posterior plasm, and by the 10th division become segregated pole cells (**fig. 3**) (13,14). These pole cells are the ancestor germ cells. In contrast to the blastoderm cells, they divide asynchronously once or twice and they enter cell-cycle quiescence at G2 until they reach the somatic gonads, which is after gastrulation has begun (15).

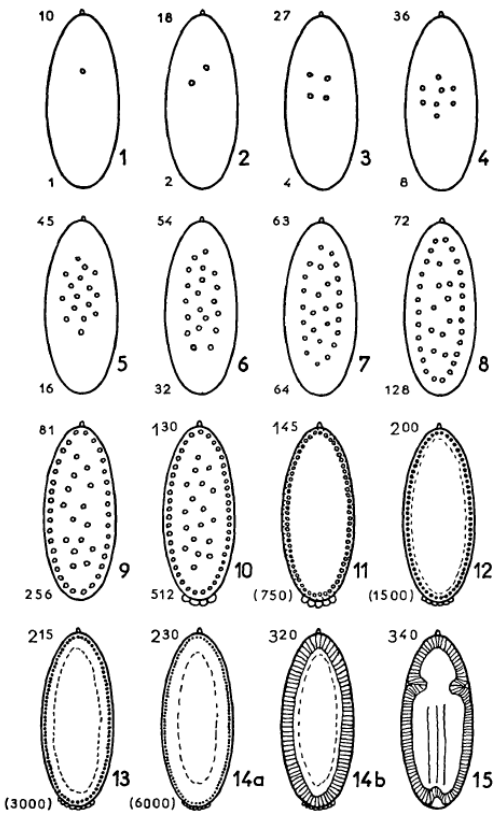


Figure 3: normal development of D. melanogaster between fertilization and onset of gastrulation. Numbers on top left of each egg refer to minutes after deposition, large numbers on the right of each egg refer to stages of division and small numbers to the left on the bottom to the number of nuclei. St. 1-9: nuclear multiplications, st. 10: pole cells, st. 14b: cellular blastoderm (from Zalokar and Erk 1976).

At 3.5h after fertilization (embryonic st. 7), gastrulation begins. Through gastrulation, the three germ layers of the animal are specified; the mesoderm, the endoderm and the ectoderm (**fig. 4**). Shape changes, cell fate decisions and simultaneous cell movements in various regions of the blastoderm take place. The mesoderm layer is formed through the inward migration of 1/6 of the cells of the ventral mesodermal side. This migration happens along with the ventral midline and the cells form a hollow tube immediately beneath the ventral ectoderm. Simultaneously, at the posterior dorsal tip the formation of endoderm begins through the invagination of the posterior midgut and ventrally the endoderm invaginates forming anterior midgut. An hour later, the stomodeal invagination occurs. The neuroblasts, which are the precursors of the Central Nervous System (CNS), begin separating from the ectoderm approximately 30min after the beginning of the gastrulation and continue to be formed for the next 2h. At the end of the gastrulation, the pole cells, that have also been included in the invagination of the endoderm, migrate through the midgut wall and reach the developing gonad (1,16).

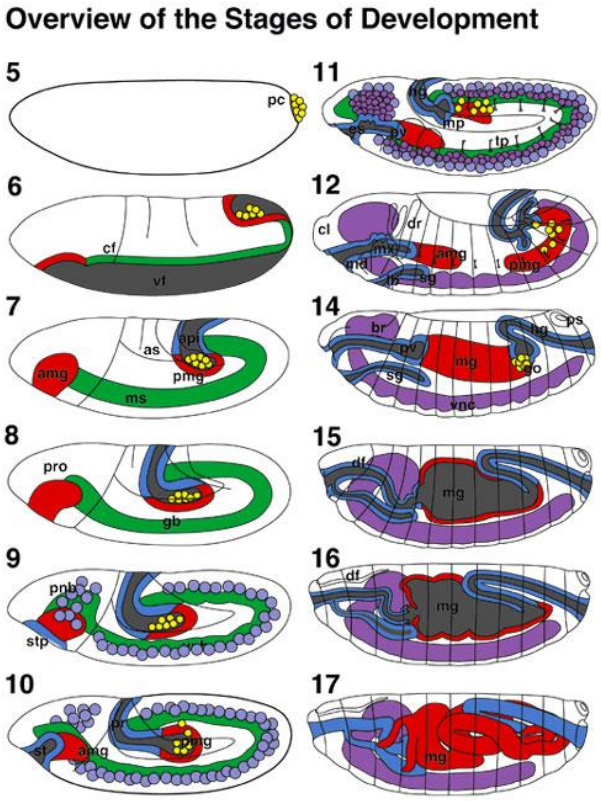


Figure 4: Stages of embryonic development of D. Melanogaster. With red is depicted the ectoderm and in later stages the midgut. With green is depicted the mesoderm. With purple is depicted the ectoderm and in later stages the CNS. With blue is depicted the anterior and the posterior part of the gut. With yellow is depicted the pole cells. In all images embryos are in lateral view and anterior is to the left (*Atlas of Drosophila Development, Volker Hartenstein*).

1.5: Neurogenesis in *D. melanogaster*

The nervous system of *D. melanogaster* comprises from the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS consists of the brain and the ventral nerve cord (VNC), while the PNS has a variety of sensory neurons that cover the whole length of the body (17,18).

Until gastrulation, the cells of the ventral ectoderm layer are all equivalent with each other. From the next embryonic stage though (st. 8 and after), the cells start differentiating either to neural progenitors, neuroblasts (NBs) that will give rise to the central nervous system, or to epidermal progenitors, epidermoblasts which will build up part of the epidermal sheath. Once the fate of each cell is determined, NBs invaginate towards inner layers of the embryo and will give rise to the CNS. On the other hand, PNS will be formed from ectodermal cells which acquire epidermal fate and are later on converted to PNS cell precursors (note that neuroectodermal cells commit to their fates by regulatory signals mediated by cellular interactions – discussed further below). Before the generation of these progenitors, patterning genes act in the anterior-posterior (AP) and in the dorsal-ventral (DV) axis, subdividing the early embryonic ectoderm. In each axis further subdivisions of expression pattern are present; the patterning genes are expressed in stripes. The result is a checkerboard pattern of expression that guides the location in which neural precursor cells are formed and also the different identities of the precursors (17). The ventral morphogen Dorsal is highly expressed ventrally and leads to the activation of the gene *short gastrulation* (*sog*). The presence of *sog* antagonizes the dorsal morphogen *Decapentaplegic* (*Dpp*) and defines molecularly the ventral neuroectoderm (19,20); the stage is now set for the expression of the remaining prepatterning and proneural genes.

Neurogenesis both in the CNS and the PNS is orchestrated by the expression of two groups of genes; the proneural and the neurogenic genes. The role of these genes is to properly distinguish the neural and epidermal cells during neurogenesis. The proneural genes encode Transcription Factors (TFs) with a basic helix-loop-helix domain (bHLH). This group of genes includes three genes of the *acheate-scute* complex (*AS-C*), which are next to each other in the genome; *achaete* (*ac*), *scute* (*sc*), *lethal of scute* (*l'sc*) and the gene *Daughterless* (*da*) as well (12). The cells where the fourth AS-C gene *asense* (*ase*) is expressed adopt a neural fate and they become either NBs if they are cells of the CNS, or sensory organ precursor cells of the PNS. Genes that are expressed in the neuroectoderm include (among others) members of the Notch signaling pathway, like the *Notch-receptor*, the Delta ligand (*Dl*) and the genes of the Enhancer of split complex (*E(spl)*) (12,21).

1.5.1: Central Nervous System (CNS) of *D. melanogaster*

As mentioned above, the NBs are ancestors of the central nervous system. The NBs of the VNC come from the ventral neuroectoderm whereas the ones of the brain come from the droso-anterior pro-cephalic neuroectoderm (18).

The formation of the CNS begins at embryonic st. 8 with the delamination of 30 NBs per hemisegment from the neuroectoderm (22,23). This segregation lasts 3h and it happens in five successive waves, S1-S5, giving rise to different kind of NBs, named S1-S5 (**fig. 5**). Three columns of NBs are produced and they derive each from one of the three columns of the ventral ectoderm; the medial columnar domain of the Ventral Neuroectoderm (mVN), the lateral columnar domain of the Ventral Neuroectoderm (lVN) and the intermediate Ventral Neuroectoderm (iVN). At first, per hemineuromere, four NBs delaminate from the mVN, four from the lVM and two additional from the iVN. These are the S1 NBs and they form a regular, orthogonal grid of three columns (medial, intermediate and lateral NBs), and four rows (A, B, C, D) in each hemisegment. Next, five S2 NBs segregate from the iVN and they are followed by six S3 NBs from the mVN and the lVN. The S4 and S5 are the waves that give rise to 12 more NBs which come mainly from the iVN (fig. 5). Besides those NBs, a double row of cells between the medial columns of each side produces neural progenitors as well, most of which end up being neurons and some of them glia cells. This double row of cells is called mesectoderm, or midline (18,22).

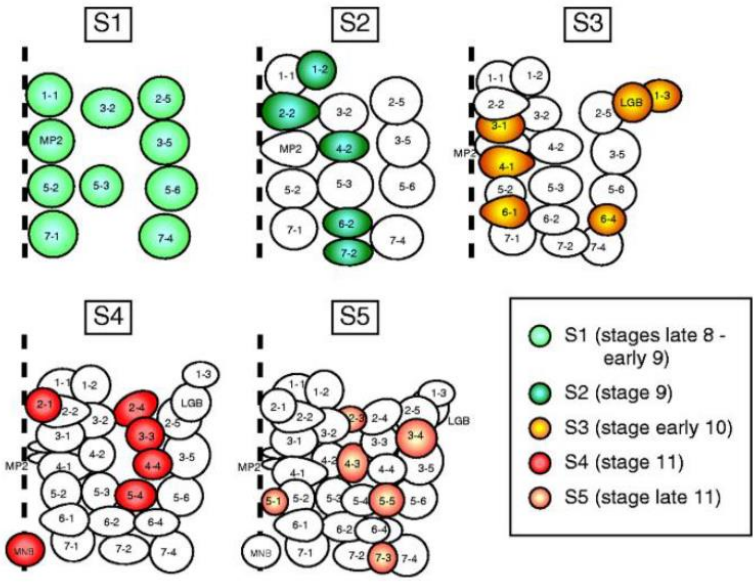


Figure 5: Spatial arrangment and temporal sequence (S1-S5) of NB creation. The higlated NB are the newly added ones during the according wave in each stage. The dotted line represents the midline and each picture the pattern of one hemisegment. Anterior is upwards (picture form Berger et al., 2001).

NBs seem to derive from 10 groups of 6-8 cells each per hemisegment that are named "poneural clusters". Prior to delamination, the proneural clusters express combination of the proneural genes *ac, sc, l'sc*. Each cluster gives rise to a single NB. Since neuroblasts and epidermoblasts arise from clusters, the selection of the cell that will become NB is mediated by a specific mechanism (28) which involves intercellular interactions that will be analyzed below. In the S1 wave, the cells that will become NBs, stop at the G2 phase of the 14th cell cycle; at this point, all of the nuclei of the cells in the cluster are located basally. When the surrounding cells start preparing for the 15th mitosis, their nucleus moves apically but the nuclei of the future NBs remains basally, postponing division. Instead, the S1 NBs start losing connection to the apical surface; they segregate and then start their mitosis (**fig 7a**). The spatial separation of cells in these clusters is followed by rapid changes in the expression pattern of proneurals. In contrast with the cells surrounding it, the cell that will become NB has elevated levels of the proneural genes *ac, sc* and *l'sc* before the S1 wave of delamination. Later on, these genes are not any longer expressed in the NBs and the expression of another gene group, the pan-neurals, takes place. This gene group includes the bHLH genes *ase*, *deadpan* (*dpn*), the zing finger gene *snail* (*sna*) and the homeobox gene *prospero* (*pros*) (18,24).

Regarding the way through which a single cell for a proneural cluster is selected to become NB, a mechanism based on cell to cell interaction, called lateral inhibition, takes place (**fig. 6**). In this mechanism, the Notch signaling pathway plays pivotal role. One cell of the cluster has higher expression levels of proneurals and that leads to increased production of the ligand Delta (*Dl*) and its activator *neur*. Dl binds to the Notch receptor of the surrounding cells; note that Notch receptor is a transmembrane protein present in all the cells of the neuroectoderm. This binding leads to the intramembrane cleavage of Notch and the nuclear translocation of the intracellular fragment (Notch Intracellular Domain – NICD),

which initiates a cascade of intracellular interactions that lead to the transcriptional activation of the genes of the *E(spl)* complex. The products of these genes are 7 bHLH transcription factors that are thought to act as repressors of proneural genes. In consequence, only one cell of the proneural cluster, the one that expresses high Dl, retains its NB potential and proceeds to actually become one (17).

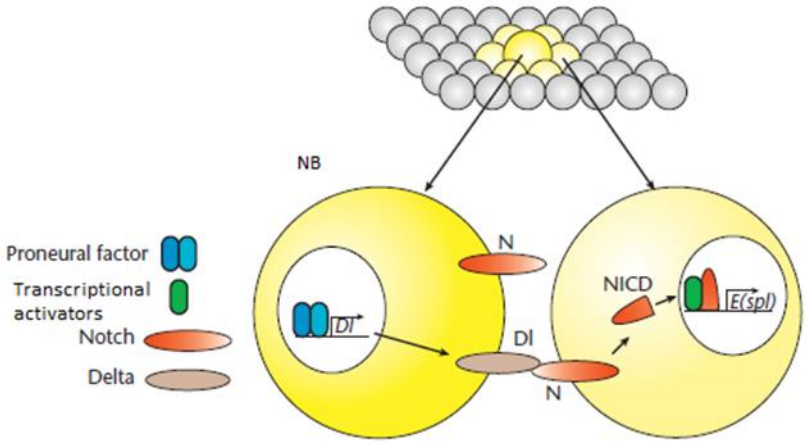


Figure 6: Notch lateral inhibition determines the NB from a proneural cluster. The cell with overexpression of proneurals expresses Dl which binds in the Notch receptor of all the neighboring cells. Notch receptor is proteolysed and the Notch Intracellular Domain (NICD) enters the nucleus where it activates the E(spl). Thus proneural genes are repressed in the neighbor cells and only one from the cluster ends up as a NB whereas the others remain ectodermal and eventually become epidermoblasts. (Image adapted from Jarman, 2013)

Once their cell fate is determined, the NBs segregate as described above and a series of asymmetrical divisions begin. Each of these cell divisions leads to a new NB and a new smaller cell, called the Ganglion Mother Cell (GMC) that acts as an intermediate precursor cell. GMCs in turn, divide once more asymmetrically and give rise either to neurons and/or glia cells (**fig 7b**). However, the midline precursor cells divide just once and generate two neurons.

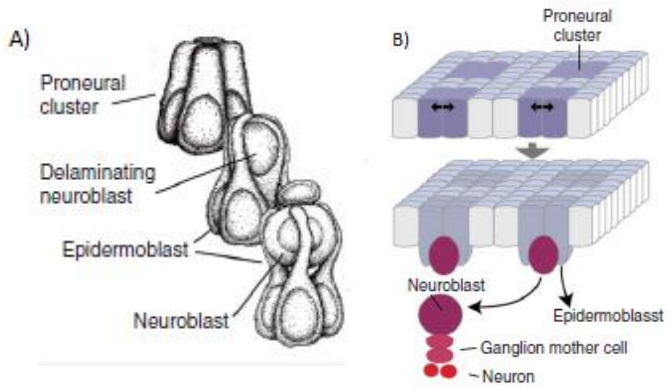


Figure 7: A) Demonstration of a proneural complex before, during and after the delamination of the NB. From each proneural cluster only one cell becomes NB whereas the others become epidermoblasts. B) Depiction of the cellular fate of each cell from the proneural cluster.

During the mitosis of an asymmetrical cell division, the determinants of a cellular fate are placed asymmetrically in the dividing cell and when cytokinesis takes place they are inherited only to one

daughter cell. This is the reason why the two daughter cells end up having different fates. In particular, there have been observed three determinants in NBs; the transcription factor *Prospero* (*pros*), which activates genes that promote differentiation and suppresses genes that promote proliferation, the *Numb* protein, which suppresses signal transduction by the N receptor, and the *Brain tumor* (*Brat*) protein, which acts as a translational regulator (18). These three cellular fate determinants are located on the basal surface of the NBs and their location there requires the presence of two extra proteins, the Miranda protein and the Partner of Numb (Pon). The Miranda protein binds with Pros and Brat, whereas Pon binds with Numb. For the accurate location both of the cellular fate determinants and their adaptors on the basal surface of the NBs, another group of proteins is required. These proteins are the ones that comprise the Bazooka (Baz) / Par3-Par6-atypical Protein Kinase C (aPKC) and the tumor suppressant proteins Lethal giant larvae (Lgl), Discs large (Dlg) and Scribble (Scrib) (18).

1.5.2: Peripheral Nervous System (PNS) of *D. melanogaster*.

The role of the Peripheral Nervous System (PNS) in insects is receiving information from the environment and transducing them to the CNS so that the animal reacts appropriately to ensure its survival and the most suitable behavior. Due to the variety of inputs, different sensory organs are required. These organs are distributed equally throughout the surface of larval and adult epidermis and they transduce the information either along the length of epidermis or towards the CNS (25).

In analogy with the cells of CNS that are derived from the NBs, all the cells of PNS originate from the ectoderm and come from a single cell type, the Sensory Organ Precursor cells (SOPs) which later divide and give rise to differentiated cells (18,25). SOPs appear in epidermal ectoderm later than NBs, at embryonic st. 10, after the delamination of NB has started in the CNS (21). In contrast with NBs, SOPs do not delaminate completely and remain connected with the epidermal ectoderm until the end of division of one of their daughter cells. Then, the sensory neurons and the glia cells migrate just underneath the surface of the epidermis (18). SOPs divide to produce two secondary precursor cells which will further divide to two cells with different identities and so on, until the formation of all cell types of this lineage are created (17).

SOPs are formed during two stages; the first happens during embryogenesis after gastrulation and produces larval sense organs, and the second takes place much later in the ectodermal tissues (imaginal discs) and will create the adult epidermal structure and produce the adult sense organs during

metamorphosis. The selection of the cell that will become SOP happens with a mechanism similar to the selection of the cells that end up being NBs; they are selected through the high level of expression of proneural genes and the mechanism of Notch lateral inhibition (described in the previous chapter). In the cells that will become SOPs, along with the overexpression of Dl in their membranes, *neur* is also expressed. That promotes higher Notch signaling in non-SOP cells. After NCID is proteolysed, it interacts with the Suppressor of Hairless Su(H) protein and promotes expression of E(spl) in the non-SOPs. E(spl) along with Groucho (Gro) – a transcriptional repressor – suppress the expression of proneural gene *ac*. In contrast, in the cell that will become SOP, Gro and Su(H) repress the expression of *E(spl)* and the expression of proneural genes is maintained. The high level expression of proneural genes is mediated by Senseless and by a positive feedback loop of the proneural factors themselves (25) (**fig. 8**).

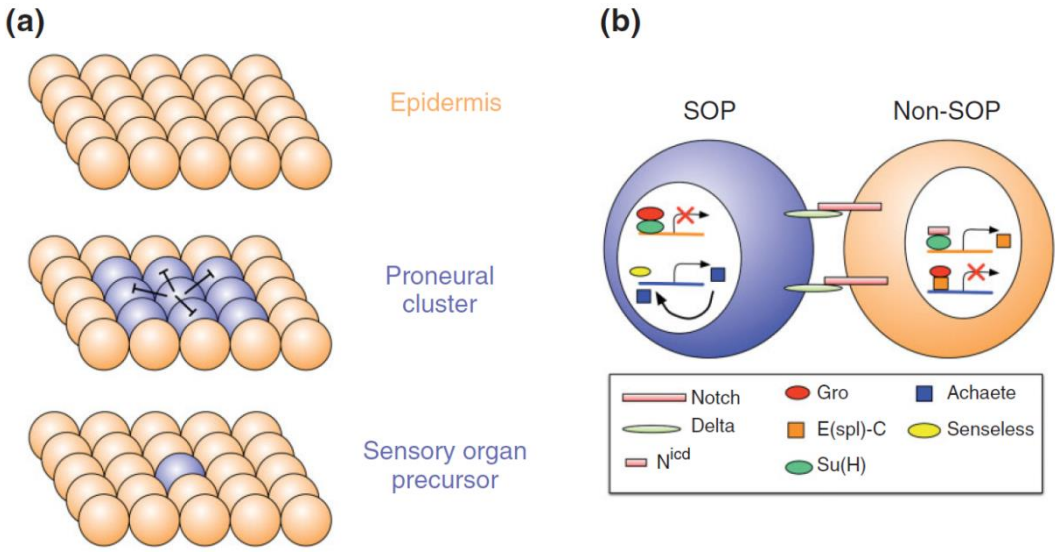


Figure 8: formation of SOPs. A) Only one cell form a proneural cluster will become SOP. B) SOPs are molecularly selected from their neighbor cells via the mechanism of Notch lateral inhibition (image from Singhania et al, 2014).

The formation of external sensory organs begins with the selection of one SOP, which undergoes four successive divisions. From the first division of a SOP two daughter cells are produced, which are named plla and pllb. From their division five cells emerge; two outer cells that derive from plla and three inner cells that derive from pllb. Sensory organs are organized into dorsal (d), lateral (l), ventral' (v') and ventral (v) clusters. These clusters are comprised of neurons that are classified as type-I and type-II. Type-I neurons have single ciliated dendrites and are those that innervate external sensory (es) organs like the mechanosensory bristles of the body and the olfactory organs of the antenna and those of the

internal chordotonal (ch) organs for proprioception and auditory reception. The type-II are multidendritic neurons and include touch- and pain-sensitive neurons which are the majority neurons in larva. More specifically, type-II neurons include the bipolar dentrite neurons (bd), the tracheal dendrite neurons (td) and the dendritic arborization (da) neurons (**fig. 9**).

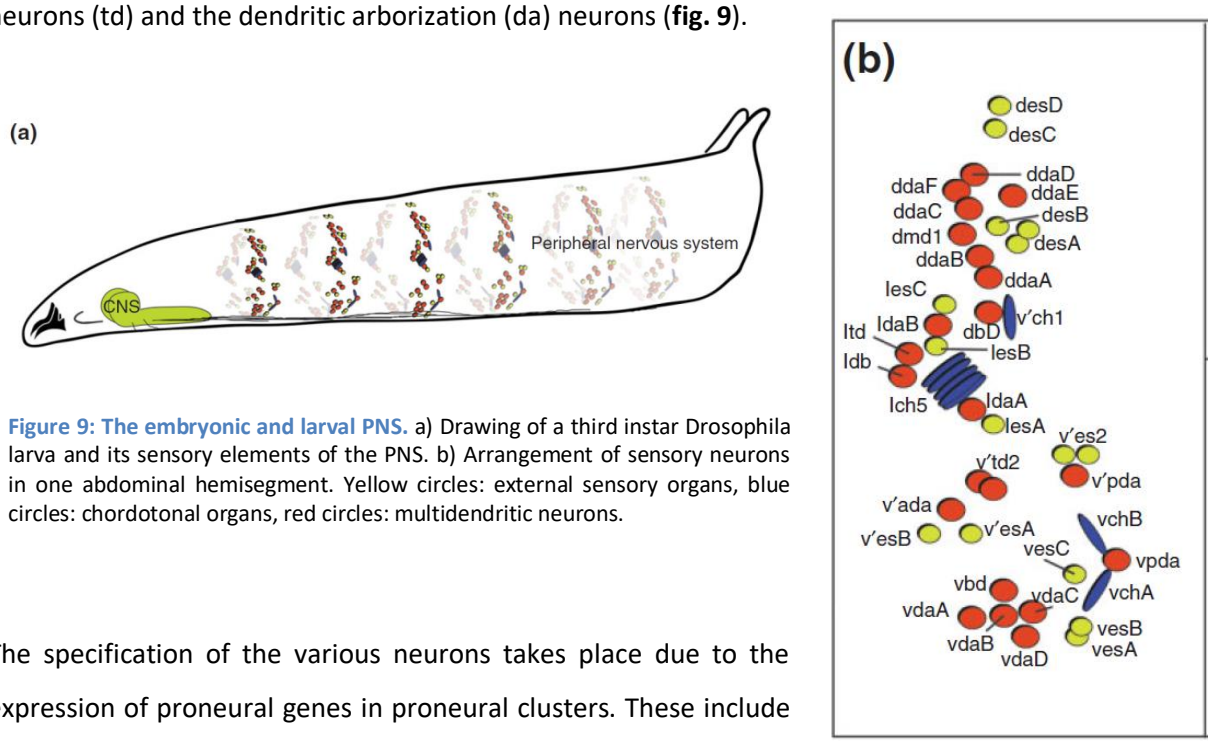


Figure 9: The embryonic and larval PNS. a) Drawing of a third instar Drosophila larva and its sensory elements of the PNS. b) Arrangement of sensory neurons in one abdominal hemisegment. Yellow circles: external sensory organs, blue circles: chordotonal organs, red circles: multidendritic neurons.

The specification of the various neurons takes place due to the expression of proneural genes in proneural clusters. These include members of the AS-C, atonal (ato) and *absent md neurons and olfactory sensilla (amos)*. The presence of AS-C is necessary in proneural complexes from which the es organs are formed and the subset of md neurons deriving from the esSOPs. That is the reason why in absence of the members of the AS-C the clusters v' and d, which mainly consist es organs, are not formed. *ato* controls the formation of ch organs and a subset of the md neurons whereas *amos* controls the rest of the md neurons (25).

1.5.3: Loss of function of Achaete Scute Complex (AS-C).

Hemizygous embryos for mutation that result in Loss of Function (LoF) of the proneural genes exhibit vividly underdeveloped CNS, with loss of segments of the ventral cord and loss of organs in the PNS, since the eso peripheral SOPs are completely lost in absence of AS-C. Unlike the complete loss of external SOPs, central NB loss is partial. Embryos that are deprived of this complex seem to have 20-25% fewer NBs than the number of NBs a wild type (wt) embryo would have (**fig. 10a**). Even the NBs that do manage to delaminate from the neuroectoderm, are smaller in size and produce fewer descendants.

Finally, the cellular apoptosis in the neural primordium exhibits an increased rate during later stages, compared to what normally happens (18,26,27). The phenotype of the mutants evolves progressively. In addition, previous work in the lab showed that in AS-C null embryos NBs have delayed divisions and expression of the majority of pan-neuroblast genes (V. Theodorou et al., submitted). However, since in the absence of AS-C many cells in the CNS and PNS develop normally but later on enter apoptosis, it is logical to assume that other genes partially substitute the role of this complex, at least during cell birth at the early developmental stages. The proteins that are produced by the AS-C and by the gene *daughterless* (*da*) are structurally and functionally similar. Also, the mutant embryos with loss of function for *da* (da^{KX13}) display similar phenotype to those with loss of function of AS-C (deletion of all four genes of the complex $Df(1)sc^{B57}$) (**fig 10bB**). Therefore, the product of *da* could be able to complement the function of AS-C. The double mutant $Df(1)sc^{B57};da^{KX136}$ has indeed more severe phenotype (**fig. 10bC**) (26). In order to generate embryos null for the AS-C genes (*ac, sc, l'sc, ase*) we use the deficiency $Df(1)sc^{B57}$, or for purpose of simplicity named B57 whereas to create embryos that lack the genes *ac, sc* and *l'sc* but *ase* is present, we used the deficiency $Df(1)sc^{19}$, or simply named sc19 (for analytical genetic map of depletions, see "results" section – fig. 55).

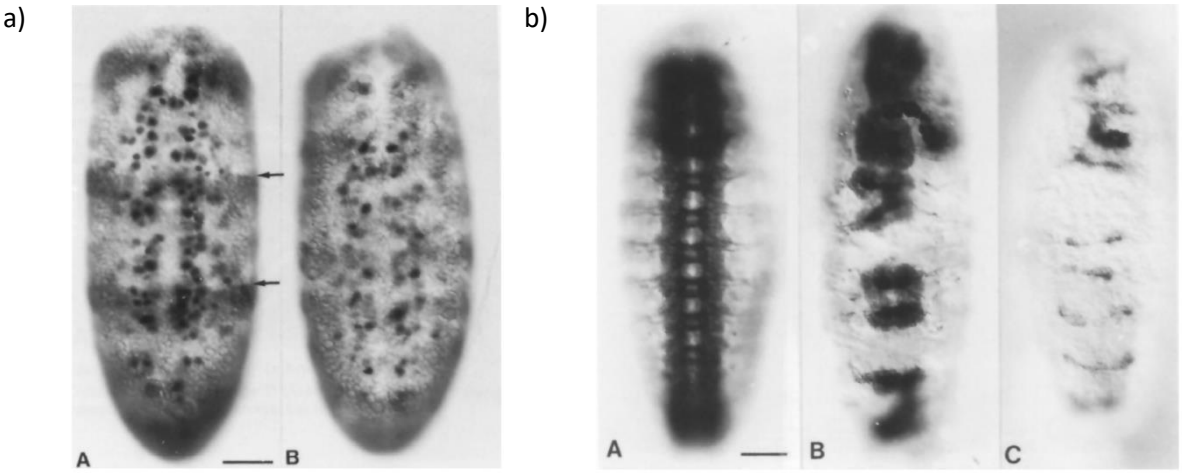


Figure 10: Morphology of st.10 embryos $Df(1)sc^{B57}$. a) WT embryo on the left, mutant with LoF for the AS-C on the right. Both of the embryos were stained with anti-β-gal antibody for phenotype selection and anti-hb antibody for selective staining of the NBs. It is visible that in the mutated embryo fewer NBs are present. The ftz stripes of I~-galactosidase (arrows) designate the wild-type genotype because the embryo carries a P element-mediated insertion of a ftz promoter-lacZ construct in the elav locus. b) ventral side of ventral cord of st. 16 embryos. A) wt embryo, B) $Df(1)sc^{B57}$ embryo, C) $Df(1)sc^{B57};da^{KX136}$ embryo. All of the embryos were stained with anti-HRP antibody, an antibody that binds specifically in neuronal cells. It is visible that this mutation causes depletion of fragments of the ventral cored and that this phenotype is more severe in the double mutant.

1.6: Expression of AS-C genes in different developmental stages and tissues

As described in previous chapters, the proneural genes of AS-C are expressed in proneural clusters, small groups of cells in the embryonic epithelium. They are expressed before the generation of a neural precursor (with the exception of *ase*, which is expressed later on and is present in the daughter cells of the precursors but is absent in neuroectoderm). However, other than their embryonic expression which determines the cell in the cluster that will become NB and will give rise to the cells of the central nervous system (CNS), genes of the AS-C are expressed in other developmental stages and tissues as well.

In the larval brain, NBs are formed de novo in the optic lobe; neuroepithelial cells divide at first symmetrically and expand their progenitor pool but later on asymmetric divisions take place and NBs that will give rise to the neurons of medulla are created. The transition from epithelial cells to NBs is marked by the expression of *l'sc* in a zone that is called the transition zone. Different signaling pathways such as epidermal growth factor receptor (EFGR) and Notch signaling orchestrate this transition (28). Also, cells in the optic lobe express *ase* and *sc* but *ac* has not been clearly detected (28,29).

Regarding the imaginal discs, the expression of genes that belong to the AS-C determines the areas where the sensory mother cells (SMCs) arise, in a manner similar to the one that leads to the creation of NBs. It has been shown that in mutants with loss of *ac* and *sc*, the sensory organ precursors (SOPs) were lost and that with generalized expression of *ac* and/or *sc* SOPs were formed in ectopic positions as well. These findings suggest that the expression of these genes determines the SOPs; *ac* and *sc* act as transcriptional regulators that activate genes responsible for differentiation, in combination with other HLH proteins like da, by forming heterodimers. More specifically, in the wing disc, in the middle of the future notum, one cell has high levels of *sc* expression and that cell later gives rise to PSA macrochaetae precursor. Later on, other clusters such as the ones for ANP, PNP macrochaetae and also the wing margin PPA and ASA regions, have elevated expression of *sc* as well (30). However, around puparium formation *sc* and *ac* expression disappears from many clusters but in all the SOPs that are formed during the pupa stages the expression of *ase* was detected. Again, *ase* was present in the precursors even after division, in contrast with the other members of the AS-C (31).

Finally, another tissue where members of the AS-C are expressed, is the adult gut. The transcription factor prospero (pros) is specially expressed in enteroendocrine (EE) cells and determines their cell fate. The expression of *pros* is promoted by AS-C; overexpression of *sc* or *ase* leads to the formation of more

EE from the intestine stem cells (ISCs), whereas conditional deletion of *sc* from pupal stages leads to the depletion of EE in the midgut. *sc* has been found to be dynamically expressed in all ISCs and it has been shown that it acts both as a cell-fate inducer and as a mitogenic factor (32).

In the following chapters we will shortly discuss the anatomy and the development of these tissues.

1.7: Larval central nervous system in *D. melanogaster*.

The larval central nervous system comprises of two brain lobes attached with each other and with a ventral nerve cord (vnc). Each lobe is derived from embryonic proecephalic neuroectoderm and can be split in two areas; the central brain and the optic lobe (**fig. 11**) (33). The VNC is created form embryonic thoracic and abdominal NBs and has different segments; three gnathal, three thoracic, seven abdominal and a non-segmented terminus (**fig. 12**) (34).

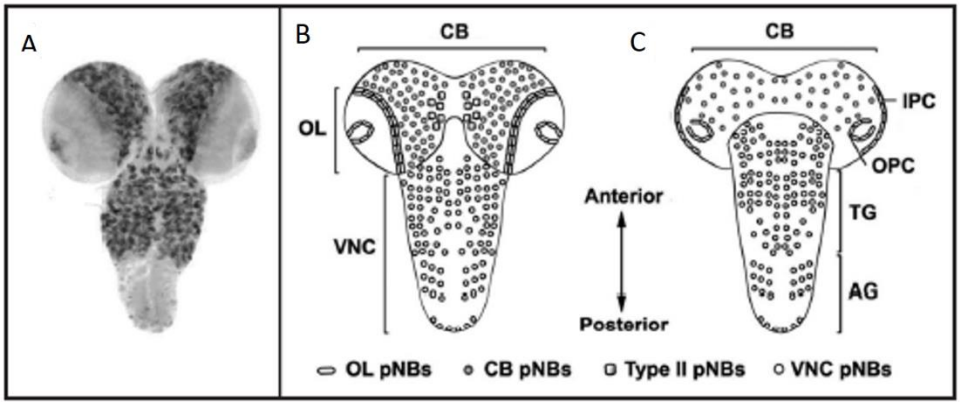


Figure 11: Morphology of larval CNS. A) 3rd instar larval CNS, B) schematic dorsal and C) ventral view. The CNS includes the (OL) optic lobes that consist of inner (IPC) and outer (OPC) proliferation centers, the central brain (CB) and the ventral nerve cord (VNC) that can be subdivided into thoracic (TG) and abdominal (AB) ganglia. pNBs: post-embryonic neuroblasts. D). Anterior is up and posterior is down. *Image from Dongwook 2009*

The larval CNS has a variety of neuroblasts most of which are born in the embryo, except for those in the optic lobes which are de novo born from the optic lobe epithelium during the larval stage. Each optic lobe has more than 800 NBs, each hemisphere of the central brain around 105 and the VNC has 30 per hemisegment, except from the gnathal and telson which have fewer (35). In general, the embryonic NBs after each division become smaller in size and they reach a point where they become quiescent. At the late 1st instar larva stage, they re-enter the cell cycle after receiving specific nutritional signals (insulin) (36), they re-grow to their parental NB size and start a new cycle of divisions and differentiation, now as pNBs. Most of the pNBs proliferate slower than the embryonic NBs and as a result they have time for

growth. However, there are two exceptions of NBs that do not enter cycle arrest; four NBs in each lobe that generate the mushroom body and one ventro-lateral NB. Also, note that the optic lobes are created in the larva with a different mechanism than the rest cells of CNS, and do not exist in embryos. Instead of delamination from the embryonic neuroectoderm, the optic lobe forms in larva with "invagination of an epithelial sheet and is expanded with symmetric divisions" (37).

There are four different types of neuroblasts in larva, each of which is formed and arrested in different stages, has a different expression pattern of temporal transcription factors, morphology, location, descendant cells and number of cells. Namely, there are type I, type II pNBs and the mushroom body NBs (**fig. 12**) (38). Type I NBs are the most abundant and each of them divides more than 50 times giving self-renewal cells and GMCs, the latter of which divide again to produce either glia or neurons. In terms of marker's expression, type I NBs are *Dpn* and *Ase* positive but they are *PntP1* negative (38). Other expressed TFs important for their detection are Klu, E(spl)mγ and wor. Type II NBs are present only in the central brain and they are much fewer in numbers (just 16) but produce the 25% of the total cells of the adult central brain due to an extra step in their lineage differentiation; they produce intermediate neural precursors (INPs) that give birth to 4-6 GMCs, which, subsequently, divide and differentiate either to neurons or glia (38,39). The expression pattern of type II NBs is different in each stage (**fig.12**). At the beginning, they express *Dpn*, PntP1 but not *Ase*, therefore they are Dpn^{+}, Ase^{-}, $PntP1^{+}$ (38), but the mature INPs start to express *Ase* as well, making them more similar to Type I NBs. It should be noted that ectopic expression of *Ase* in Type II NBs is sufficient to transform them to Type I (40), and in analogy, ectopic expression of *PntP1* in Type I NBs transforms them to Type II (41). Both Type I and II express also *worniu* (*wor*), *E(spl)mγ* and *klu*. Another pNBs category shortly mentioned above is the one giving rise to the mushroom bodies. It consists of only four NBs that divide in a type I mode (but give rise to over 2500 progeny) which have not entered quiescence from embryo and probably that is the reason for the large size of their neuronal lineages. They are located in the dorsal central brain and in the adult fly they produce the Kenyon cells which participate in olfactory learning and memory storage (42). The last type of NBs observed in larva, are the Type 0 pNBs which divide asymmetrically as well but they give rise only to neurons without having any intermediate precursor (42).

All NBs eventually stop dividing, once they have created all their progeny. The way and the time that this happens vary, again, from type to type. The abdominal Type I NBs are the first to stop, at larva stage, and they do so through entering apoptosis. Type II NBs follow but through cell cycle arrest, and the last

to stop proliferating are the mushroom bodies (MB) NBs, which stop in pupa by entering apoptosis as well. Finally, note that all NBs are *Dpn*$^{+}$.

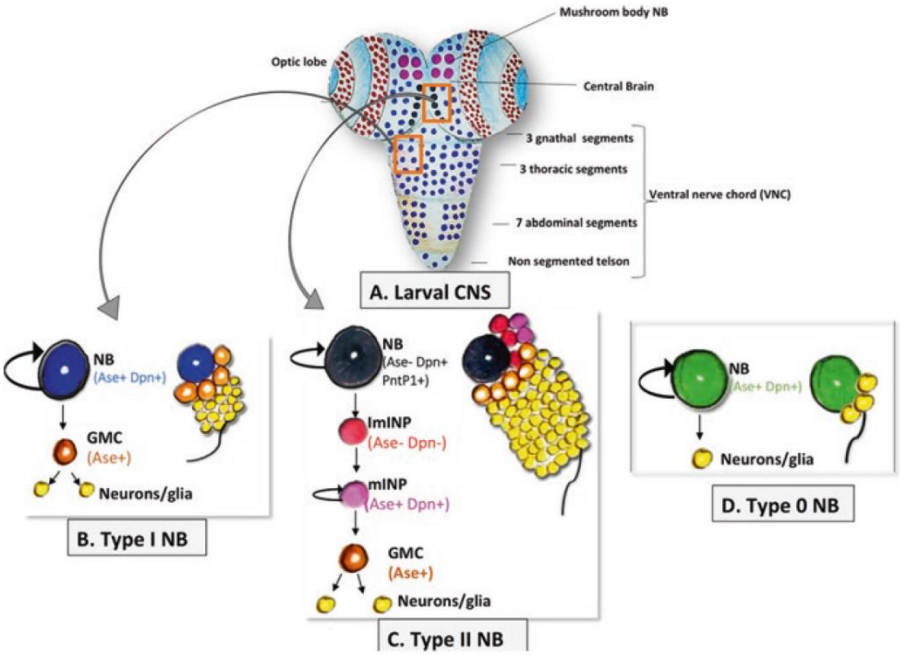


Figure 12: Different type of NBs in the larval CNS. A) larval CNS and location of each type of NBs. Asymetric division of b) type I NB that gives rise to GMC and after to neurons/glia, c) type II NBs that produce the intermediate neural precursor (IMP) which after maturation (mIMP) divides again to produce GMCs which, in turn, will create neurons/glia and d) asymmetric division as well of Type 0 which rise to neurons/glia without any intermediate type of cell.

Image from Akanksha 2019

1.8: Wing imaginal disc of *D. melanogaster*.

Imaginal discs are sacs of epithelial cells in the larvae of insects that after the pupation create the adult external structures. The name derives from the Latin "imago" and it is the term by which the adult form of insects is described. They are comprised of two layers of cells, the peripodial – a layer of wide and flat cells, and the columnar epithelium, a layer of tall and thin cells (43). The latter is mainly responsible for the adult structure formation, whereas the first is necessary for the merge of discs. Imaginal discs are a great model for studying development since the transformation from this 2 layer structure to whole organs and adult structures with numerous cell types and functions requires enormous changes and includes most aspects of developmental biology.

In *D. melanogaster*, the precursors of the imaginal discs are formed in each thoracic segment during germ band extension in embryonic development. The larva has 8 bilateral pair of discs from which the adult external structure are derived; the head and thorax derive from labial, clypeolaberal and eye-antenna discs whereas the limbs derive from humeral, wing, haltere and 1st/2nd/3rd leg discs. Finally, the genitalia are formed through the medial sac and therefore the larva has in total 19 imaginal discs (**fig. 13**) (44,45). In contrast with most larval cells, the ones of the imaginal discs have high proliferation rates

and the wing disc for example, which is the largest, consists of ~50.000 cells (46). Simultaneously with the cell proliferation, the determination of the adult cell types takes place.

During metamorphosis, the discs evert from larva through the larva wall into the empty space of the pupal case. They unfold and elongate due to cell shape changes; the cells are initially compressed but when the tissue elongates they acquire their rounder state (47). At the same time when these movements take place, cells continue to differentiate towards their final identity that they have in the adult fly. In pupa, fusion of the bilateral pair of discs begins; the pairs come together and the pairs of wings and antenna discs fuse with the ones of the legs and form an epithelium of adult fly shape.

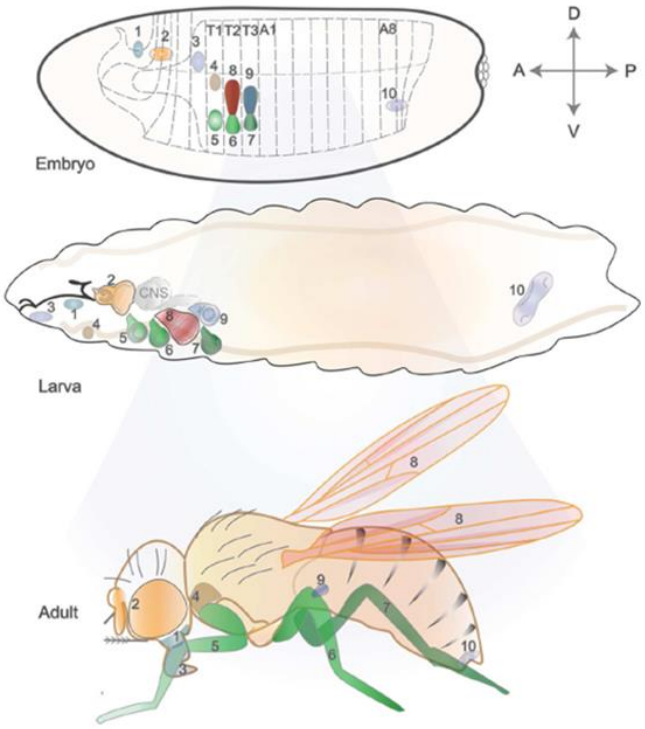


Figure 13: schematic representation of the embryonic origin of the larval imaginal discs and their adult products. The location of the precursors of the imaginal discs is represented in the embryonic cellular blastoderm (top), with corresponding numbers in larval (middle) and adult (bottom) stages. T1-T3: Thoracic segments, A1-A8 abdominal ones. The adult structures of the head, thorax and appendages come from 9 pairs of bilateral discs and the genitals from a middle sac disc. 1: clypeolaberal, 2: eye-antenna, 3:labial, 4: humeral, 5:1st leg, 6:2nd leg, 7:3rd leg, 8:wing, 9:haltere, 10:genital.

Image from: Beira, 2010.

The adult structures appear in precise positions and the information for their formation comes from the discs which are subdivided in specific domains – compartments. Each cell belongs to a specific compartment and its descendants cannot enter another domain. These boundaries are determined by the expression of many transcription factors and by morphogens, which emit long and short diffusible signals, forming organizing centers. Morphogens control transcriptional activators and repressors and their interactions determine the formation of the axes in the imaginal discs (48).

Regarding the wing disc specifically, as mentioned above it is the largest imaginal disc present in larva. It originates from the same set of imaginal precursors as the leg, which are located between the A/P stripes of wingless (wg) protein expression and the horizontal bands of cells expressing *Decapentaplegic (Dpp)* (49,50). Wing disc though, in contrast with the leg disc, is regulated by the gene *vestigial* and

previous experiments have shown that ectopic expression of *vestigial* in discs converts them to wing tissue (51). Its anterior-posterior boundaries are formed during early embryogenesis. Posterior compartment is characterized by the expression of *engrailed*, which is a transcription factor and in animals with loss of function of *engrailed* all disc cells become anteriorized (52,53). In short, *engrailed* activates the expression of *hedgehog (hh)* which is expressed in the posterior compartment (54) and activates, in turn, the expression of *Dpp* in anterior cells. In the posterior cells, the expression of *en* prevents the activation of *Dpp*. Therefore, *Dpp* establishes the anterior-posterior axis (**fig. 14**) (55,56).

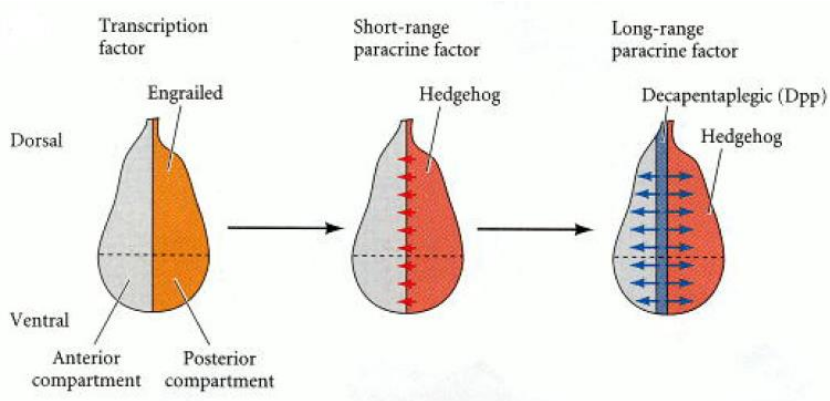


Figure 14: Configuration of the A-P axis in wing disc. Mechanism described in the text.

Regarding the formation of the Dorsal-Ventral axis (DV), it takes place later than the formation of the AP axis, during the 2nd instar larva. The boundary here is the future wing margin and it separates the upper (dorsal) from the lower (ventral) surface of the wing (53). The gene that regulates this boundary is *apterous,* another transcriptional factor, and in experiments with loss of function all cells became ventral. *apterous* is expressed dorsally and it activates the expression of *serrate (ser)* which is a ligand of the Notch receptor. Notch receptor is expressed ubiquitously and it binds with dorsally expressed Ser, determining in this way the wing margin (43,51).

The anatomy of the wing disc and the structure that it gives rises to in the adult wing are presented schematically in **fig. 15**.

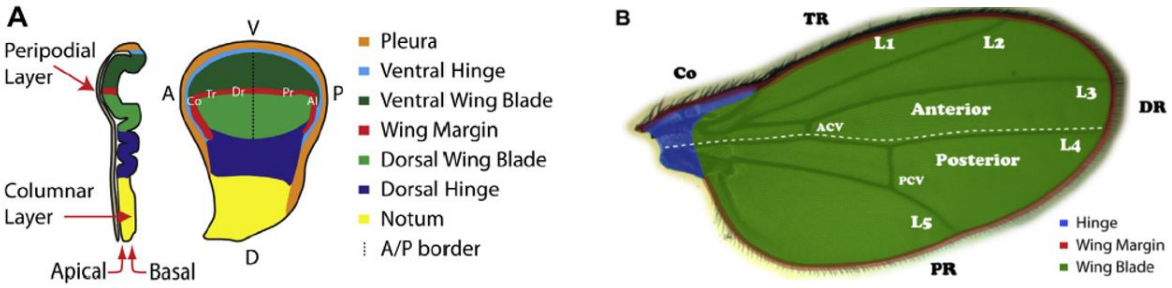


Figure 15: anatomy of the wing disc and the corresponding regiongs in the adult wing. The regions of the disc are color-coded with the structures in the adult wing. L1-L5 are named the wing veins, ACV: Anterior Crossvein, PCV: posterior cross vein, Co: Costal, Tr: Triple row, Dr: double row, Pr: posterior row, Ar: Alula Row. *Image from Hartl, Scott 2014*

1.9: The gut of the adult *D. melanogaster.*

Another tissue where proneural factors of the AS-C have been shown to play a role is the adult midgut. The midgut in adult D. melanogaster is the analogous of the mammalian small intestine. It is not a simply passive tube and it has numerous roles. Apart from digesting and absorbing nutrients, the gut represents the first level of defense towards pathogens and it also transmits signals relevant to food intake and nutrient storage (57,58). It is a complex tissue with specific compartmentalization and plasticity but it has been understudied, although recently it has started to gain attention.

Regarding its compartmentalization, it is composed of three main domains, with different developmental origins; the foregut, which is formed from the ectoderm, the midgut, which originates from the endoderm and the hindgut, which is produced from the ectoderm as well. Each of these regions is subdivided in further domains. The foregut is subdivided in the esophagus, the crop, which stores food before entering the midgut, and the proventriculus, which is also known as cardia. The midgut is protected by the peritrophic matrix (PM) and it is separated in numerous segments that have different metabolic and digestive functions. Some of them are surrounded by sets of muscles that act as sphincter in order to control the movement of food. Between the midgut and the hindgut are found the malphigian tubes, that control osmosis and secretion of hormones and enzymes (**fig. 16a,b**) (58).

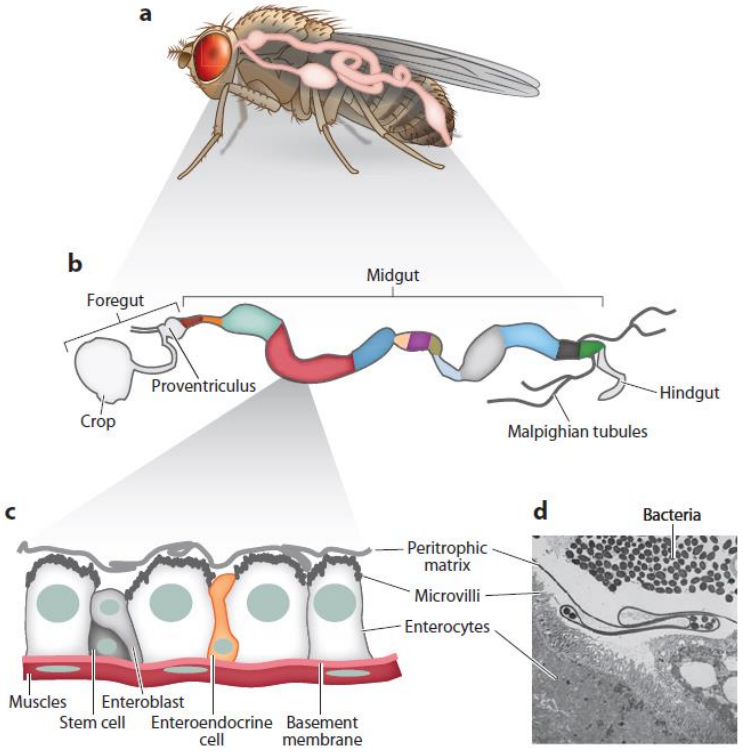


Figure 16: representation of the adult gut, its regions and its cell types. A) the gut in the adult fly and b) its domains and subdomains (depicted with different colors). C) The different cell types of the gut and their relative position within the tissue. D) the cells seen on electron microscopy. *Image from Lemaitre, 2013*

All the different cell types of the midgut derive from the same precursor; the Intestinal Stem Cells (ISCs). These self-renew with each division and give rise to intermediate precursor cells, the enteroblasts (EB) or the enteroendocrine precursor cells (EEP). EBs are large, polyploid cells and differentiate into enterocytes (EC) which have an absorptive role. The EBs are distinguishable by their large size and by the fact that they express *esg* whereas EC are characterized by the expression of POU domain protein (Pdm1) and are *esg* negative. The EEPs will finally differentiate into two different type of enteroendocrine cells that express different TFs and produce hormones. EEPs are detectable through *pros* expression (**fig 17**). Their relative position in the tissue is depicted **in fig. 16c**. The ISCs are located basally in contact with the basal membrane and their descendants move away from the basement membrane upon division. EC have great morphological variety across the gut but they are the largest cells. EE are disperse in the gut. The underlying visceral muscle contacts the entire epithelium.

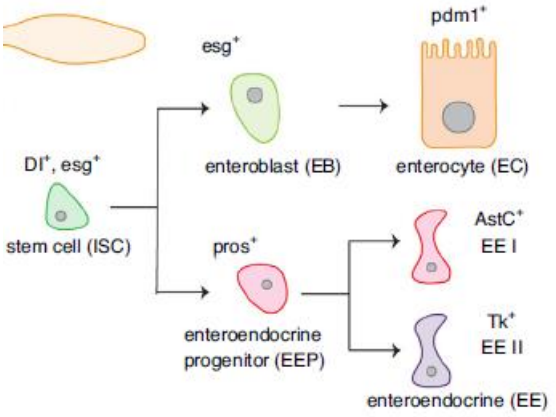


Figure 17: The different cell types of the adult gut and their origins. Description in text. *Luchetta, 2012*

The ISCs become EB or EEP depending on a mechanism based on Notch signaling (**fig. 18**). All ISCs express Dl, but in different concentrations and therefore activate in a different way the Notch pathway on their daughter cells. ISCs with a high level of Dl expression send strong N signal to their descendants and that differentiates them into ECs, whereas ISCs with low level of Dl give a weaker N signal to their daughter cells and that makes them EEP. Once again, Notch pathway is pivotal for the differentiation (59,60).

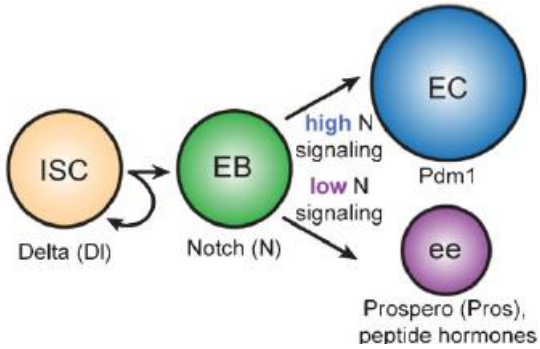


Figure 18: Notch signaling determines the cell fate of the ISCs *Lucchetta, 2012*

1.10: Enhancers

During the development of an organism form a single cell, the fertilized egg, a big variety of cells with different morphology and functions arises. This diversity is not a result of genome differences among the cells, but a consequence of the different expression pattern of the same genes in each cell and the interactions of the transcriptional active genes. The orchestration of this expression pattern is mediated through interactions of different transcriptional modulators with the DNA which lead to transcriptional activation. For the successful initiation of transcription, the activity of a group of cis-regulatory elements plays crucial role; these elements are called enhancers, since in their absence, transcription is drastically reduced (61).

The activation through enhancer is a fundamental mechanism of gene activation in eukaryotes (62). The first enhancer was discovered almost 40 years ago and it was characterized as a sequence of 72bp in the genome of the virus SV40. Since then, many studies regarding enhancers have taken place and today it is known that they are sequences situated upstream or downstream the sequence of the target gene and that involve short patterns of DNA which are used as binding sites of various transcriptional factors. Their action though does not depend on the distance from their target gene and their orientation towards it; they seem to work perfectly in distance of hundreds of kilobases or even megabases. This happens due to various folding patterns of the DNA, which can make distant location interact. Finally, their activity is independent of the sequence of their target gene (61,62).

Regarding the chromatin structure, enhancers seem to lack nucleosomes, which makes them more easily accessible to TFs. The nucleosomes that surround them present characteristic post-translational modifications like mono-methylation of lysine 4 in histone 3 (H3K4me1) and acetylation of lysine 27 in histone 3 (H3K27ac) in their N terminus (61). These modifications as well as the binding of TFs have been used to predict enhancers in the level of genome.

Chip-seq (Chromatin Immunoprecipitation followed with deep sequencing) for proneural transcription factors of the Acheate-Scute Complex (AS-C) was performed, and regions with altered chromatin structure where the products of the AS-C genes bind were found and characterized as enhancers. We wanted to study the expression pattern of these enhancers and for that reason we checked their expression in different developmental stages and tissues.

2. Purpose of the study

Although the importance of the differentiation of NBs and SOPs in *D. melanogaster* for the CNS and PNS formation is widely known, there is very little knowledge for the chromatin changes that take place during cell fate determination.

After ChIP-seq (Chromatin Immunoprecipitation followed by deep sequencing) for proneural TFs and K27-acetylated histone H3 in the neuroectoderm of embryos st. 8-11, that was performed previously in the lab by Vasiliki Theodorou, the proneural TFs were found to bind in numerous regions in the genome. Regions where TFs are bound, can be, but not necessarily are, enhancers. In order to study the hypothesis that these regions were, indeed, working as enhancers of these specific TFs, 10 different regions whose protein product showed proneural dependency in mutated embryos were used to create transgenic lines of *Drosophila melanogaster* by Florentia Romanou. Each of those lines carried a transegne sequence where the proneural TFs bound linked to a reporter gene – lacZ.

In order to monitor the activation of the hypothetical enhancers, an antibody that targets the protein β-galacotsidase, the product of the transcription of the gene lacZ, was used. The transcription of this gene is a result of the activation of the enhancer, and therefore, detecting β-galactosidase would mean detecting the pattern of activation of the enhancer of interest.

These enhancers have already been studied in embryos, with and without the presence of the AS-complex, by previous lab members. It was shown that in embryos lacking the genes of the AS-C (*sc, l'sc, ac, ase*) the enhancers were not expressed (V. Theodorou et al, submitted). Now, we wanted to see whether the expression of these enhancers relies on some gene members of the AS-C (ac, sc and l(1)sc) by studyin their expression in $Df(1)sc^{19}$, which removes these three genes, but spares *ase.*

Furthermore, we wanted to investigate whether these enhancers would be expressed in other tissues and developmental stages that also have high expression of AS-C. We checked the larva CNS, imaginal discs and the adult gut.

3. Materials and methods

3.1: Growth and handling of *Drosophila melanogaster*

All of the flies used for the following experiments were grown in standard culturing conditions. The tubes were held in controlled temperature, at 25°C when it came to crosses, cages, or flies for immediate use (for example dissections) or at 18°c for long-term culturing. In the first case, flies were flipped every 2-3 days and in the latter, every 21 days approximately. For the maintenance of the lines, the older flies were sacrificed and the ones that had recently enclosed were transferred into a tube with fresh food. Usually there were two back-up tubes where flies of intermediate age were kept.

When anesthesia was required, the flies were sedated by using CO_2 (with exception flies that were to be used for gut dissections – see section 3.6.1.); CO_2 was inserted from tanks to the tube through the cotton cap with the help of a needle, attached to a plastic tube. The flies were then laid on a portable porous table from which CO_2 was also emitted, and observed in the stereoscope. Note that the duration of exposure in CO_2 is rather important since overexposure can have side effects on flies and even lead to death (63).

The flies that were not needed for experiments or further maintenance were disposed in a mixture of soap and water.

3.2: KV lines

As explained in the previous section (2: purpose of the study), the regions that were suspected to be enhancers were fused to a region that encoded for the reporter gene lacZ and then used to create transgenic animals. The lines that were created were named KV-x, were –x stands for different numbers. Here, a table of the name of the enhancers and KVs that were used in the present study is presented.

Inscutable	KV 1.1.1	**Embryonic lethal abnormal vision / appl**	KV 16.12.1
Phylopod	KV 4.3.1	**Scratch 1**	KV 19.15.1
Brain tumor	KV 8.22.1	**Target of Poxn**	KV 21.7.2
Scratch 2	KV 10.17.1	**Deadpan**	KV 23.4.1
Nervy 1	KV 14.16.1	**Worniu**	KV 29.9.4
Nervy 2	KV 15.8.3		

3.3: Crosses and cages

Embryos that had the ablation of the genes *ac, l'sc, sc* (named Dfsc19) and the expression of lacZ under the control of the desired enhancer (KV-), were created by performing crosses. In each cross, virgins Dfsc19/FM7Kr>GFP were mated with young males of the KV lines. Virgins can be selected by the presence of meconium in their abdomens.

The virgin females with the desired phenotype (sc19/FM7kr>GFP) were selected from a tube where flies of the following genotypes were present; FM7Kr>GFP/sc19 (female heterozygous with red colored bar eyes), FM7/FM7 (homozygous female with red ultra-bar, thinner eyes), FM7/Y (hemizygous male with red colored ultra-bar eyes). FM7 is a balancer chromosome. Balancer chromosomes are essential for maintenance of genetically modified flies and mating schemes, since they prevent the recombination with their homologous chromosomes, during meiosis. That is achieved through the significant rearrangement of the position of the genes due to the multiple inversions the balancers carry. As a result, any product of recombination is lethal because of deletions or duplications of chromosomal fragments (64). Also note that sc19/sc19 and sc19/Y are embryonic lethal genotypes and that FM7/FM7 female flies are infertile.

Regarding the males, the genotype of each was homozygous KV/KV or heterozygous KV/cyo. CyO is another balancer which in homozygous form is lethal, whereas in heterozygous form is leads to a phenotype with curly wings. For the desired crosses, males homozygous for the KV were used (males with straight wings), so that it was sure that the F1 embryos would receive the lacZ reporter.

For the crosses that would be used for embryo collection the ratio of males: females was 1:3 and the females were around 60-80 individuals. The crosses were grown at the cylindrical tubes with solid food described above at 25^{o}C for 2 days and then they were transferred in cages. The cages consisted of petri dishes containing a medium of agar and fruit juice (recipe below) where the eggs were laid, and a urine collector, with small holes for air was used as a container. In the petri dish a small amount of fresh yeast was placed and the cages with the flies were put in 25^{o}C. The petri dishes were changed every 24h or more often, depending on the desired developmental stages of the embryos (the embryogenesis lasts ~24h). Normally, the egg laying was higher after the second day of the cage and it would last up to a week before the amount of eggs laid significantly declined.

Cherry juice medium recipe

Materials:

- 8gr agar
- 250ml commercial cherry juice

Procedure:

1. Dilute the agar in the juice with the help of a magnet in a >500ml Erlenmeyer flask suitable for boiling
2. Place the flask in a rotator with heat
3. Wait until the mixture turns darker and starts producing foam
4. Divide the mixture in ~22 petri dishes of 5cm diameter and wait for it to cool down
5. Store the petri dishes at 4-8^{o}C

3.4: Embryo Collection

3.3.1: Dechorionation

Materials:

- Solution of commercial bleach diluted in ddH_2O 1:1

Procedure:

1. Rinse the petri dish that has the embryos with tap water and carefully detach the embryos with the help of a brush
2. Transfer the embryos in special nets with pores with smaller diameter than the size of the embryos, so that water can go through but the embryos stay on the net
3. Immerse the nets with the embryos in bleach solution for 2 minutes in order to remove the chorion (when chorion is removed the embryos will rise to the surface of the solution)
4. Rinse extensively the embryos with tap water to remove bleach

Note: embryos should stay hydrated at all times.

3.3.2: Fixation of the embryos

Materials:

- Bi-phase fixative solution:
  - 1200ml Phosphate Buffered Saline (PBS) – 0.13M NaCl, 0.007M Na_2HPO_4, 0.003M $NaHPO_4$
  - 800μL Formaldehyde 10%
  - 2ml Heptane
- Methanol

Procedure:

1. Transfer the dechorionated embryos with the help of a brush in 2ml, cylindrical, glass bottles that have the fixative solution.
2. Place the bottles with the embryos in a shaker for 16min and shake vigorously at 260rpm at RT.
3. The embryos should be and the interface of the bi-phase solution. With a Pasteur pipette remove the lower phase that contains the formaldehyde.
4. Add 2ml of methanol and shake vigorously by hand for 45sec.
5. The majority of embryos should sink to the bottom of the glass bottle. Collect them with the help of a Pasteur pipette and transfer them in a 2ml, sterile eppendorf.
6. Perform 3 washes with 1ml of methanol in order to remove completely the excess of the fixative.
7. Label carefully the eppendorf and store it at -20^{o}C.

3.5: Embryo staining – Immunochemistry fluorescence staining

Immunofluorescence is a method which allows the acquisition of information about the spatial distribution of specific molecular targets and the cellular structure (65). The first report of usage of antibodies with the purpose of marking and detecting target molecules is in 1941 from Albert H. Coons (66). In summary, the tissue or cell studied is fixed, permeabilized and exposed in antibodies designed to bind to a specific target molecule. The binding of the primary antibody with the target molecule is detected with the incubation in a secondary antibody which recognizes the primary one and it is conjugated with a fluorochrome. The fluorochromes have the ability to absorb light in specific wave

lengths and emit in bigger ones. That produces a visible signal and the results are observed through fluorescent microscope (65).

Materials:

- PT solution
  - 100ml PBS 1x
  - 1 ml Triton 20x
- PBT solution
  - 0.05g BSA (albumin)
  - 10ml PT
- Solution with primary antibody diluted in PBT in desired concentration
- Solution with secondary antibody diluted in PBT in desired concentration
- 55μL NPG (n-propyl gallate – 80% glycerol in 1x PBS) for fluorochrome protection

Procedure:

1st day of staining

1. Remove methanol from the embryos (leave ~50μL, don't let the embryos dry out).
2. Add carefully drop by drop 1ml of PT.
3. Perform 3 washes with 1ml of PT to remove excess methanol.
4. Perform 3 15-20min washes with 2ml of PT, shaking mildly to fully remove methanol from the embryos.
5. Add 2ml of PBT for blocking. Leave 2-3h while embryos are mildly shaking. The BSA contained in PBT prevents the non-specific binding of the antibody.
6. Remove PBT.
7. Dilute primary antibody in 80μL of PBT and add it to the embryos.
8. Incubate O/N at 4^{o}C

2nd day of staining

1. Remove the PBT-antibody solution (you can store it at 4^{o}C with thimerosal for next use).
2. Perform 3 short washes with PT to remove excess antibody.
3. Perform 3 20min washes with PT to fully remove the primary antibody,

4. Dilute the secondary antibody in 100µL of PBT, in the desired concentration.
5. Protect tube from light to avoid photobleaching and incubate for 2-3h, shaking vertically, at RT.
6. Remove secondary antibody and perform 3 short washes with PT.
7. Perform 3 20min washes with PT to remove any leftovers of the secondary antibody*.
8. Remove PT.
9. Add 90 µL of NPG.
10. Incubate O/N at 4°C, without shaking.

* if you want to visualize nuclei by using DAPI (4',6-diamidino-2-phenylindole, a fluorescent that strongly binds to adenine-thymine rich regions of DNA), perform a first 15min wash with PT, then add 30µL of DAPI in 1ml of PBS and incubate shaking for 20min, and, finally, perform a 20min wash with PT.

<u>3rd day of Staining</u>

1. Aspirate embryos 3 times up and down with a Gibson pipette in order to separate the embryos from each other.
2. Transfer embryos in NPG to a microscope slide.
3. Cover them with a coverslip.
4. Seal with nail polish and let it dry for ~20min.
5. Store the slide in -20°C in dark for future observation.

<u>Antibodies used in the following embryo staining</u>

Primary antibodies for:

- sc19 / FM7Kr>GFP x KV-:
    1. goat anti-GFP (1:50) → marks the KrGFP, meaning the embryo didn't get the sc19
    2. rabbit anti-bgal (1:50) → β-galactosidase
    3. mouse anti-sxl (1:50 + 1:50) → sex determination, active in females only

Secondary antibodies for:

- sc19 / FM7Kr>GFP x KV-:
    1. donkey anti-rabbit 555 (1:50)
    2. anti-goat 488 (1:50)
    3. donkey anti-mouse 647 (1:50)

3.6: Dissection and Immunostaining for *Drosophila* Tissues

3.6.1: Dissection and fixation of adult gut

Preparation

One day prior to the dissection, flies are put into empty glass vials that instead of solid food they have a Whatman paper soaked with 10% sucrose. That way, the gut will be clear of solid food remnants when the time for dissection comes. Also, ideally relatively young flies (< 1 week) are used for the dissection. For each strain aim to have in the end around to 4-5 intact guts, therefore start with around 6-8 individuals.

Materials

- Sylgard dish
- Forceps
- ice

Solutions

- Fixative solution (4% formaldehyde / 1x PBS → 400μL / 600 μL)
- PBS 1x

Procedure

1. Place drops of PBS 1x in the Sylgard pate in which the whole fly should be immersed.
2. Anesthetize the flies by immersing the vials in ice.
3. Put the flies on the lid of a petri dish on ice.
4. Under a stereoscope, decapitate with the forceps the flies and remove the wings and the legs.
5. Immerse the fly in the drop of PBS.
6. Grab the fly abdomen with one pair of forceps, while holding the thorax with the other pair of forceps. Slowly separate those two and the gut will be visible and the crop will probably be attached to the thorax. *
7. Carefully remove the tissue around the gut so as only the gut will remain in the end (thorax and abdomen will be removed, like “undressing” the gut).
8. Remove the foregut, Malphighian tubules, hindgut and ovaries leaving bare midgut.

9. Transfer the tissue in a glass plate containing 400 µL of the fixative solution. Immerse the tissue and incubate for 20min at RT, with very mild shaking.
10. Remove fixative (be careful to not let the tissue fry out) and perform 3 rinses with 1ml of 1x PBS. Be careful in the washes to not touch or suck the gut with the Gibson pipette. **
11. The tissue is ready for immunostaining.

Notes:

* an alternative method for the dissection, is to grab with the forceps the posterior abdomen and the edge of the anteriorly open cuticle with the other pair of forceps. While being very careful to not destroy the protruding tissue, pull very gently the posterior end away until the entire gut has been pulled out of the abdominal cavity. Note that in case of large ovaries and / or crop, extra opening of the posterior cuticle may be needed.

** For Delta staining, these extra steps should be followed:

1. After the 3rd wash with PBS, add 20 µL PBS and drop by drop 400 µL of MetOH.
2. Remove the solution, add 200 µL of pure MetOH and shake mildly for 5min.
3. Add drop by drop 400 µL of PBS
4. Remove the solution, add 200 µL of PBS and shake mildly for 5min.
5. Proceed normally with PBT.

*** Dissections should be performed in <20min before placing the tissues in the fixative.

3.6.2: Dissection and fixation of *Drosophila* larva CNS and discs

For the purpose of this study, late 3rd instar larvae from different KV lines were used. The goal is to end up with around 8 brains and disks pairs, so start with around 10 individuals which you will remove from the vial by using the forceps. Be careful not to damage the larvae; grab them from the posterior end so that the head will remain intact.

Materials

- Forceps
- Glass plates

Solutions

- Fixative (400μL formaldehyde : 600μL PBS 1x)
- PBS 1x

Procedure

1. Collect around 8 larvae and place them in a glass plate with 1x PBS, under a stereoscope.
2. Remove the 1/3 of the posterior part of the larvae. The animal will try to curl.
3. Gently press on the side of the abdomen so the most organs start to come out of the incision. Carefully remove them with the forceps.
4. Turn the larvae inside out ("like a shock") with the help of the forceps; use one to stabilize the larva from the head, near the mouth hooks, and the other to push it around the first forcep, so that you end up with an inside out larva.
5. Remove the remaining organs carefully so as to leave the CNS and the disks intact. Ideally, if you are about to perform staining in the disks, gently detach the trachea from the body so as the disks will be fully immersed in the antibodies in the next steps.
6. Immerse the larvae in a 2ml tube with fixative solution and incubate for 20min, with mild shaking, at RT.
7. Remove the fixative by placing the waste in an appropriate bottle and rinse 3 times the larvae with 1ml of 1x PBS. Gilson pipettes can be used, but carefully so that you avoid aspirating the fixed larvae.
8. The tissues are ready for staining.

3.6.3: Immunostaining in *Drosophila* tissues

Solutions:

- PBT
- PT
- NPG
- Primary antibodies in desired concentration, diluted in PBT
- Secondary antibodies in desired concentration, diluted in PBT

Procedure:

After you have the desired tissue (gut or CNS) fixed, proceed to the following steps. Note that the staining process for guts is performed in the very same glass dishes where the dissection took place, whereas for the CNS it is performed in 2ml eppendorfs.

With **blue** are the time / quantities / steps for **gut** samples and with **orange** are the ones regarding the **CNS**. All the others are common.

1. Block and permeabilize tissue by incubating in 1ml of PBT at RT, with mild shaking (**30min-1h** / **1h**).
2. Remove PBT and add the primary antibody which is diluted in PBT (**200μL** / **80μL**). Incubate at 4^{o}C overnight (14-18h) with mild shaking (in the CNS, shake vertically the eppendorfs).
3. Remove the primary antibodies and store them with thymerosal for next use.
4. Perform 3x 10min washes with PT to remove excess antibody.
5. Incubate with appropriate secondary antibody diluted in PBT. Incubate for 1-2h at RT, shaking, in the dark to avoid photobleaching.
6. Perform 3x 5min washes with PT, shaking, to remove excess antibody*.
7. **Perform 2x 5min washes with PBS**
8. Mount the tissue;
   - **Guts**: place 80μL of NPG in a microscope slide and transfer there the guts. Try to unfold them before you cover them with the coverslip. Seal the coverslip with nail polish.
   - **CNS**: detach the CNS and the disks from the larva body, carefully by using forceps. Transfer them in a microscope slide with 30μL of NPG and seal with nail polish.
9. Store the samples in -20oC in the dark for future use.

* If visualizing the nuclei is desired, in the second wash, add Dapi diluted in PBS (3:100) and incubate shaking for 15-20min. Perform the 3rd wash with PT for 15-20min, and proceed normally.

Notes:

- Dissections should be completed within 20min.
- In the following study, Dapi was used only in gut samples and not in CNS.
- In this series of experiments, different antibodies were used for the CNS and different for the disks. Therefore, for each KV line, the larva dissection was performed twice: one for a CNS staining, and once for disks staining, since disks and CNS cannot be separated before the addition of antibodies.

Antibodies used in the following stainings:

a) Guts:

Primary antibodies	Secondary antibodies
Rabbit anti-bgal (1:80)	Donkey anti-m555 (1:50)
mouse anti-pros (1:50)	goat anti-rb488 (1:50)

b) CNS:

Primary antibodies	Secondary antibodies
Guinea pig anti-dpn (1:100)	Donkey anti-m555 (1:50)
Rabbit anti-bgal (1:50)	goat anti-rb488 (1:50)
Mouse anti-repo (1:50)	Goat anti-gp647 (1:50)

c) Discs:

Primary antibodies	Secondary antibodies
Mouse anti-cut (1:80)	Donkey anti-m555 (1:50)
Rabbit anti-bgal (1:50)	Goat anti-rb (1:50)
Guinea pig anti-sens (1:50)	Goat anti-gp (1:50)

4. Results

As mentioned above in the chapter "purpose of the study", transgenic lines of *D. melanogaster* were created by previous lab members in order to investigate if some regions of the genome bound by AS-C TFs were working as enhancers in the embryo. These lines were named KV (analytical table in materials and methods) and they contain lacZ as a reporter gene of the enhancer's expression (more details in the "purpose of the study"). Here, we wanted to investigate the expression pattern of these enhancers in tissues where genes of the AS-C are known to be expressed in larva and adult flies, in order to investigate if the expression pattern of these enhancers is dependent from the AS-C genes. Also, we wanted to see whether their expression pattern in sc19 embryos is different than in B57 embryos and therefore if the presence of *ase* alone is sufficient to bypass any putative requirement of the earlier expressed genes of the complex and drive the expression of the enhancers.

4.1: Expression pattern of enhancers in CNS of 3rd instar larva of *D. melanogaster*

It is known that *ase* is present in all type I NBs in the late larva brain and that other members of the AS-C are expressed broadly in the optic lobe. We wanted to see whether the enhancers whose expression seemed to be depleted in embryos lacking AS-C genes are expressed in the larva brain where these genes are normally present. Since brain is a rather thick and dense cell-wise tissue, results will be presented as separate stacks of sections of dorsal and ventral regions of the brain. Anti-Dpn was used to mark the NBs, anti-repo for glia and anti-βgal for the enhancer expression.

4.1.1: Expression pattern of the *inscutable* (enhancer KV1)

The transcriptional product of the gene *inscutable (insc)*, is an adaptor protein which is necessary for the asymmetrical cell division (67). In CNS, insc is expressed in large amounts in NBs and in GMCs and it is vital for the polarity of the cytoplasm during metaphase, the orientation of cell division and therefore the asymmetrical division of the cell (68). Regarding the PNS, insc is expressed in the apical pole of pIIb cells regulating the polarity of their future division (69).

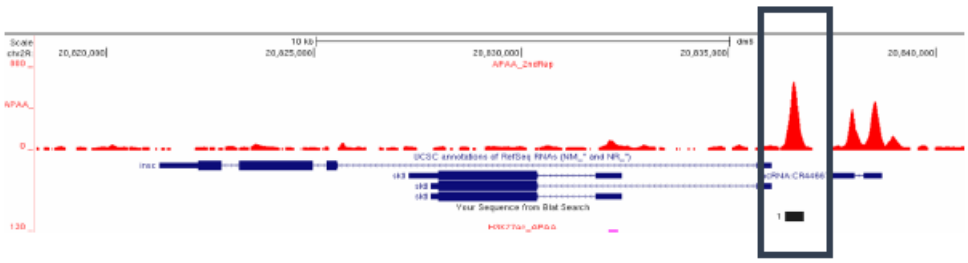
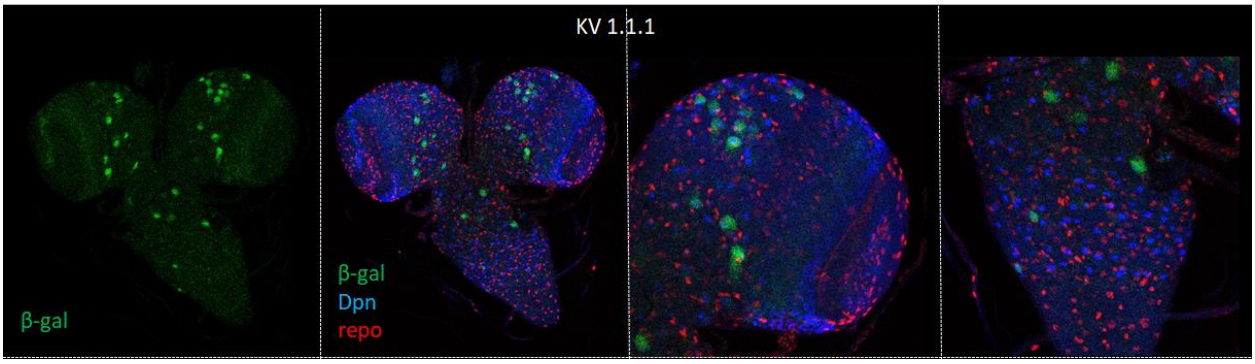


Figure 19: Results of ChipSeq analysis of TFs in neuroectoderm of embryos st. 8-11, performed by Vasiliki Theodorou. In the boxed sequence the most enriched binding of proneural TFs was observed and therefore it was selected for further analysis.

a) ventral view



b) dorsal view

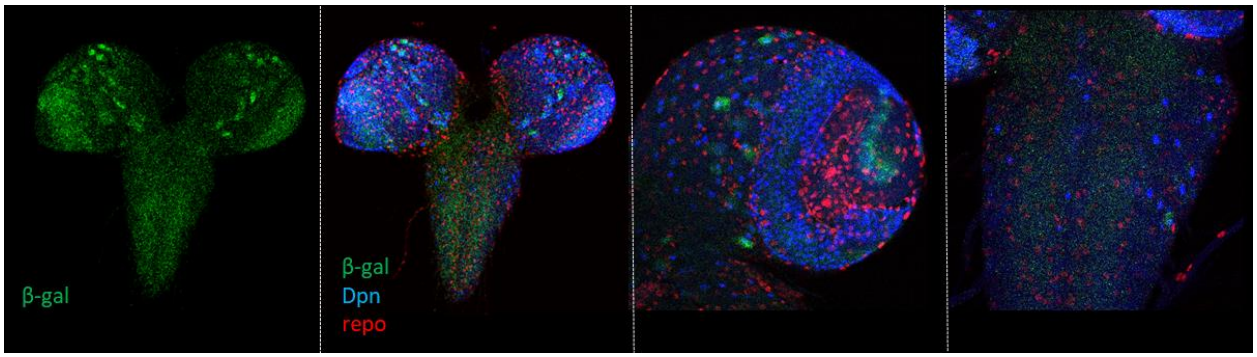


Figure 20: Pattern of expression in CNS of 3rd instar larva with inserted *inscutable* KV1 enhancer upstream of lacZ reporter. A) dorsal b) ventral view. The last two pictures in each are zooms. Stained with rabbit anti-bgal (green), mouse anti-repo (red) and guinea pig anti-dpn (blue).

We can conclude that insc enhancer is expressed in CNS of larvae. If the cells in which it is expressed are dpn$^+$, they are NBs and if they are repo$^+$, they are glia. Dorsally, we can see that the enhancer is activated in NBs and in glia in the central brain and also in the VNC in thoracic segments. Ventrally, it is expressed mainly in the optic lobe (for more analytical pictures see **Sup. Fig. 1**).

4.1.2: Expression pattern of *phyllopod* (enhancer KV 4.3.1).

The protein of the gene *phyllopod (phyl)* is an adaptor protein which participates in many pathways among which is the negative transduction of signal, the induction of protein degradation by the proteasome and the development of sensory organs (70,71). The protein phyl seems to play a role in the orchestration of cell division of SOPs and the determination of the cell fate of their descendants. This

procedure is connected with degradation of proneural proteins and requires the presence of ligase-ubiquitin E3 complex which includes the protein Seven in absentia (Sina) and Phyl. In addition, it has been previously observed that phyl is a transcriptional target of Ac and Sc (70,71).

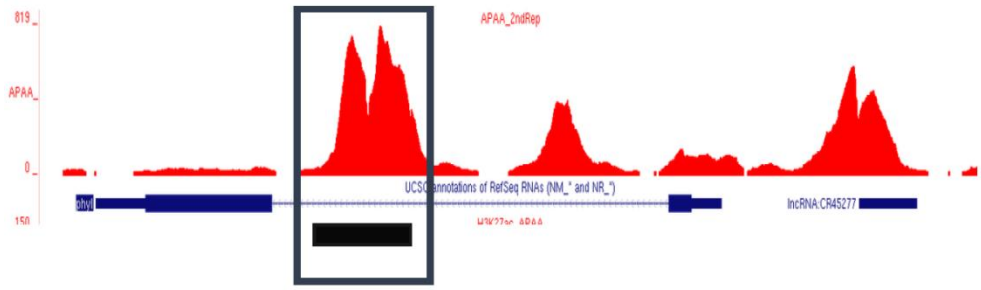
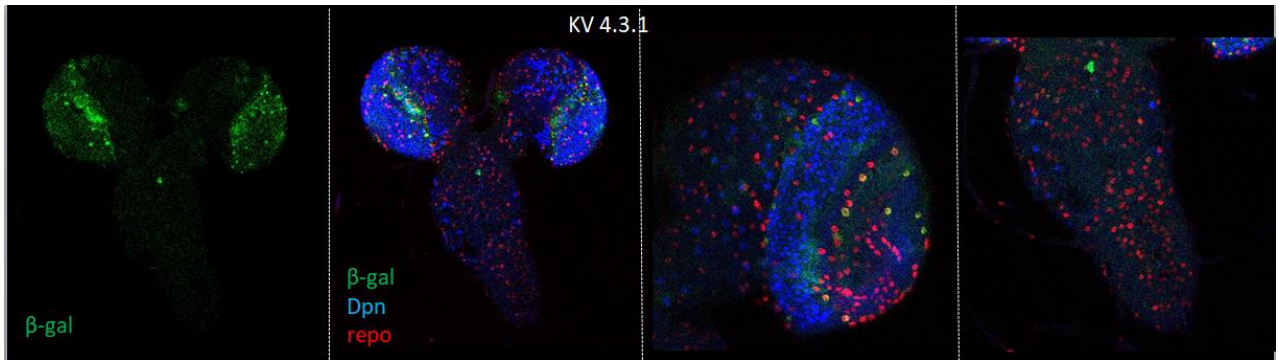


Figure 21: Results of ChipSeq analysis of TFs in neuroectoderm of embryos st. 8-11, performed by Vasiliki Theodorou. In the boxed sequence the most enriched binding of proneural TFs was observed and therefore it was selected for further analysis.

a) ventral view



b) dorsal view

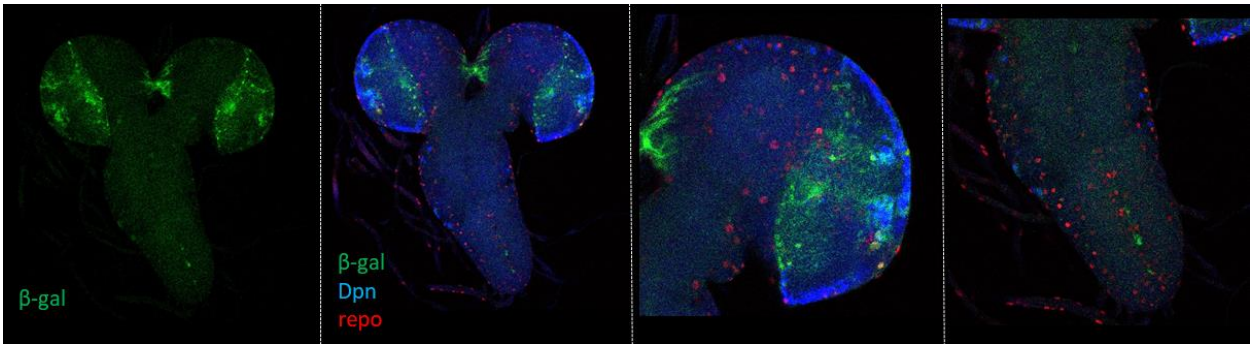


Figure 22: : Pattern of expression in CNS of 3rd instar larva with inserted *Phyllopod* enhancer upstream of lacZ reporter. A) ventral b) dorsal view. The last two pictures in each are zooms. Stained with: rabbit anti-bgal (green), mouse anti-repo (red) and guinea pig anti-dpn (blue).

Thinking as previously, we can conclude that the Phyllopod enhancer is also expressed in the larval CNS and more accurately, based on the known anatomy, it is mostly expressed in the optical lobe and the brain commissural neurons, seemingly in cells that are located between NBs and glia. Regarding the

VNC, it is expressed in abdominal hemisegments and in the telson, in 1-2 cells which are not dpn^{+}, and therefore are not NBs (**Sup. Fig. 2**).

4.1.3: Expression pattern of *brain tumor* (enhancer KV8).

The protein produced by *brain tumor (brat)* is an NHL translational repressor (72) which in NBs has several targets, included Dpn. In larva, it is asymmetrically localized in the dividing NBs and transferred to only one daughter cell whose self-renewal arrests (73).

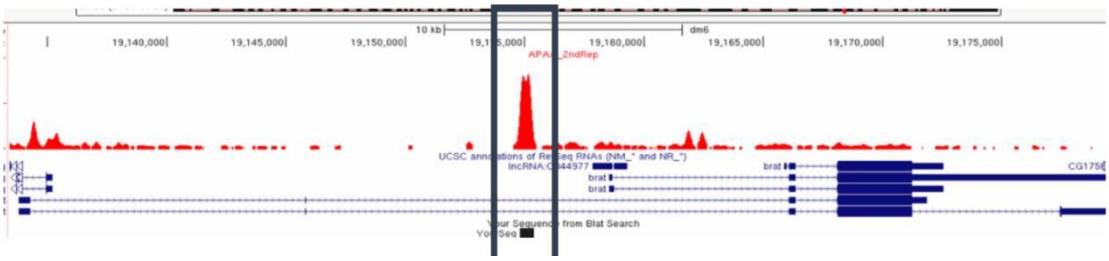
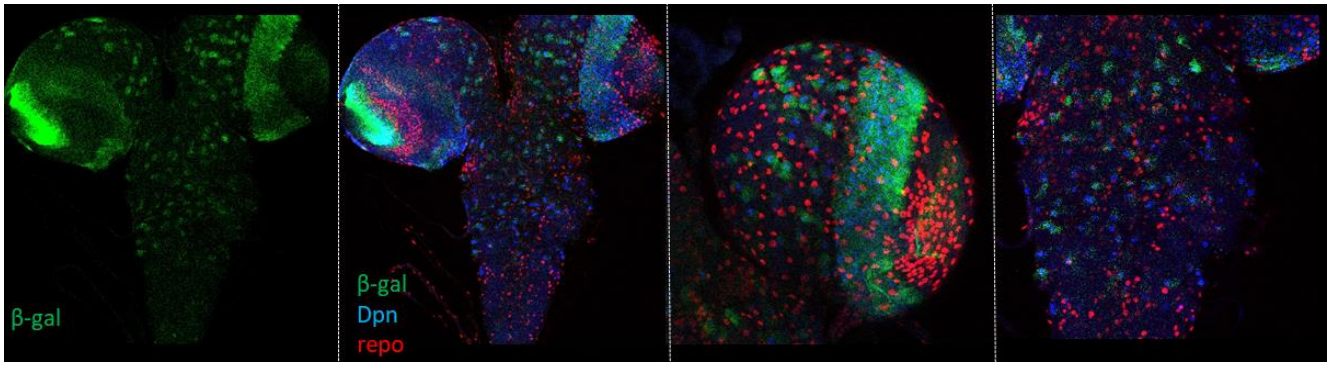


Figure 23: Results of ChipSeq analysis of TFs in neuroectoderm of embryos st. 8-11, performed by Vasiliki Theodorou. In the boxed sequence the most enriched binding of proneural TFs was observed and therefore it was selected for further analysis.

a) ventral



b) dorsal

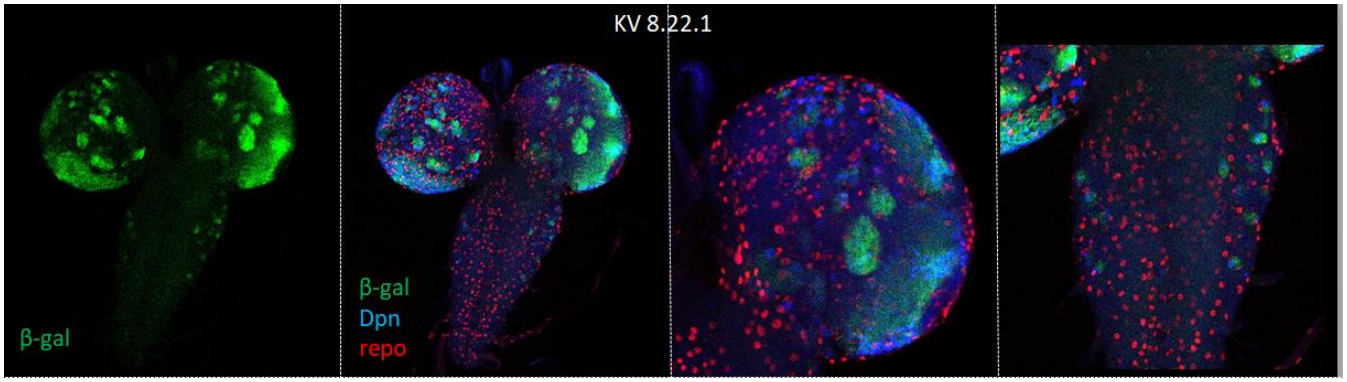


Figure 24: Pattern of expression in CNS of 3rd instar larva with inserted *Brain tumor* enhancer upstream of lacZ reporter. A) ventral b) dorsal view. The last two pictures in each are zooms. Stained with: rabbit anti-bgal (green), mouse anti-repo (red) and guinea pig anti-dpn (blue).

As we can see, brat produces a very strong signal of expression in the optic lobes, a less strong but still notable signal in the central brain and in the VNC except for the telson. Its signal is co-localized with the one of some NBs but not exclusively; it seems like in the central brain it is expressed in some NBs daughter cells too (**Sup. Fig. 3**).

4.1.4: Expression pattern of *scratchII & scratchI* (enhancer KV10 & KV19).

Scratch (scrt) is a pan-neural gene; it is expressed in all differentiated, intermediate cells of CNS and PNS. Its product is a zinc-finger transcription factor which is related with snail. It promotes neural growth by repressing genes that promote the non-neural cell fate (74).

The Chip-seq gave more than one peaks and two of them were selected as probable enhancers of *scrt*.

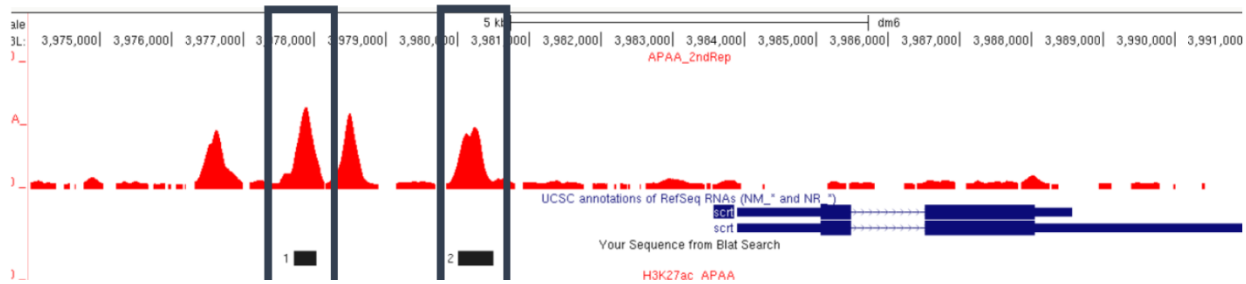
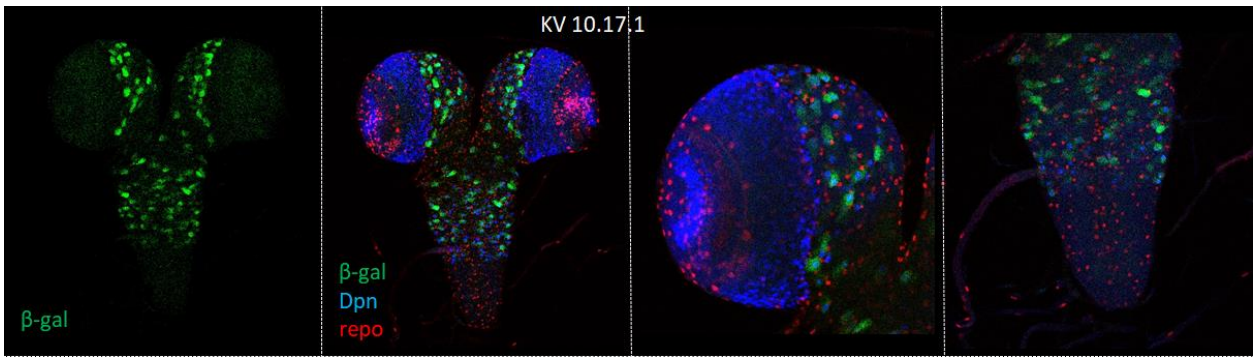


Figure 25: Results of ChipSeq analysis of TFs in neuroectoderm of embryos st. 8-11, performed by Vasiliki Theodorou. Two were selected for further analysis

a) ventral



b) dorsal

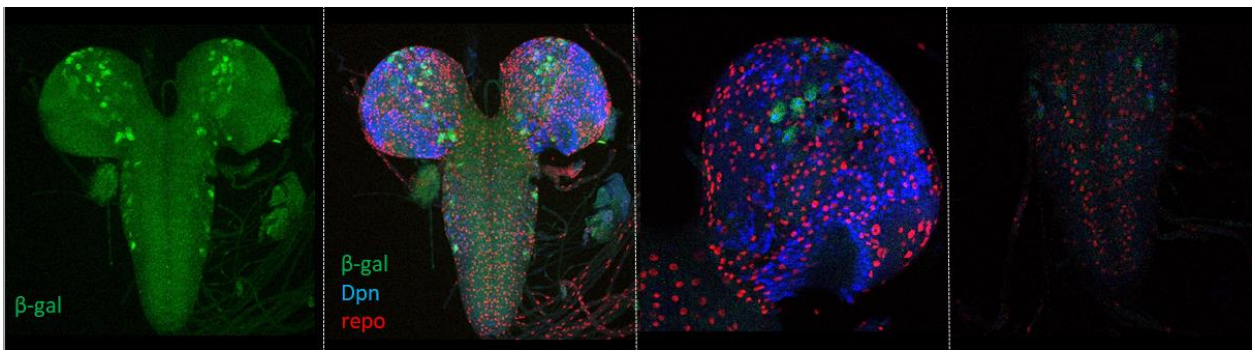
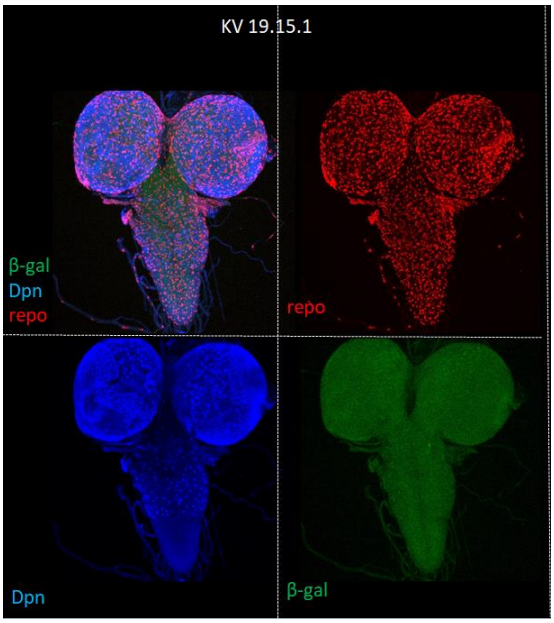


Figure 26 Pattern of expression in CNS of 3rd instar larva with inserted *Scrt2* enhancer upstream of lacZ reporter. A) ventral b) dorsal view. The last two pictures in each are zooms. Stained with: rabbit anti-bgal (green), mouse anti-repo (red) and guinea pig anti-dpn (blue).

It is clear that this enhancer is not expressed in the optic lobe but it is vividly expressed in the central brain and the VNC in the gnathal and thoracic segments (ventrally it is expressed in a couple abdominal NBs as well). In the central brain it is expressed in NBs and seemingly in NBs alone, whereas in the VNC it varies; ventrally, it is expressed in NBs and other cell types, but dorsally only in NBs (**Sup. Fig. 4**).



In contrast with *Scrt2* enhancer, *Scrt1* is not expressed anywhere in the larval CNS.

Figure 27: Pattern of expression in CNS of 3rd instar larva with inserted *Scrt1* enhancer upstream of lacZ reporter. A) dorsal b) ventral view. The last two pictures in each are zooms. Stained with: rabbit anti-bgal (green), mouse anti-repo (red) and guinea pig anti-dpn (blue).

4.1.5: Expression pattern of *nervy1 & nervy2* (enhancer KV14 & KV15).

Nervy (nvy) belongs to the family of myeloid translocation genes (MTGs) whose members act as transcriptional co-repressors in the nucleus. nvy produces a transcriptional repressor and a kinase anchoring protein (AKAP) (source: flybase).

In the Chip seq two high peaks were observed, and therefore studies as potential enhancers.

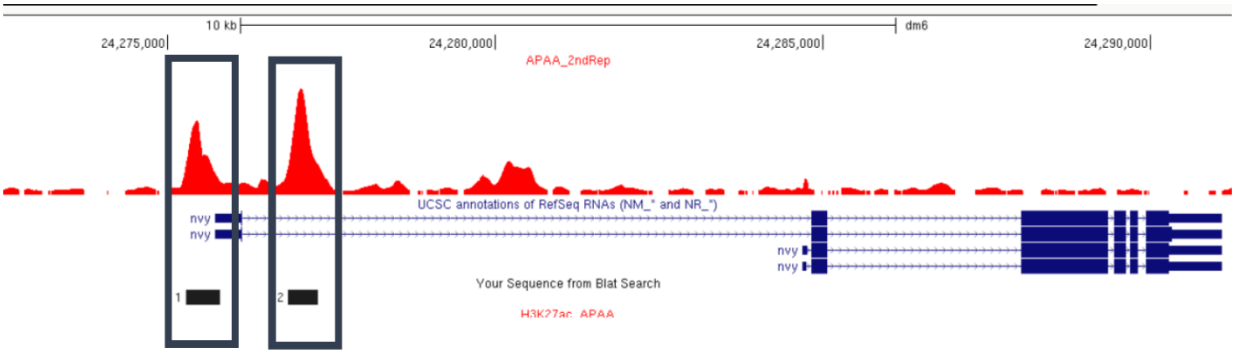
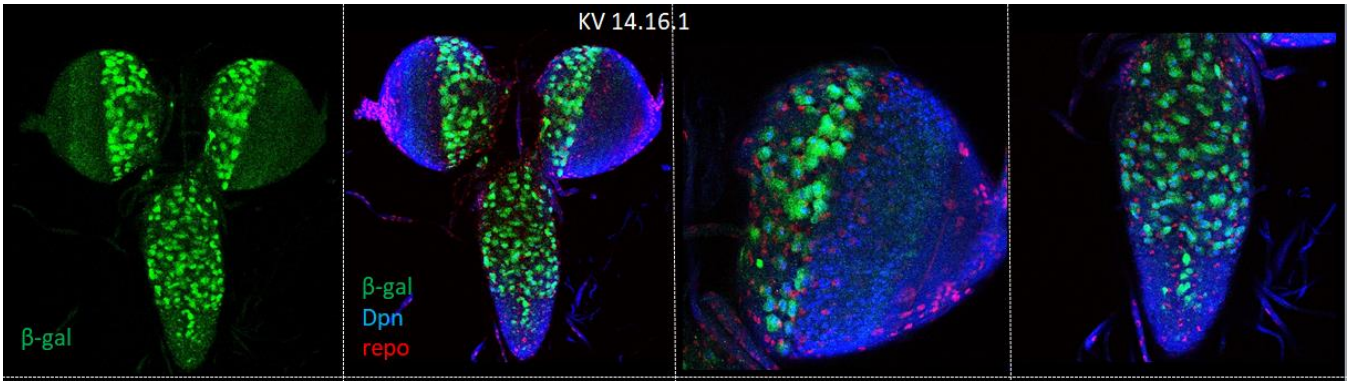


Figure 28: : Results of ChipSeq analysis of TFs in neuroectoderm of embryos st. 8-11, performed by Vasiliki Theodorou. In the boxed sequences the most enriched binding of proneural TFs were observed and therefore it was selected for further analysis

a) ventral



b) dorsal

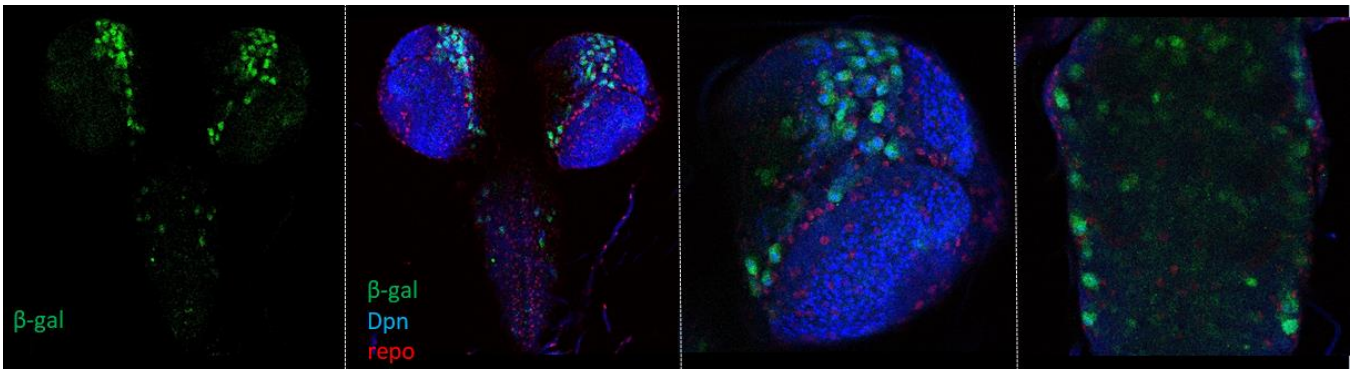
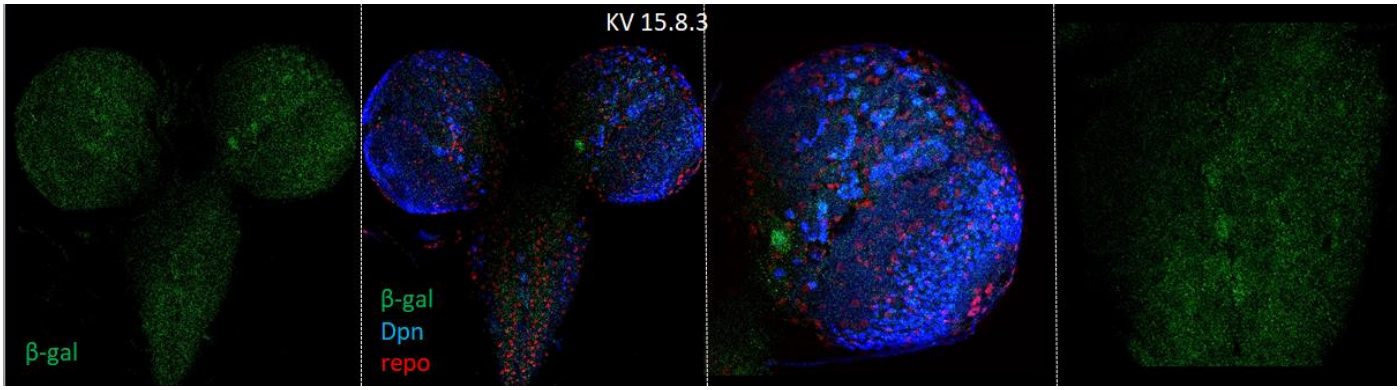


Figure 29: Pattern of expression in CNS of 3rd instar larva with inserted *nervy1* enhancer upstream of lacZ reporter. A) ventral b) dorsal view. The last two pictures in each are zooms. Stained with: rabbit anti-bgal (green), mouse anti-repo (red) and guinea pig anti-dpn (blue).

As we can observe, nervy1 enhancer is strongly expressed both in the central brain and the VNC. It is expressed in NBs but not NBs alone. Note that in the VNC it seems to be expressed in all segments (including the telson). Ventrally the signal is much weaker but still present, mostly in the central brain (**Sup. Fig. 4**).

a) ventral



b) dorsal

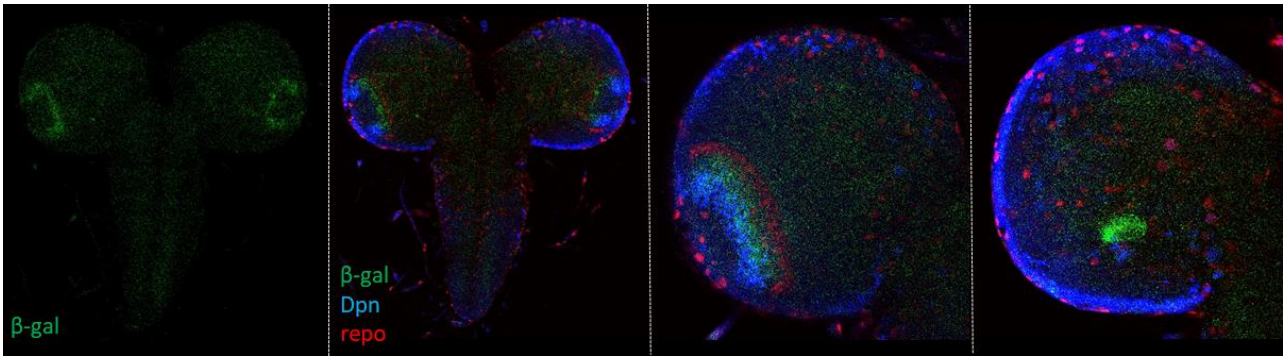


Figure 30: Pattern of expression in CNS of 3rd instar larva with inserted *nervy2* enhancer upstream of lacZ reporter. A) ventral b) dorsal view. The last two pictures in each are zooms. Stained with: rabbit anti-bgal (green), mouse anti-repo (red) and guinea pig anti-dpn (blue).

It is obvious that nervy2 is a much weaker enhancer than the previous ones. Ventrally, it is not expressed in the VNC and it has a weak but detectable expression in the outer proliferating center (OPC) of the optic lobe. Ventrally, there could be a very weak signal in the central brain and in a couple of cells in the VNC, but the signal is barely detectable.

Since nervy1 and nervy2 are enhancers of the same gene, they are comparable; the first one, in contrast with the second, is much stronger but it is not expressed in the optical lobe at all (**Sup. Fig. 5**).

4.1.6: Expression pattern of *embryonic lethal abnormal vision / appl* (enhancer KV16)

embryonic lethal abnormal vision (elav) is also a pan-neural gene which encodes a protein expressed in all neurons in the first stages of development and is essential for their differentiation. elav2 is a member of a protein family that binds in the RNA and play crucial role in post-transcriptional control, the Hu family (75). Note that this enhancer was in the 1st intron of appl gene, which is near to elav and that it was not bound by AS-C proteins, but by Dpn instead.

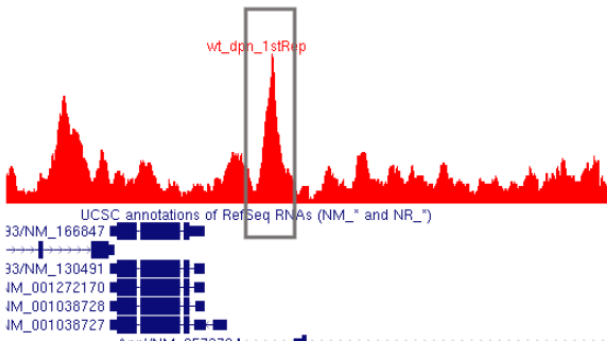


Figure 31 Results of ChipSeq analysis of TFs in neuroectoderm of embryos st. 8-11, performed by Vasiliki Theodorou. In the boxed sequence the most enriched binding of *Dpn* was observed and therefore it was selected for further analysis

a) ventral

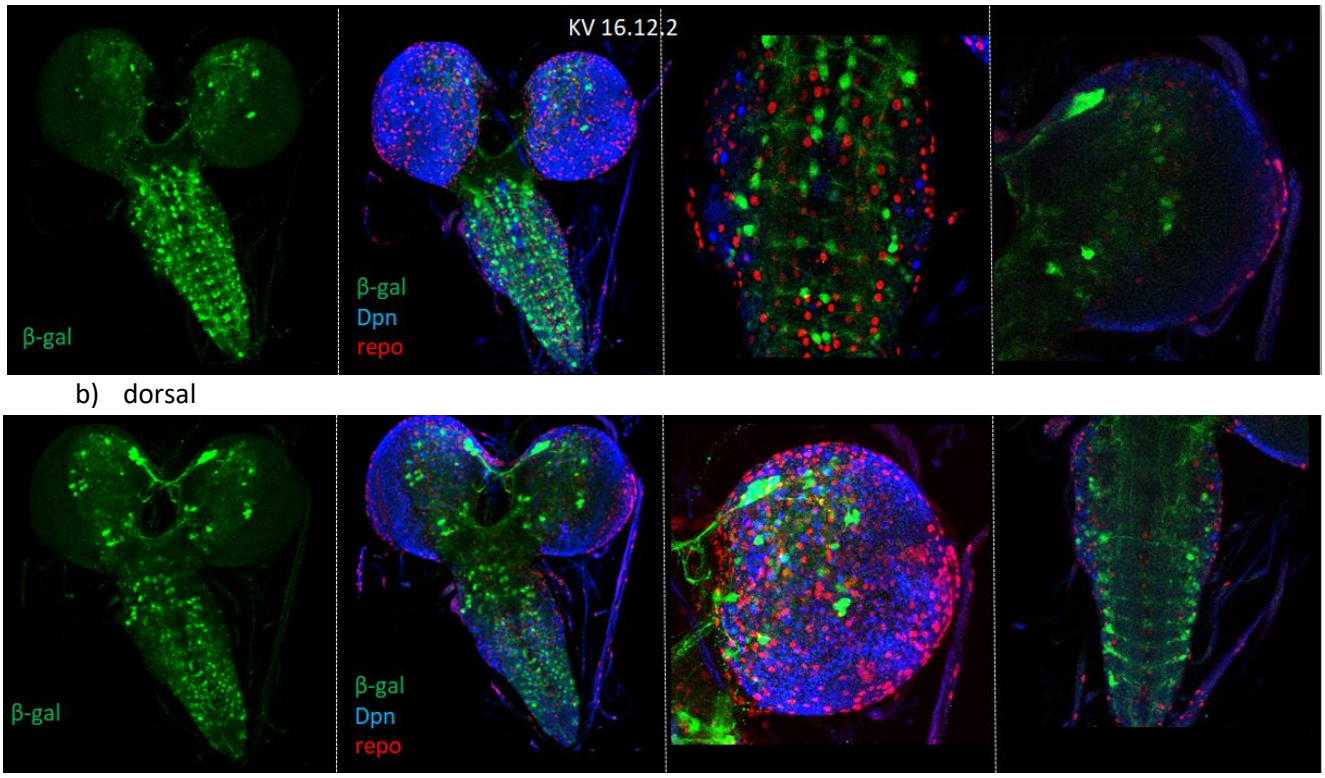


Figure 32: Pattern of expression in CNS of 3rd instar larva with inserted *elav2* enhancer upstream of lacZ reporter. A) ventral b) dorsal view. The last two pictures in each are zooms. Stained with: rabbit anti-bgal (green), mouse anti-repo (red) and guinea pig anti-dpn (blue).

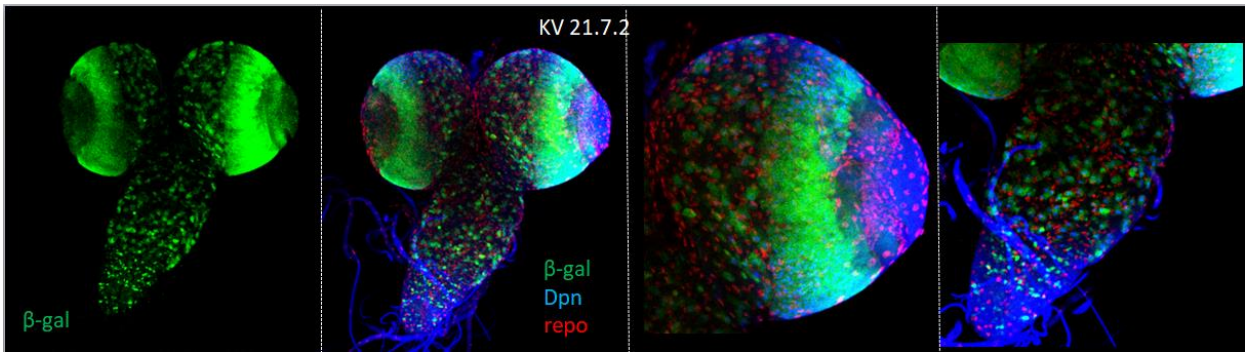
Here we can observe a rather different pattern of expression than the previous enhancers; elav/appl enhancer is expressed very strongly in a subset of the neurons, especially in the central brain and through all the length of the VNC. In the central brain it seems to be expressed in some other cell types as well, which, however, do not appear to be NBs (**Sup. Fig. 7**).

4.1.7: expression pattern of target of *Poxn* (enhancer KV21).

The protein encoded by the target of Poxn (tap) is a bHLH transcription factor which is expressed in a small population of neurons when they differentiate. It is expressed after the proneural genes and in mature neural cells in the CNS, just before they exit the cell cycle. In the PNS it is exclusively expressed in one of the neurons of chemosensory organs of the larva (76,77).

Figure 33: Results of ChipSeq analysis of TFs in neuroectoderm of embryos st. 8-11, performed by Vasiliki Theodorou. In the boxed sequence the most enriched binding of promeural TFs was observed and therefore it was selected for further analysis

a) ventral



b) dorsal

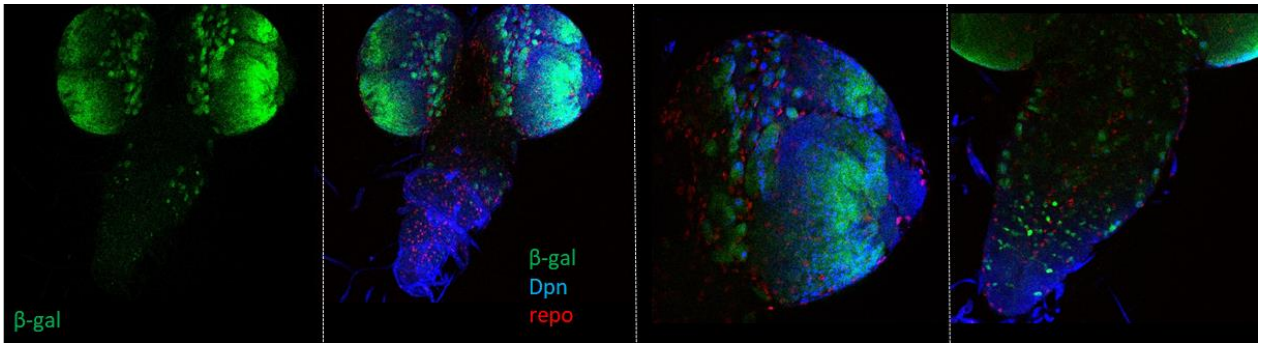


Figure 34: Pattern of expression in CNS of 3rd instar larva with inserted *tap* enhancer upstream of lacZ reporter. A) ventral b) dorsal view. The last two pictures in each are zooms. Stained with: rabbit anti-bgal (green), mouse anti-repo (red) and guinea pig anti-dpn (blue).

The enhancer of tap appears to be strongly expressed throughout the CNS: it is strongly expressed in the optic lobes, the central brain and all the VNC. It is expressed in NBs but not exclusively. It is also expressed in some VNC neurons, where βgal can be seen in their axon tracts (**Sup. Fig. 8**).

4.1.8: expression pattern of *Deadpan* (enhancer KV23).

Deadpan (dpn) is a pan-NB gene expressed almost in every developing NB of the CNS and some PNS precursor cells. It produces a bHLH transcription factor which is necessary for the self-renewal of the NBs and the repression of *Ase* when NBs exit quiescence. It is widely used as a NBs marker (78,79).

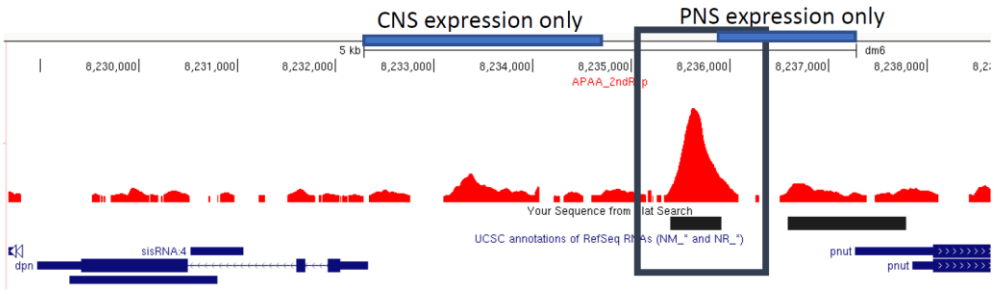


Figure 35: Results of ChipSeq analysis of TFs in neuroectoderm of embryos st. 8-11, performed by Vasiliki Theodorou. In the boxed sequence the most enriched binding of proneural TFs was observed and therefore it was selected for further analysis

a) ventral

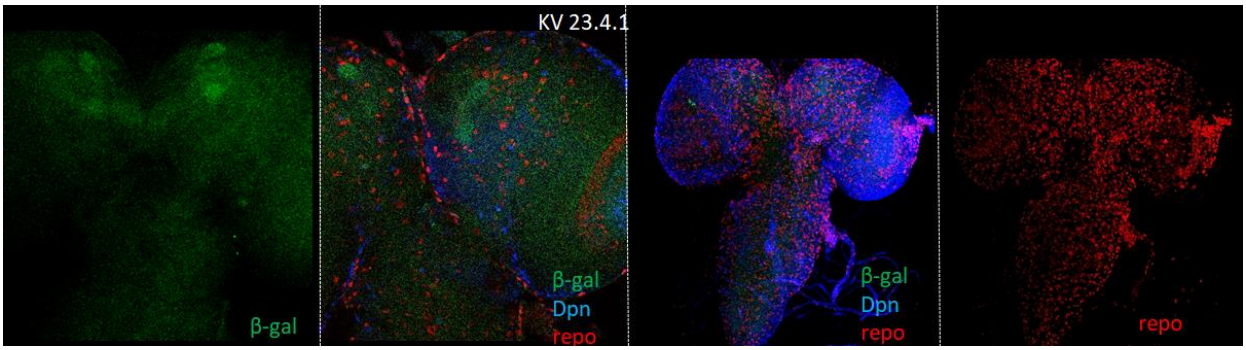


Figure 36: Pattern of expression in CNS of 3rd instar larva with inserted *Dpn* enhancer upstream of lacZ reporter. Dorsally no signal was detected and therefore only dorsal view is depicted. The first two pictures are zooms. Stained with: rabbit anti-bgal (green), mouse anti-repo (red) and guinea pig anti-dpn (blue).

Dpn enhancer KV23 appears to be very weakly expressed in only 4 regions of the central brain, which seem to be the mushroom body. It is not expressed in the VNC and ventrally it is not expressed anywhere. This specific enhancer has proved to be very strong in embryos in previous experiments and it could be only embryo specific (**Sup. Fig. 9**).

4.1.9: Expression pattern of *worniu* (enhancer KV29).

worniu (wor) is similar with scrt in the sense that they both belong in the snail family. The encoded proteins are zinc fingers TFs which participate actively in the development of the CNS. More specifically, wor is pivotal for the development of the brain since it is required for the asymmetrical division of the NBs (23).

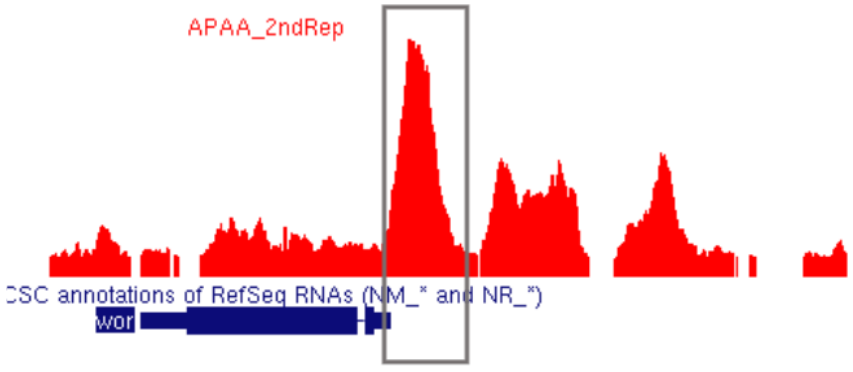
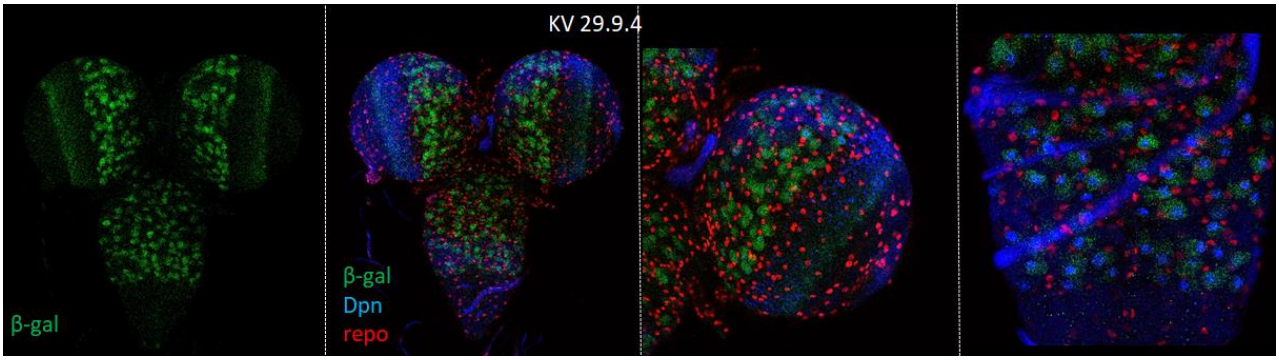


Figure 37: Results of ChipSeq analysis of TFs in neuroectoderm of embryos st. 8-11, performed by Vasiliki Theodorou. In the boxed sequence the most enriched binding of proneural TFs was observed and therefore it was selected for further analysis

A) Ventral



b) Dorsal

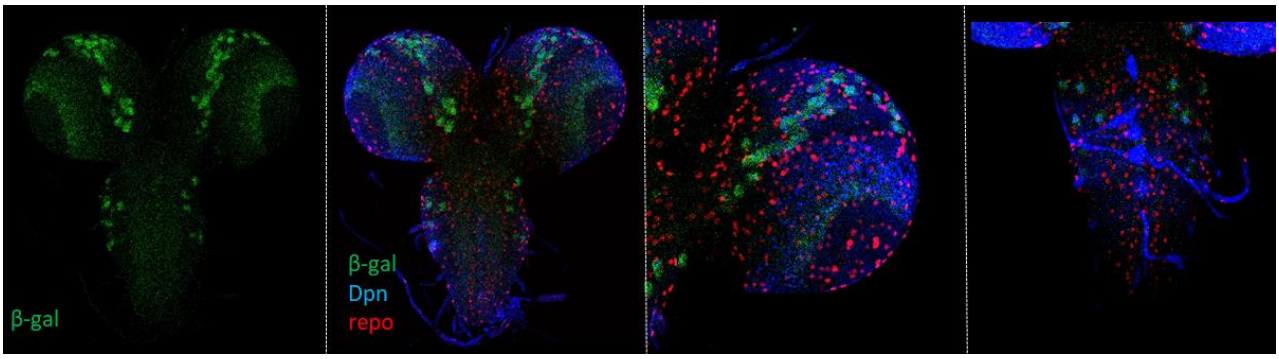


Figure 38: Pattern of expression in CNS of 3rd instar larva with inserted *wor* enhancer upstream of lacZ reporter. The last two pictures are zooms. Stained with: rabbit anti-bgal (green), mouse anti-repo (red) and guinea pig anti-dpn (blue).

This enhancer appears to be expressed in NBs specifically. It is expressed in the central brain, but in the optic lobe the signal is much weaker and more selective. In the VNC it is expressed in the gnathal and thoracic segments but not in the abdominal and the telson (**Sup. Fig. 10**).

In summary, the results of expression pattern of each enhancer in the larval CNS are presented in the table below.

		CENTRAL BRAIN		OPTIC LOBE		VNC	
Target gene	**KV enhancer**	**S.I.**	**Cell types**	**S.I.**	**Cell types**	**S.I.**	**Cell types**
insc	1.1.1	++	NBs, glia	+	NBs (?)	++	NBs, glia, other (gnathal, thoracic)
phyl	4.3.1	++	neurons	++	glia, other	+	other (abdominal, telson)
brat	8.22.1	+++	NBs and others	++++	NBs (?)	++	NBs (gnathal, thoracic)
scrt2	10.17.1	++++	NBs	-		++++	V: NBs, D: NBs, other (gnathal, thoracic)
nvy1	14.16.1	++++	NBs, other	-		++++	NBs, other (everywhere)
nvy2	15.8.3	-		+	NBs (?), OPC	-	
Appl, elav	16.12.1	++++	Neurons, axons	-		++++	Neurons (everywhere)
scrt1	19.15.1	-		-		-	
tap	21.7.2	+++	NBs (all), weak brain commissural neurons	++++	NBs (?)	+++	NBs and other (everywhere), NBs and some glia (abdominal)
dpn	23.4.1	++	Mushroom body	-		-	
wor	29.9.4	++	NBs	+	some NBs		NBs and other (gnathal, thoracic)

Table 1: Results of enhancers' expression pattern in 3rd instar larva in the CNS. OPC: outer proliferating center, S.I: signal intensity, ("+" to "++++" corresponds to "low" to "very strong", "-" means not detected), parenthesis in VNC column: segments of expression.

We can conclude that, as expected, the expression patterns varies from enhancer to enhancer, both regarding the anatomic regions of the CNS and their subdivisions, and the cell types where they are expressed.

Please note that no quantification of signal intensity has been made and therefore the scale is entirely indicative

4.2: Expression pattern of enhancers in wings discs of 3rd instar larva of D. Melanogaster.

Discs are another tissue where expression of AS-C genes is known to take place. Again, we wanted to see whether the enhancers were expressed in the presence of these genes. Since wing discs are a much thinner structure than the CNS, vertical and dorsal view will not be presented separately. Note that in contrast with larval CNS, here *Dpn* will not be detected; Dpn was used as a NBs marker and NBs are the precursors of the CNS. Disks will give rise to the PNS and therefore, Sensory Organ Precursors (SOPs) will be detected, which express *ase* but not *Dpn*. Regarding the antibodies, anti-βgal, anti-cut and anti-sensless were used. B-gal indicated, as before, the expression of the enhancer. *cut* is a TF present in the SOPs of the external sensory organs (ESOs) which are mechano- and chemosensory bristles. It is also present in the wing margin as in muscular precursor cells located below the notum primordium (70). Its expression is also *ase/sc* dependent in the SOPs. Note that is expressed form the SOP stage to the final division of those cells. *senseless (sens)* is another TF that stimulates the expression of proneurals and it is necessary and sufficient for the development of the PNS. It is expressed in all the SOPs, ESOs and ISOs (internal sensory organs), like organs of the dorsal radius and it does not stay active after mitotic divisions. For information of each enhancer gene read section 4.1.

4.2.1: Expression pattern of inscutable (enhancer KV1)

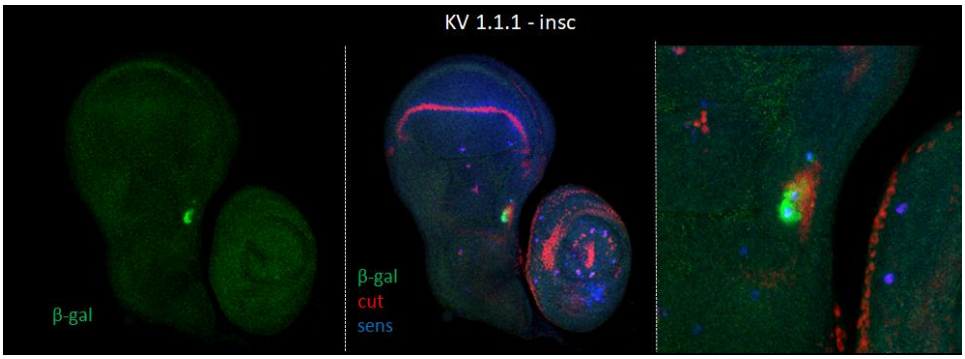


Figure 39: Pattern of expression in wing disc of 3rd instar larva with inserted insc enhancer upstream of lacZ reporter. The last picture is zoom. Stained with: rabbit anti-bgal (green), mouse anti-cut (red) and guinea pig anti-sens (blue).

The enhancer appears to be expressed only in two *sens*$^{+}$ cells, and therefore SOPs, in the notum-wing junction, called tendula (**Sup. Fig. 11**).

4.2.2: Expression pattern of phyllopod (enhancer KV4).

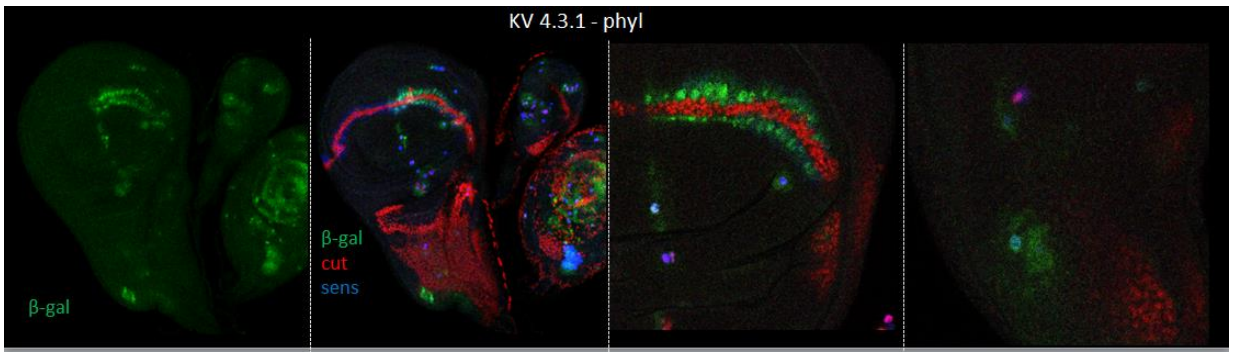


Figure 40: Pattern of expression in wing disc of 3rd instar larva with inserted *phyl* enhancer upstream of lacZ reporter. The last two pictures are zoom. Stained with: rabbit anti-bgal (green), mouse anti-cut (red) and guinea pig anti-sens (blue).

We can observe that the phyl enhancer appears to be rather active in the wing disc; it is expressed in SOPs of two rows of chemeosensory organs above and below the wing margin, in dorsal radius (hinge), in the L3 vein (between the DR and the WM), in a few cells on the wing pouch outside the WM and a few in notum (**Sup. Fig. 12**).

4.2.3: Expression pattern of brain tumor (enhancer KV8).

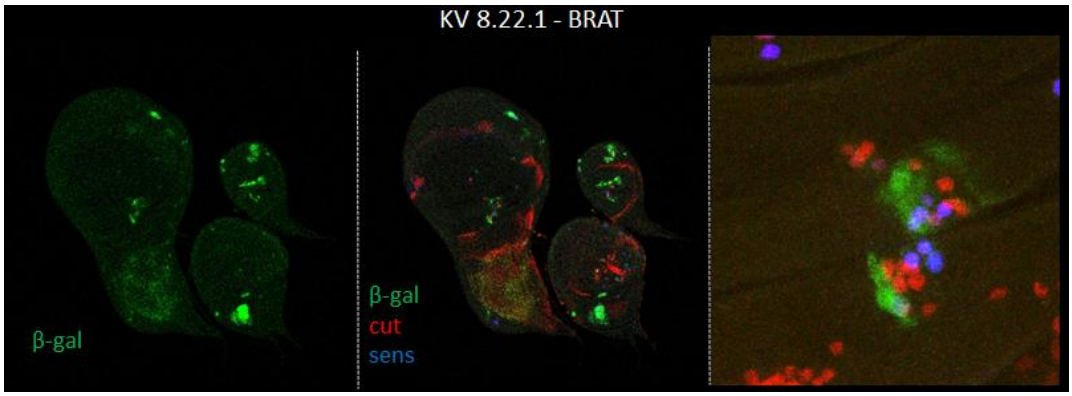
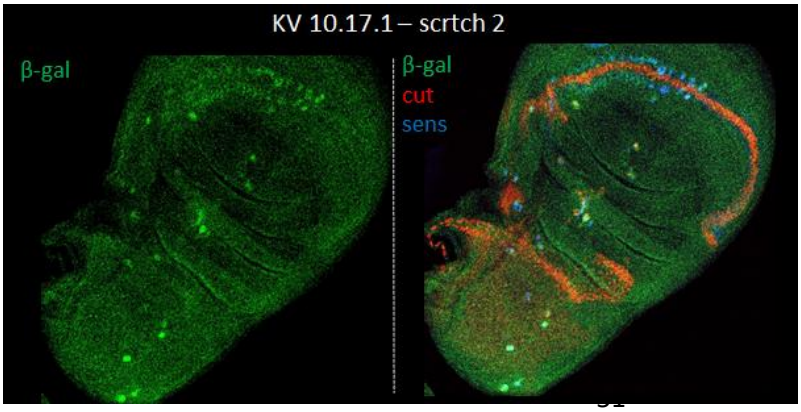


Figure 41: Pattern of expression in wing disc of 3rd instar larva with inserted *brat* enhancer upstream of lacZ reporter. The last picture is zoom. Stained with: rabbit anti-bgal (green), mouse anti-cut (red) and guinea pig anti-sens (blue).

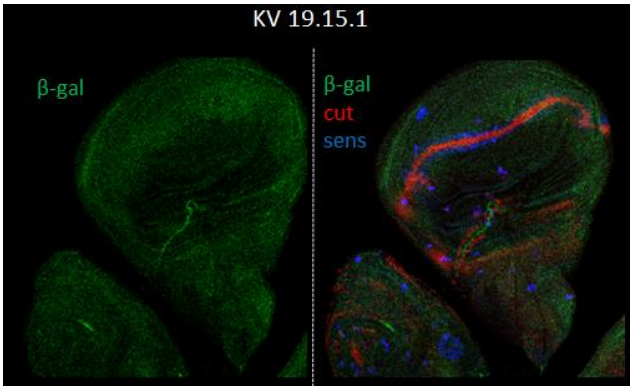
Here, the brat enhancer is strongly expressed in some SOPs, in the dorsal and ventral radius, in the wing margin SOPs and the twin sensillum of the margin (TSM) in the dorsal wing blade. Some cells appear to have lacZ expression but without being *sens*$^{+}$ (zoomed picture on the right). That could be explained if their nucleus is in different position from the cytoplasm due to the natural bending of the disc. However, most likely these cells are dividing ones, and since *sens* becomes inactive after the division, that could explain the reason why *sens* is inactive in the one cell (daughter cell) and has a very weak signal in the other (maternal cell) (**Sup. Fig. 13**).

4.2.4: Expression pattern of scratch1 & scratch2 (enhancers KV10 & KV19).



Scratch2 enhancer seems to be vividly expressed in all SOPs (**Sup. Fig. 14**).

Figure 42: Pattern of expression in wing disc of 3rd instar larva with inserted *scratch2* enhancer upstream of lacZ reporter. Stained with: rabbit anti-bgal (green), mouse anti-cut (red) and guinea pig anti-sens (blue).



On the contrary, the scratch1 enhancer does not seem to be expressed at all in the wing disc.

Figure 43: Pattern of expression in wing disc of 3rd instar larva with inserted *scratch1* enhancer upstream of lacZ reporter. Stained with: rabbit anti-bgal (green), mouse anti-cut (red) and guinea pig anti-sens (blue).

4.2.5: Expression pattern of nervy1 & nervy2 (enhancers KV14 & KV15).

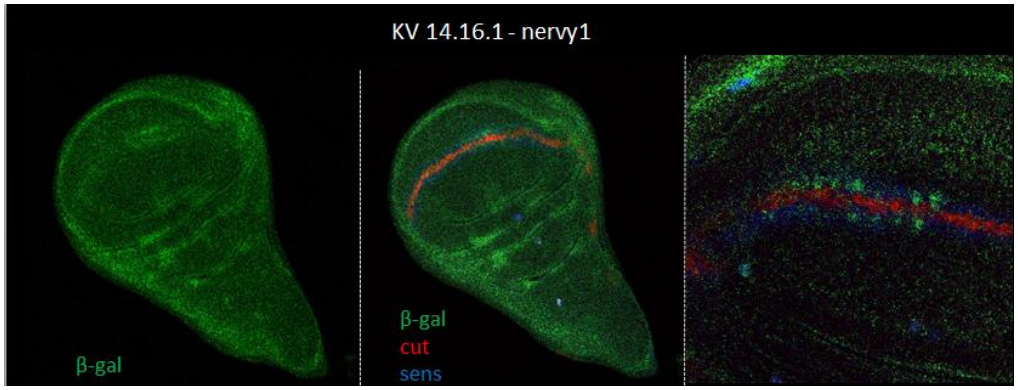


Figure 44: Pattern of expression in wing disc of 3rd instar larva with inserted *nervy1* enhancer upstream of lacZ reporter. The last picture is zoom. Stained with: rabbit anti-bgal (green), mouse anti-cut (red) and guinea pig anti-sens (blue).

The nervy1 enhancer presents a weak signal of expression in the SOPs above and below the wing margin and in a few other SOPs, in the notum (**Sup. Fig. 15**).

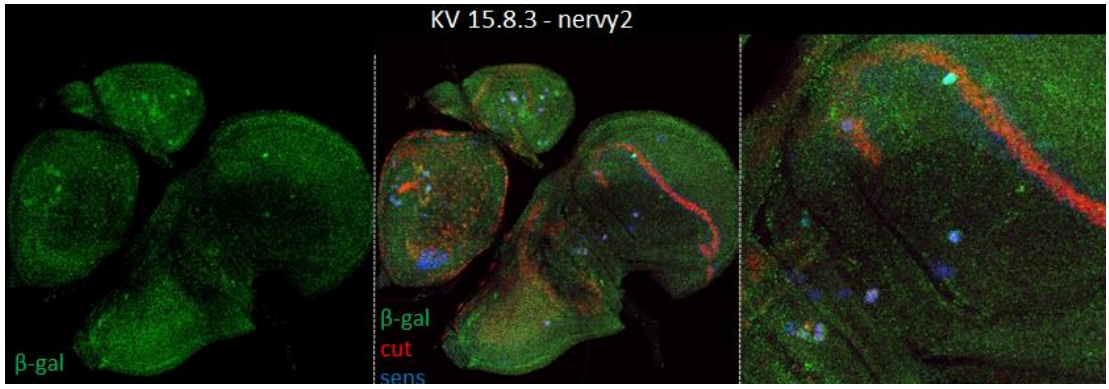
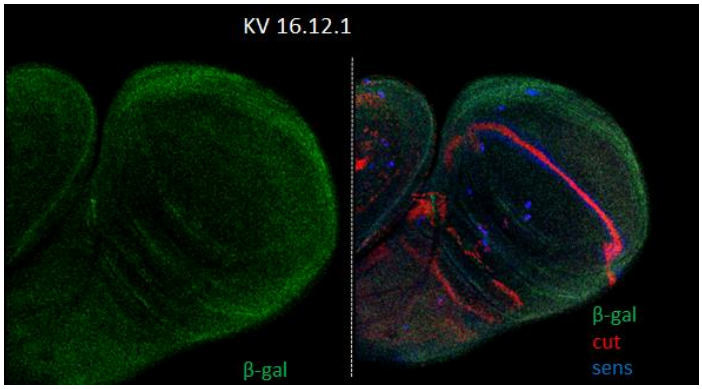


Figure 45: Pattern of expression in wing disc of 3rd instar larva with inserted *nervy2* enhancer upstream of lacZ reporter. The last picture is zoom. Stained with: rabbit anti-bgal (green), mouse anti-cut (red) and guinea pig anti-sens (blue).

The nervy2 enhancer is expressed in the tegula and the central thorax. It is active in some SOPs (**Sup. Fig. 16**).

4.2.6: Expression pattern of elav / appl (enhancers KV16).



The elav/appl enhancer does not seem to be expressed in the wing disc. This result is logical considering the fact that it is expressed in neurons, which, in PNS appear in the pupa stage. Maybe these are precursor cells that do not express it yet.

Figure 46: Pattern of expression in wing disc of 3rd instar larva with inserted *scratch1* enhancer upstream of lacZ reporter. Stained with: rabbit anti-bgal (green), mouse anti-cut (red) and guinea pig anti-sens (blue).

4.2.7: Expression pattern of target of poxn (enhancer KV21).

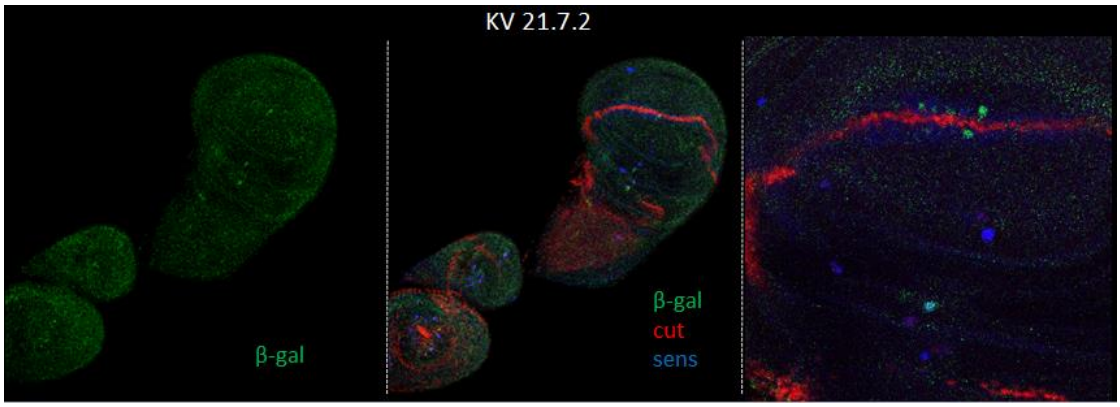
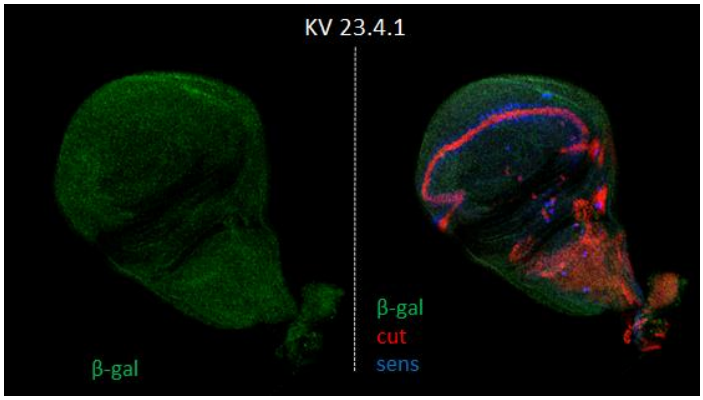


Figure 47: Pattern of expression in wing disc of 3rd instar larva with inserted *tap* enhancer upstream of lacZ reporter. The last picture is zoom. Stained with: rabbit anti-bgal (green), mouse anti-cut (red) and guinea pig anti-sens (blue).

The tap enhancer appears to be expressed in SOPs above and below the wing margin and in dorsal and ventral radius SOPs (**Sup. Fig 17**).

4.2.8: Expression pattern of Dpn (enhancer KV23).



The expression of the Dpn enhancer is not detected, so it is not expressed in the imaginal discs.

Figure 48: Pattern of expression in wing disc of 3rd instar larva with inserted *Dpn* enhancer upstream of lacZ reporter. Stained with: rabbit anti-bgal (green), mouse anti-cut (red) and guinea pig anti-sens (blue).

4.2.9: Expression pattern of worniu (enhancer KV29).

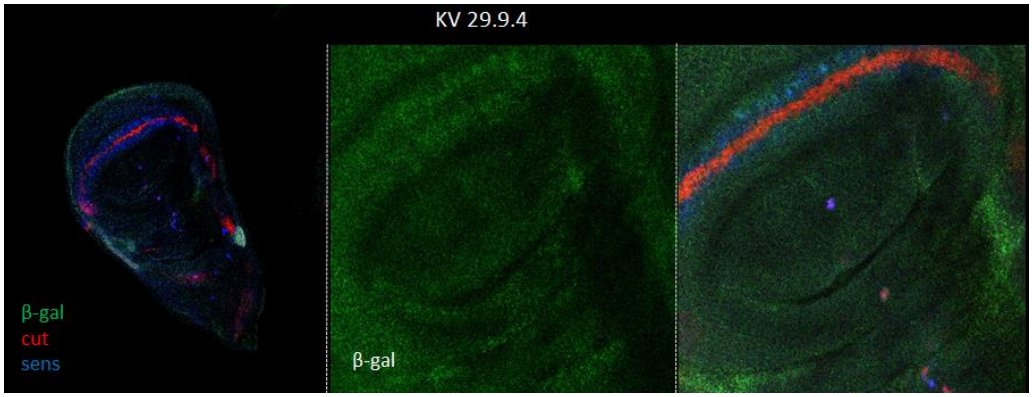


Figure 49: Pattern of expression in wing disc of 3rd instar larva with inserted *wor* enhancer upstream of lacZ reporter. The last picture is zoom. Stained with: rabbit anti-bgal (green), mouse anti-cut (red) and guinea pig anti-sens (blue).

This enhancer seems to be expressed only in one cell that is also *sens*$^{+}$, above the wing margin (**Sup. Fig. 18**).

In summary, the results of expression pattern of each enhancer in the PNS are presented in the table below:

Table 2: Results of enhancers' expression pattern in wing disc of 3rd instar larva. S.I: signal intensity ("+" to "++++" corresponds to "low" to "very strong", "-" means not detected), parenthesis: region of expression. CSO: chemosensory organs, MSO: mechanosensory organs. SOPs: sensory organ precursors.

Target gene	KV enhancer	S.I.	Cell types
insc	1.1.1	++++	SOPs (only 2 cells in tegula)
phyl	4.3.1	+++	SOPs: CSOs (2 rows of above and below the wing margin) MSOs, hinge, L3 vein, wing pouch, notum
brat	8.22.1	++++	SOPs (dorsal and ventral radius, wing margin, twin sensillum in dorsal blade)
scrt2	10.17.1	+++	All SOPs
nvy1	14.16.1	+	SOPs (wing margin, notum)
nvy2	15.8.3	++	some SOPs (tegula, central thorax)
appl, elav	16.12.1	-	
scrt1	19.15.1	-	
tap	21.7.2	++	SOPs (wing margin, dorsal and ventral radius)
dpn	23.4.1	-	
wor	29.9.4	+	SOP (only one, above the wing margin)

Most of the enhancers are expressed in the wing disc in various regions mainly in SOPs (except scrt2 which is also expressed in muscular cells).

Please note that no quantification of signal intensity has been made and therefore the scale is entirely indicative

4.3: Expression pattern on enhancers in adult gut of *D. melanogaster*.

Another tissue that was studied for its expression pattern of the enhancers in interest was the adult gut. Here, it is known that sc is necessary of the production of enteroendocrine cells (32). The gut consists of five type of cells; intestine stem cells (ICSs), enteroblasts (EB) that give rise to enterocytes (EC) and eneteroednocrine precursors (EEP) that give rise to two different types of enteroendocrine cells (EE). EC are distinct due to their big size and their big nucleus, since they are polyploid, and EE are marked with *pros*. ICSs have a triangular shape. In the staining performed in the dissected tissues anti-βgal was used to detect the expression of enhancers, Dapi to mark the nuclei and *pros* to mark the EE and EEP.

4.3.1: Expression pattern of phyllopod (enhancer KV4).

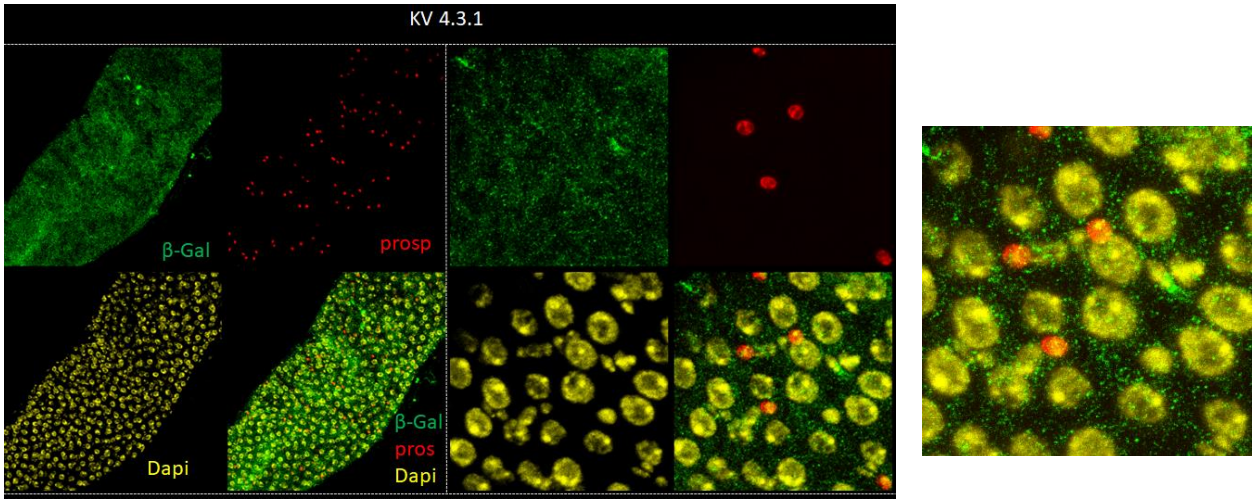


Figure 50: : Pattern of expression in adult gut with inserted *phyl* enhancer upstream of lacZ reporter. The middle and last pictures are zoom. Stained with: rabbit anti-bgal (green), mouse anti-prosp (red) dapi (yellow).

The phyllopod enhancer seems to be active in a few cells of the adult gut which are prosp- and therefore are not enteroendocrine cells. It is not expressed in cells with large nuclei (EC) either so we can't be sure for the cell type that it is expressed (further antibodies should be used).

4.3.2: Expression pattern of brat (enhancer KV8).

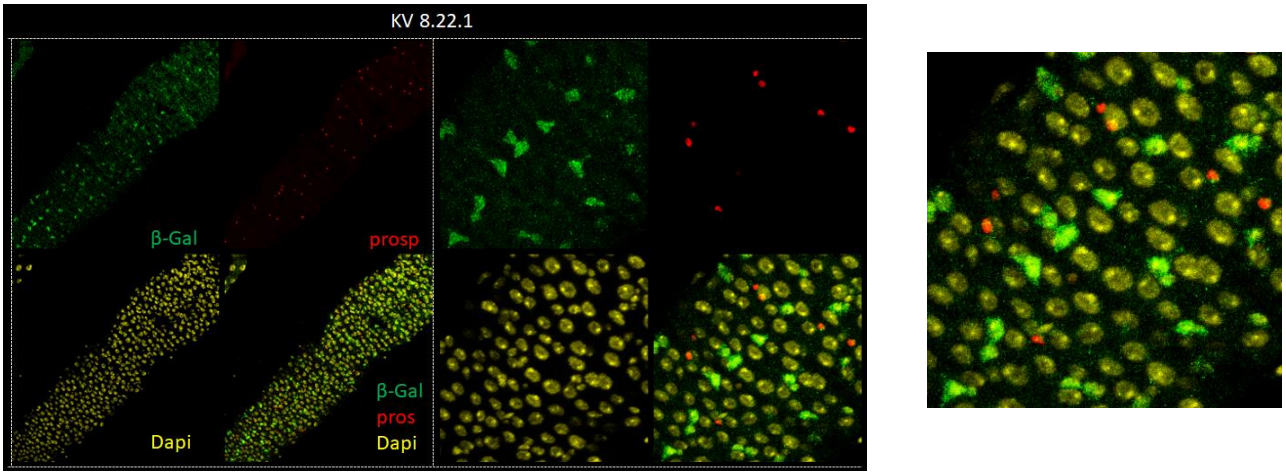


Figure 51: Pattern of expression in adult gut with inserted *brat* enhancer upstream of lacZ reporter. The middle and last pictures are zoom. Stained with: rabbit anti-bgal (green), mouse anti-prosp (red) dapi (yellow).

The Brat enhancer seems to be more vividly and widely expressed in the adult gut. The cells that have β-gal signal have a characteristic triangular shape are *pros* negative and therefore they probably are ISCs.

4.3.3: Expression pattern of scratch (enhancer KV19).

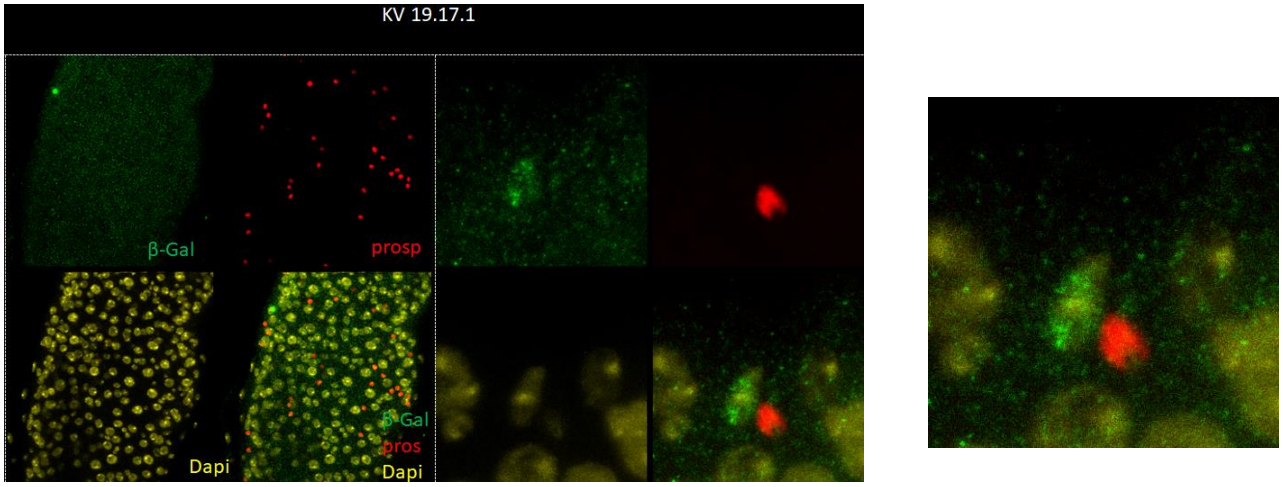


Figure 52: Pattern of expression in adult gut with inserted *phyl* enhancer upstream of lacZ reporter. The middle and last pictures are zoom. Stained with: rabbit anti-bgal (green), mouse anti-prosp (red) dapi (yellow).

The scrtch1 enhancer is mildly expressed in the gut. A dividing cell is depicted in zoom. Since it has neither big nucleus nor prospero, it could be ISC but we can't be sure via the present staining. .

4.3.4: Expression pattern of Dpn (enhancer KV23).

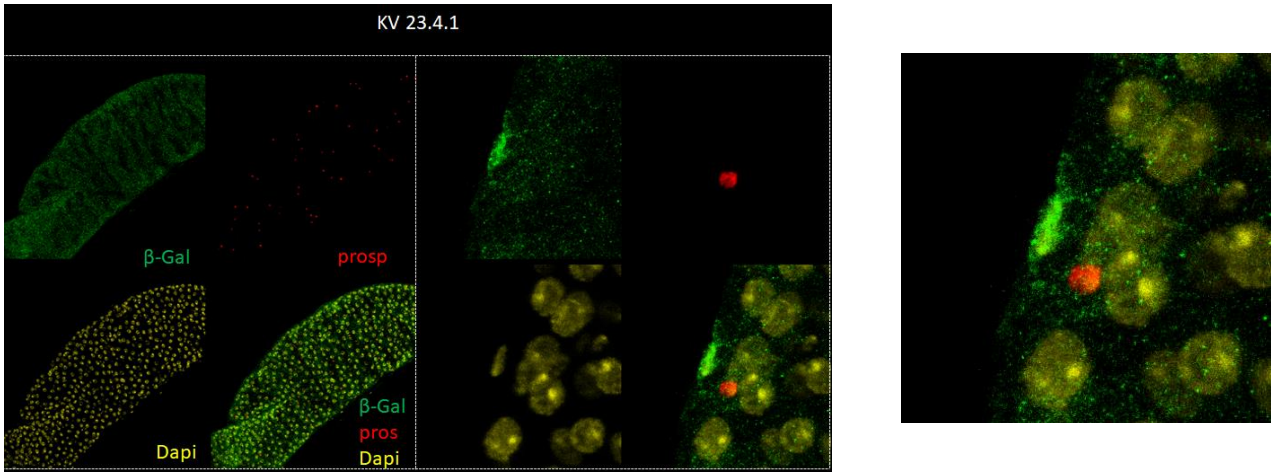


Figure 53: Pattern of expression in adult gut with inserted *Dpn* enhancer upstream of lacZ reporter. The middle and last pictures are zoom. Stained with: rabbit anti-bgal (green), mouse anti-prosp (red) dapi (yellow).

Finally, the Dpn enhancer is expressed but even less than the scratch1 enhancer. Signal was found in merely one cell that is in the end of the gut and therefore it is not visible as a whole due to the natural folding. As a consequence, no speculation of the type of the cell can be made.

4.4: Expression pattern of the KV enhancers in *Df(1)sc^{19}* embryos.

As previously mentioned, the enhancers, in which proneural TFs bind, were first identified by Chip-seq in Drosophila embryos and then the KV lines were created. The choice of these specific enhancers for creation of the transgenic flies was based in part on the proneural dependency the nearby gene expression displayed in AS-C mutant embryos. The expression pattern of each enhancer had been studied in WT embryos and in embryos lacking the whole AS-C (*Df(1)sc^{B57}*). For the latter, the crosses that were used were the following: *Df(1)*sc^{B57}/FM7Kr>GFP x KV/KV. The endogenous GFP signal from Kr was used as a tool to detect the non-mutated embryos. In mutants lacking all the proneural genes of the complex, the expression of the enhancers was lost (with the exception of KV1 that rebounded in later stages) which meant that the proneural genes were necessary for the normal expression of the enhancer (**fig. 54**). Embryos of st. 10 were studied, since this is the stage where the S3 wave of NB appears, in which proneurals are required (although as it has been shown, in complete absence of the AC-S complex the embryo is not aneural, more information in chapter 1.5). The mutant embryos had "stalled" NBs which later on overcame this arrest, but they could not sustain their stem cell competence

and lead to hypoplastic nerve cord. It was concluded that proneurals are necessary for the proper initiation time of the neural stem cell program (V. Theodorou et al., submitted).

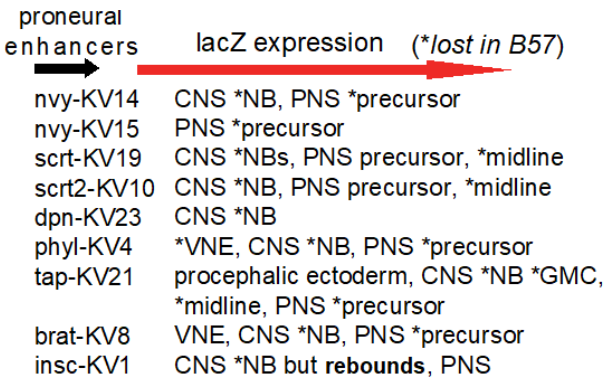


Figure 54: Expression of KV enhancers in embryo st. 10 in WT and B57 mutants. Unpublished data, image from V. Theodorou.

It is known that *ase* is a member of the AS-C complex but differs from the other proneurals (ac, sc, l'sc); it is not expressed in the embryonic ectoderm and it is only expressed in neural precursors, resembling the pan-NB genes, like *dpn* and *wor*. It is activated later in the development, right after the S1 phase, when the other proneurals cease to be expressed, being activated downstream of them. Also, it has been shown that *ase* can bypass the requirement of *ac* and *sc* for the formation of imaginal sense organs (31) and central NBs as well (Theodorou et al., submitted). The question that was raised in the present study was whether the expression of these enhancers would be altered from $Df(1)sc^{B57}$ in the presnsce of *ase*. In other words, we wanted to investigate if $Df(1)sc^{B57}$ displayed a different expression pattern than $Df(1)sc^{19}$ embryos, which lack *ac*, *sc* and *l'sc* but *ase* is present (**fig. 55**).

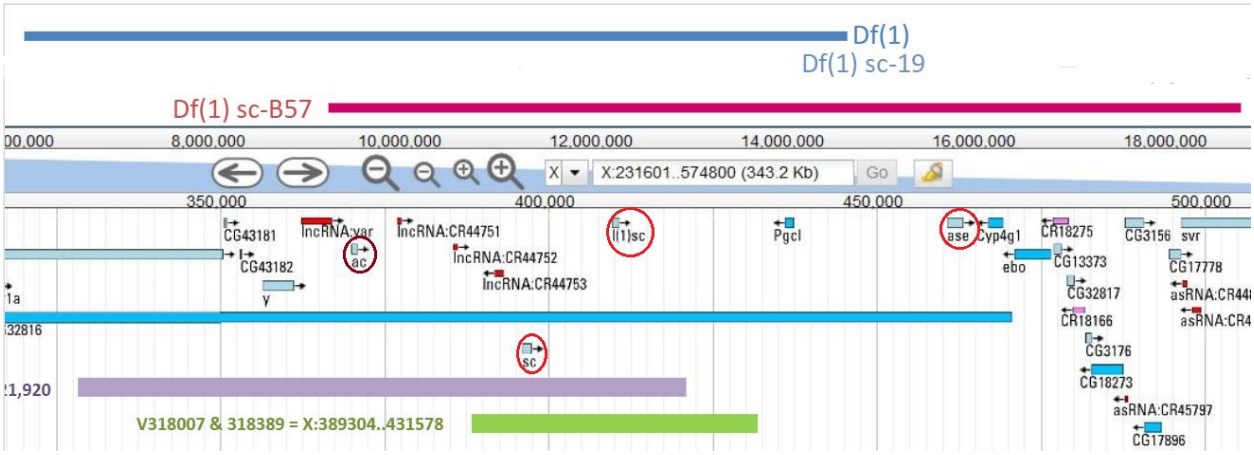


Figure 55: genetic map of AS-C genes and the mutations B57 and sc19.

For that reason, the following crosses were used: sc19 / FM7Kr>GFP x KV/KV. Again, the expression of GFP is used to determine the non-mutated embryos. The antibodies that were used are the following: goat anti-GFP, rabbit anti-βgal (detects the enhancer expression) and mouse anti-sxl (X-dosage dependent gene, expressed only in females). Females will be heterozygous for sc19, since it is located in

the X chromosome, whereas males negative for KrGFP will be hemizygous mutants. The crosses were performed for three KV lines and the results are presented below.

For comparison, the results of sc19 vs WT will be presented next to the B57 vs WT (images from V. Theodorou). The antibodies used in the B57 stainings were the following: anti-βgal to detect enhancer expression, anti-ase to detect the presence of proneurals and anti-prospero to mark the GMCs.

Experiments for three enhancers were performed: phyl (KV4), brat (KV8) and tap (KV21). The rest will be completed in the future.

4.4.1: Expression pattern of *phyl* KV4 enhancer in B57 and sc19 embryos.

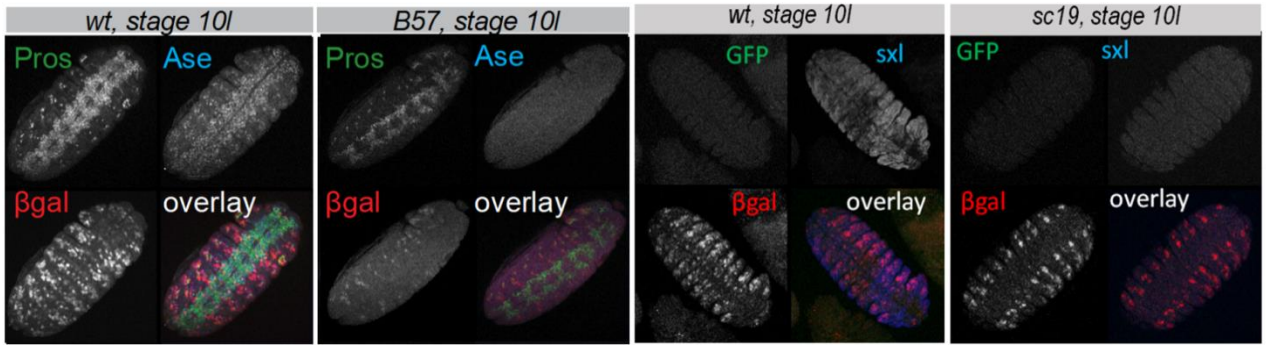


Figure 56: Expression pattern of *phyl* enhancer in WT, B57 and sc19 embryos. First two pictures stained with: anti-pros (GMCs) anti-ase (presence or absence of the AS-C) and anti-βgal (enhancer expression). Last two pictures stained with: anti-GFP (to detect the presence of the FM7, Kr>GFP chromosome), anti-sxl (female embryos, non-mutated) and anti-βgal (enhancer expression). The first two pictures are sumbitted data of V. Theodorou et al.

In B57 embryos, the enhancer expression is drastically decreased. In sc19 however, there is a visible improvement; in the PNS it is activated but in the CNS it remains inactive (it still is less than WT embryos). Therefore, we can conclude that *ase* does bind in this specific enhancer and its expression level and localization seems to be dependent on the presence of *ase* specifically in PNS precursors.

4.4.2: Expression pattern of brat KV8 enhancer in B57 and sc19 embryos.

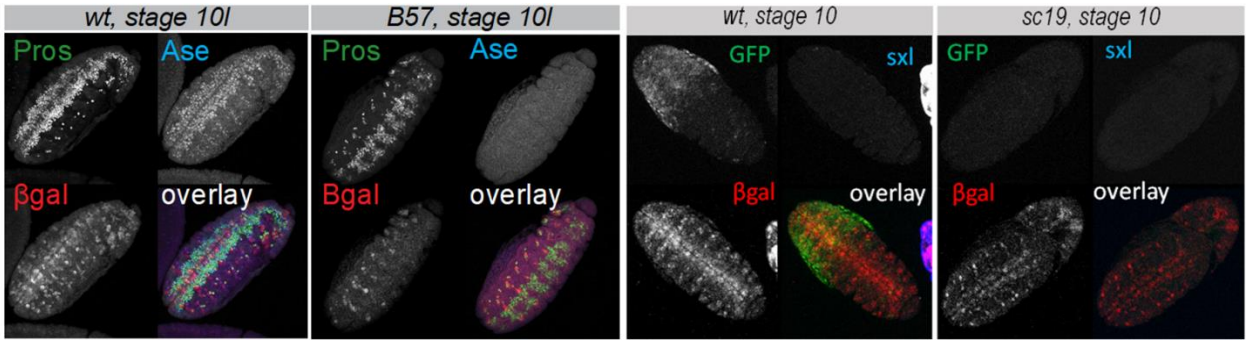


Figure 57: Expression pattern of *brat* enhancer in WT, B57 and sc19 embryos. First two pictures stained with: anti-pros (GMCs) anti-ase (presence or absence of the AS-C) and anti-βgal (enhancer expression). Last two pictures stained with: anti-GFP (to detect the presence of the FM7, Kr>GFP chromosome), anti-sxl (female embryos, non-mutated) and anti-βgal (enhancer expression). The first two pictures are submitted data of V. Theodorou et. al.

Regarding the expression of *brat* enhancer, we can see that in B57 is greatly reduced whereas in sc19 it is present both in CNS and PNS which means that the presence of *ase* is adequate for the enhancer to be expressed, almost to its full extent; the number of cells in the PNS is significantly reduced whereas in CNS almost no effect is observed. GMCs numbers are reduced in both mutants.

4.4.3: Expression pattern of tap KV21 enhancer in B57 and sc19 embryos.

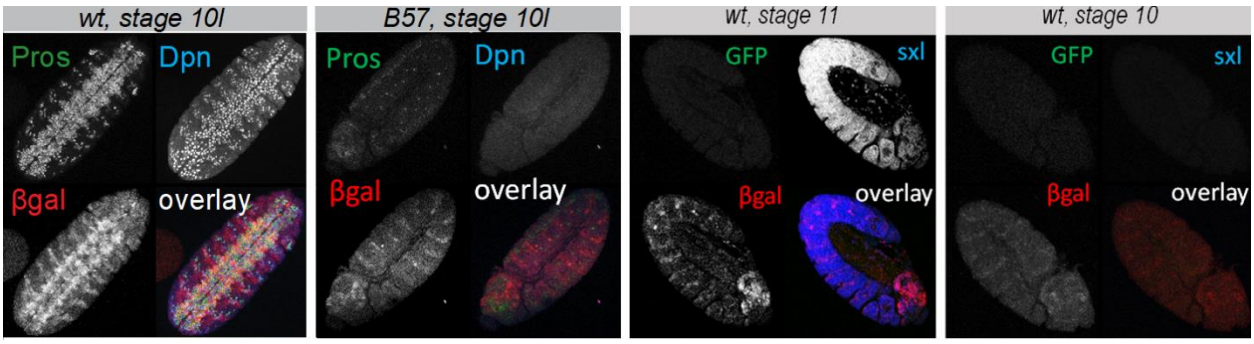


Figure 58: Expression pattern of poxn enhancer in WT, B57 and sc19 embryos. First two pictures stained with: anti-pros (GMCs) anti-ase (presence or absence of the AS-C) and anti-βgal (enhancer expression). Last two pictures stained with: anti-GFP (to detect the presence of the FM7, Kr>GFP chromosome), anti-sxl (female embryos, non-mutated) and anti-βgal (enhancer expression). The first two pictures are submitted data of V. Theodorou et. al.

The *tap* enhancer KV 21 in B57 embryos is much less expressed compared with the WT ones. In sc19, we can see that it is expressed in the brain detectably and weakly in the rest embryo but compared to B57 no big difference is observed. However the staining was not very successful and we cannot draw any conclusions as to whether *ase* binds in this enhancer or not – the experiment should be repeated.

In the following table are summed up the results for all the enhancers, tissues and embryos examined. "-"represents experiments not executed, the check-mark marks the presence of expression of the enhancer in question and "x"for its absence.

Table 3: Summary of the expression of the enhancers in the examined tissues and sc19 embryos.

Target genes	KV enhancers	LARVA CNS	LARVA DISCS	ADULT GUT	Embryo sc19
insc	1.1.1	✓	✓	-	-
phyl	4.3.1	✓	✓	?	✓ (PNS only)
brat	8.22.1	✓	✓	✓	✓
scrt2	10.17.1	✓	✓	-	-
nerv1	14.16.1	✓	✓	-	-
nerv2	15.8.3	✓	✓	-	-
appl, elav	16.12.1	✓	x	-	-
scrt1	19.15.1	x	x	✓	-
tap	21.7.2	✓	✓	-	?
dpn	23.4.1	✓	x	?	-
wor	29.9.4	✓	✓	-	-

5. Discussion

It is known that proneurals tightly regulate the differentiation of the nervous system in *D. melanogaster*. They are thought to act as activators of the next level of genes, like the pan-NB genes in the CNS and the pan-SOP genes in the PNS. Several of these genes have been shown to contain ASC-dependent enhancers. Previous experiments conducted in the lab showed 10 regions that act as enhancers where proneurals bind and transgenic flies with these enhancers driving a reporter gene were produced. In embryos lacking all of the genes of the AS-C ($Df(1)sc^{B57}$) the expression of these enhancers was highly reduced or eliminated in the CNS primordium. The proteins encoded by some of the nearby genes were accordingly initially absent, but were expressed later on (scrt, wor, tap). This means that these genes are activated by other transcriptions factors as well which seem to function later on. These findings together indicate that the genes of this complex are necessary for the correct spatiotemporal expression of these enhancers and the timely activation of the according genes. Here we showed that one out of two enhancers in which *sc, l'sc, ac* were depleted but *ase* was present ($Df(1)sc^{19}$) showed expression that was lost in B57 embryos, suggesting that the presence of *ase* alone is sufficient to bypass the other AS-C genes that are previously expressed and activate KV8 expression.

In the first part of the study, tissues from different developmental stages (laval CNS, discs and adult gut) in which the presence of AS-C TFs has been documented, were examined for these enhancers' expression. The results showed that most of them are very widely expressed in all the tissues tested whereas a small minority is expressed in larval CNS but not in discs. This strengthens the hypothesis that these enhancers are AS-C dependent not only in embryo, but throughout the development of *D. melanogaster* and could be part of the mechanism through which proneurals control the differentiation in the level of chromatin. Further expreriments need to be performed to esablish this theory; all of the enhancers should be tested in gut as we did in the larval CNS and discs. Also, it would be interesting to see if the protein product is depleted / stalled in these tissues and developmental stages in the absence of the AS-C to see if there is a direct connection between the complex's genes and the proper spatiotemporal expression of the enhancer's protein product, as it is in embryos. For this experiment, clones of cells in which the depletion happens selectively in specific time and in some controlled regions should be made, since the B57 embryos are not viable and will never reach the next developmental stages.

At this point, it would also be interesting to raise the question whether the cells in which these enhancers seem to be active in embryos are related with the cells in which the enhancers are active in larva or, in other words, if these cells are related by lineage. Could it be that the same cells that we see the enhancer expression in the embryo brain are the ones with the enhancer expression in the larval CNS? If that is indeed the case, we could monitor through live imaging or tracing whether the enhancers that are activated in embryos remain active throughout the development, up to the larval tissues or if they stop being expressed at some point and are re-activated later on. Here we should also note that GFP that was used as a marker of the enhaver's expression has the disadvantage of being detected even after the gene expression has stopped – the protein has a half-life of 26h and also the folding to its active fluorescent form is fairly slow and occurs in the order of hours, making it thus an unsuitable marker for real time observation and fast transcriptional activation (80).

The role of proneurals varies in different chromatin backgrounds (embryo ectoderm, embryo mesoderm, disk epithelium, larval optic lobe ectoderms, larval NBs, adult intestine) and leads to different cell fates, depending on the tissue and the time they are expressed. However, different combinations of gene members of the AS-C seem to be expressed in the different tissues/cells; the larval OL express l'sc, sc and ase but not ac, larval type I NBs express ase, imaginal discs express ac, sc and ase but not l'sc and in adult gut only sc and ase have been detected. Maybe this different combination of these proneurals that act as TFs enables them to access different part of chromatin and induce different cell fates.

In addition, in this study we showed that different enhancers seem to be bound by proneurals in different developmental stages and cell types, therefore being active in different chromatin context. The common that these enhancers had, was the binding of the proneurals. It would be interesting to see whether these enhancers bind other TFs or merely the presence of proneurals is enough for their activation. One way to determine that would be to created animals in which these binding site of the proneural TFs would be mutated and see whether the enhancer expression is still present. Another approach, would be bioinformatically; we could check the sequence of these enhancers, especially the promiscuously expressed ones (like KV8 and KV1), to determine what other TFs may bind. Recently a tool named TFcoop was created by Vandel et al (81) and it enables the discovery of the TFs combinations involved in the binding of many genetic regions, including enhancers, in different cell types. It needs a Chip-seq of a single TF (for example sc) and it can predict the combinations that this TF has with other identified TFs and the binding sites of the presumptive TF complex as well. This tool could

be useful to furhter elusidate the TF role of the proneural genes and see wheter they use the same cohort of enhancers in all the cell types where they are expressed. Another approach would be in vitro, cell-free gene assays to determine whether the presence of proneurals is sufficient for the activation of the gene they control; the enhancer of interest could be expressed upstream of luciferase gene and this tool can be used to check whether proneural TFs either alone or in different combinations can ativate the enhancer and thus the expression of the luciferase marker by fluorescence detection (82).

Furthermore, while conducting this study, preliminary data showed that the examined enhancers, especially the *brat* enhancer, were robustly and specifically expressed in gut cells that appear to be intestine stem cells. This should be futher investigated by stainings with ISCs specific antibodies (such as Delta) and if this indication is validated, we may have in our hands valueable new tools for marking the ISCs which has been proven tricky in the past.

Finally, the fact that almost all of these enhancers appear to require the presence of proneurals for their function and the effective activation of trancription of their gene targets, in such different tissues and developmental stages, focuses attention in the conserved epigenetic mehcanisms for successful differentiation throughout the development. We could use the binding sites of the proneural TFs detected in *D. melanogaster* embryos and align them within different species of Drosophilidae to see if they are conserved and up to what extent. Also, it is known that *scute* acts as an input-output gene; its expression is regulated by a complex mechanism of upstream genes, and *sc* on its turn controls a different number of genes which have simpler enhancers and activation (83). The enhancers detected and studied here could be the mechanism through which proneural TFs act in their output and determine differentiation in different developmental stages and cell types.

Aknowledgements

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