universität wien

MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

"RNAi screen for essential genes in the central complex in *Drosophila*"

verfasst von / submitted by

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angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien, 2021 / Vienna, 2021

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	UA 066 878
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Masterstudium Verhaltens-, Neuro- und Kognitionsbiologie
Betreut von / Supervisor:	Univ.-Prof. Dr. Thomas Hummel

Table of contents

1 Abstract

Symmetry can be found throughout wildlife from the physical appearance of big animals to the cytokinesis. However, if inspected closely small asymmetric features are visible through all living things, because perfect symmetry is only possible on paper. Even bigger asymmetric features can be observed across the body of animals like the inner organs of humans or extremities like the bigger claw of a fiddler crab. Brains across the animal kingdom are prone to asymmetry, just because functional asymmetry proves to have an benefit on signal transduction and specialization. This brain lateralization however is not completely understood and recent findings of an asymmetric structure the asymmetrical body (AB) in *Drosophila* could help to shed light onto the development of brain lateralization. This AB is a small neuropile, located between the fan-shaped body and the ellipsoid body in the central complex of the *Drosophila melanogaster* brain. It is structurally asymmetric in its size difference across both hemisphere with the neuropile on the right hemisphere being significantly larger. This seems to have a functional matter, as phenotypical mutant flies with same size neuropiles, also called "double AB", have a lack of long-term memory formation.

In this study a knockdown of candidates via RNAi was done to determine key genes involved in the development of the asymmetry of this AB. Most interestingly a "left" phenotype, which mirrored the wildtype phenotype through the brain midline and a "middle" phenotype, which had a innervation on the brain midline, could be observed. Neither of those phenotypes have been reported. Strikingly those phenotypes could be induced via knockdown in glia cells as well as in neurons. The AB seems to be essential for an adult fly, as no AB-absent phenotype could be observed.

A high penetrance of non-wildtype phenotypes could be observed in the knockdown of the genes *Wnt4* and *Tetraspanin 96F*, both seem to be essential for the correct guidance for the axonal growth cones to form the asymmetric neuropiles.

2 Zusammenfassung

Symmetrie ist überall in Flora und Fauna zu sehen, von der physischen Erscheinung großer Tiere bis hin zur Zytokinese. Bei genauem Hinsehen sind jedoch bei allen Lebewesen kleine asymmetrische Merkmale zu erkennen, denn perfekte Symmetrie ist nur auf dem Papier möglich. Selbst größere asymmetrische Strukturen lassen sich am Körper von Tieren beobachten, zum Beispiel an den inneren Organen des Menschen oder an den Extremitäten, wie zum Beispiel die größere Klaue eines Winkerkrebses. Gehirne im gesamten Tierreich neigen zur Asymmetrie, weil sich die funktionelle Asymmetrie als vorteilhaft für die Signalübertragung und Spezialisierung erweist. Diese Lateralisierung des Gehirns ist jedoch noch nicht vollständig verstanden, und die jüngsten Entdeckungen einer asymmetrischen Struktur, des asymmetrical bodys (AB) bei *Drosophila*, könnten dazu beitragen, Licht in die Entwicklung der Lateralisierung des Gehirns zu bringen. Dieser AB ist eine kleine Neuropile, die sich zwischen dem fan-shaped body und dem ellipsoid body im zentralen Komplex des Gehirns von *Drosophila melanogaster* befindet. Es ist strukturell asymmetrisch in seiner Größendifferenz zwischen beiden Hemisphären, wobei das Neuropile auf der rechten Hemisphäre deutlich größer ist. Dies scheint eine funktionelle Bedeutung zu haben, da phänotypisch mutierte Fliegen mit gleich großen Neuropiles, auch "double AB" genannt, keine Langzeitgedächtnisbildung aufweisen.
In dieser Studie wurde ein Knockdown von Kandidaten via RNAi durchgeführt, um Schlüsselgene zu bestimmen, die an der Entwicklung der Asymmetrie dieses AB beteiligt sind. Interessanterweise konnte ein "linker" Phänotyp, der den Wildtyp durch die Hirnmittellinie widerspiegelt, und ein "mittlerer" Phänotyp, der eine Innervation an der Hirnmittellinie aufweist, beobachtet werden. Es gibt keine Berichte über diese beiden Phänotypen. Auffallend ist, dass diese Phänotypen durch Knockdown sowohl in Gliazellen als auch in Neuronen induziert werden können. Das AB scheint für eine erwachsene Fliege essentiell zu sein, da kein AB fehlender Phänotyp beobachtet werden konnte.
Eine hohe Penetranz von Nicht-Wildtyp-Phänotypen konnte beim Knockdown der Gene *Wnt4* und *Tetraspanin 96F* beobachtet werden, die beide für die korrekte Leitfunktion der axonalen Wachstumskegel zur Bildung der asymmetrischen Neuropiles wesentlich zu sein scheinen.

3 Introduction

3.1 Asymmetry in biology

Symmetry is perceived as beauty or nice shape and is most commonly to be found in biology [1]. Symmetry in physical appearance could be a sign of good gene hypothesis or principal of highest reproductive value phenotypically [2]–[5]. However asymmetric features across the body, like inner organs in humans, cells in early development in mammalian embryos and whole-body plans like the bigger claw of a fiddler crab, can be found throughout nature [6], [7]. Brains are no exception to that. Their anatomy is almost symmetrical, however brain lateralization or functional asymmetry, which could also lead to size difference of hemispheres is essential for a normal functioning brain [8], [9]. Asymmetrical structures in the brain like in the zebra fish forebrain [10] and in general the asymmetries in the vertebrae forebrain [11] show that there are still more asymmetrical structures in brains to be found. The asymmetry in brains is generally due to a higher throughput of information and the specialization of certain brain regions e.g. speech [12],[13].

However, the specific developmental genes, which encode this functional asymmetry in both hemispheres could not be found.

Luckily in the fruit fly *Drosophila melanogaster (Drosophila mel.),* a Fasciclin 2 (FasII) positive asymmetric brain structure, the asymmetrical body (AB), has been found, which was associated with long term memory.

This feature could be used to further analyse the development of the AB. Flies with symmetrical AB are associated with a lack of long-term memory, whereas short term memory isn't impaired [14].

Since the release of the initial AB study, a Gal4-expression line repository for *Drosophila mel.* was made, which enabled the manipulation of specific cells [15]. This followed by finding specific Gal-4 drivers, which most certainly are AB cell specific Drivers, enabled the characterization of this structure. More interestingly through new findings in the lab, this structure seems to be a novel evolutionary step, since only close relatives to *Drosophila mel.* have a FasII positive AB [16].

To further characterize it, a knockdown of specific genes could help to understand the underlying developmental pathway building this structure. Looking at other species, asymmetric neuronal development is prominent in animals like *Caenorhabditis elegans* (*C. elegans)* and or zebrafish [10], [17].

Therefore, in this study I analysed the function of candidate gene in AB development via targeted RNA interference, which could have an impact on building this structure asymmetrically in the brain in *Drosophila mel.*. Through this screen I wanted to determine

how many genes are involved in the development of this structure and if this structure is established through asymmetric expression of those genes.

3.2 AB morphology

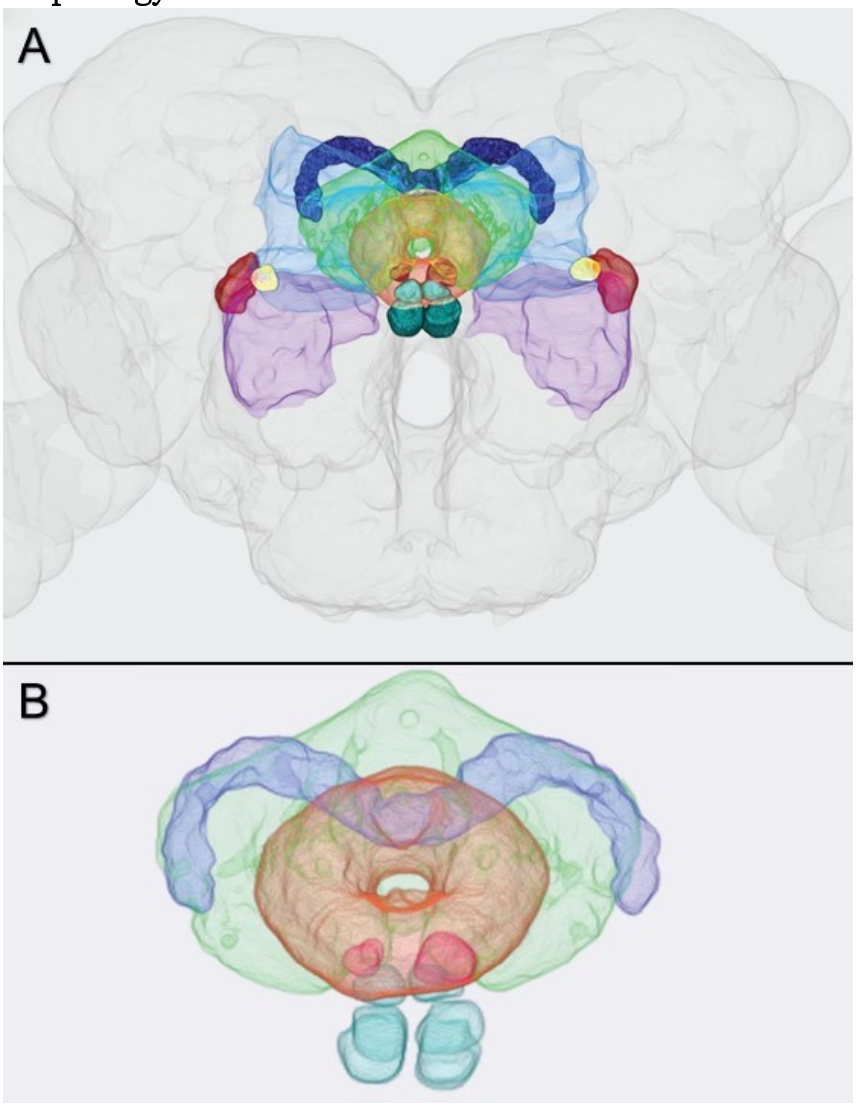

Figure *1: Central complex morphology*

The central complex of Drosophila consist of multiple neuropils as can be seen in A and B. View is ventral on the front. The neuropiles are the protocerebral bridge (blue), the ellipsoid body (orange), the fan-shaped body (green), the asymmetrical body (rose) and the noduli (teal). This picture was taken from Wolff and Rubin (2018).

The asymmetrical body (AB) is the 5th neuropile of the central complex in *Drosophila mel.*, which is shown with the other neuropiles in **Figure 1** [18]. It is bilateral with neuropiles on both hemispheres. However, the size asymmetry of its innervation is bigger in the right hemisphere as on the left hemisphere as can be seen in **Figure 2**. The main and bigger structure seems to be located on the right hemisphere. This structure gets signals from its pre-synapses at the protocerebrum, which is involved in perception of stimuli and sends processed information to the post-synapses at the central complex connecting to the fan-

shaped body. This could suggest that it is involved in signal transduction of visual stimuli into behavioural outputs [19], [20].

Furthermore, the developing neurons are located ventrally, the growth cones split at the height between the fan-shaped body and the ellipsoid body as can be seen in **Figure 2** and **Figure 1** with the first axons crossing the midline, if their origin was on the left hemisphere, or innervate small post synapse regions radial to the midline, if their origin was on the right hemisphere [18]. The other growth cones converge to the dorsal side of brain where they have a large innervating pattern in the protocerebrum.

It has already been described that the structure is bilateral and has asymmetry in its innervation and that in brains which are bilateral that mostly the innervation on the right hemisphere is bigger, whereas the innervation on the left hemisphere seems to be fragmented [18], [22].

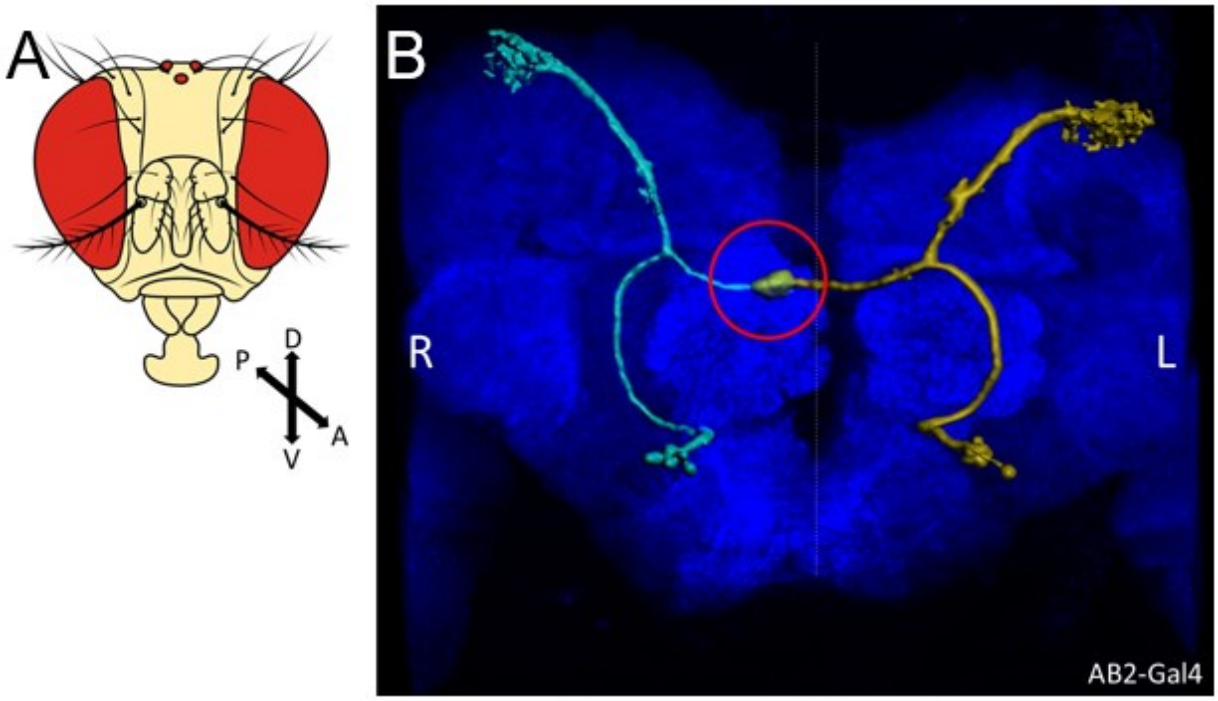

Figure 2: ***Asymmetrical body morphology***

(A) is a reference of a Drosophila head with frontal view and a axis (P for posterior, A for anterior, D for dorsal, V for ventral), which shows the orientation of the brain pictures thought the thesis. (B) A picture of a brain with the AB2-Gal4 expression was used, which shows the whole neuron morphology of the AB (red circle) next to the midline (dotted), with the left (L) and right (R) hemisphere. The neurons are mostly bilateral, with only the innervation of the AB on the right hemisphere. NCAD-staining (blue) was used as a background thought the screen to determine the orientation of the brain. This 3D image was made by Johann Markovitsch using Imaris® Software [21].

3.3 RNAi-based screen

The RNA-Interference (RNAi) is a process in which a targeted messenger RNA (mRNA) is cut and later destroyed to regulate the translation of a specific gene or altogether stop it

(**Figure 3**). The process was observed in many different eukaryotes and specifically analysed in *C. elegans* [23]. The process starts with a double stranded RNA (dsRNA) binding to the enzyme Dicer which cuts the dsRNA into single-interfering RNA (siRNA) fragments, with the approximate length of 21 nucleotides. The siRNA unwinds and binds to the RNA-induced silencing complex (RISC). The passenger strand is degraded, now the guide strand is a single stranded RNA (ssRNA). The guide strand ssRNA then binds to its targeted complementary mRNA, which induces the ARGONAUT protein (AGO) to cleave the targeted mRNA. This cleavage pathway is also used by microRNA(miRNA), however miRNA can target multiple mRNAs and thus regulate multiple genes, which is also their function in a normal cell. This method enabled the knockdown of many genes in many organisms, cells or tissues in those organisms, to further characterize gene function in these specific subsets of cell clusters. In this study the ssRNA was introduced to the fly brain via the GAL4/UAS system. The corresponding ssRNA to the targeted gene mRNA was expressed in either all neuronal cells or in midline

Many of those targeted genes have a function in development, cell signalling or cell maintenance.

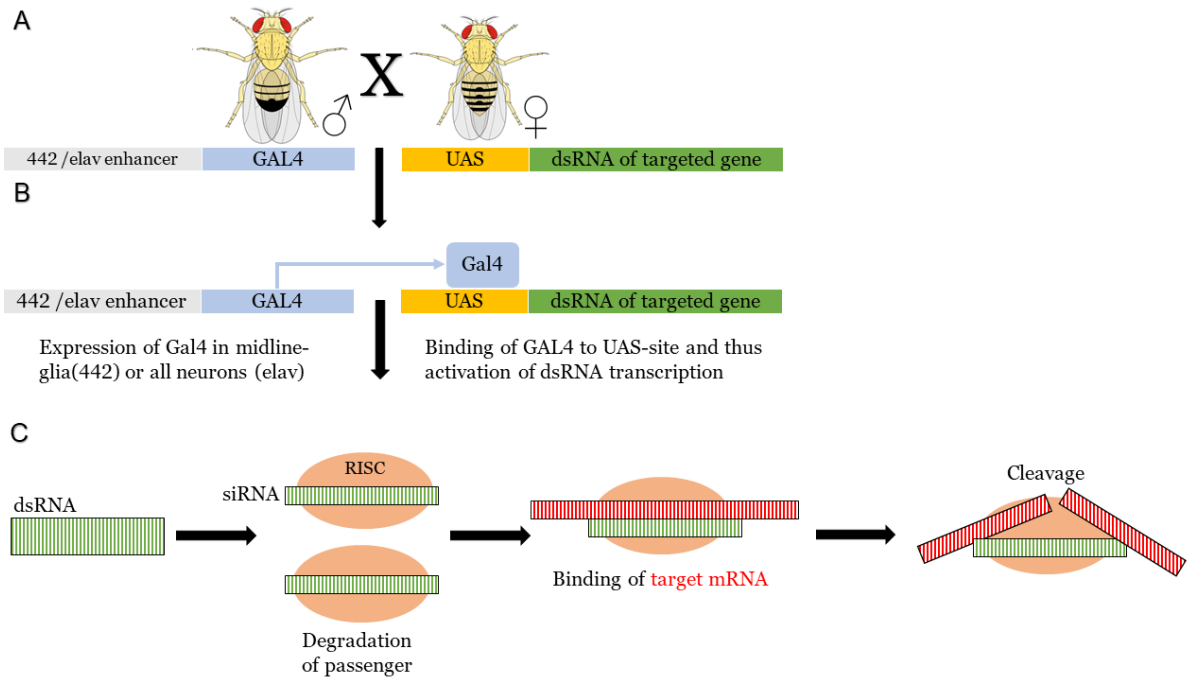

Figure 3: Scheme of GAL4/UAS based RNAi

This study used the GAL4/UAS system to induce RNAi in drosophila brains. A: Female flies, which express the transgene GAL4 in cells where either the enhancer for 442 or elav are active, were crossed with male flies having the UAS site with the desired dsRNA. In the offspring B GAL4 get expressed and binds to the UAS-site, which activates the transcription of the dsRNA of the targeted gene. C the transcribed dsRNA splits during RISC formation and the resulting siRNA binds to the targeted mRNA, which gets cleaved, thus a knockdown of the targeted gene is done.

3.4 Hypothesis

The brain asymmetry could be formed during development via 2 principal mechanisms, which induce AB asymmetry as can be seen in **Figure 4**.

The first mechanism is the asymmetry formation, which induced greater innervation on one hemisphere to the other.

The second mechanism is the directionality of this asymmetric innervation to be either left or right.

The goal of this study is to determine developmental genes involved in forming this asymmetry in the AB via RNAi knockdown.

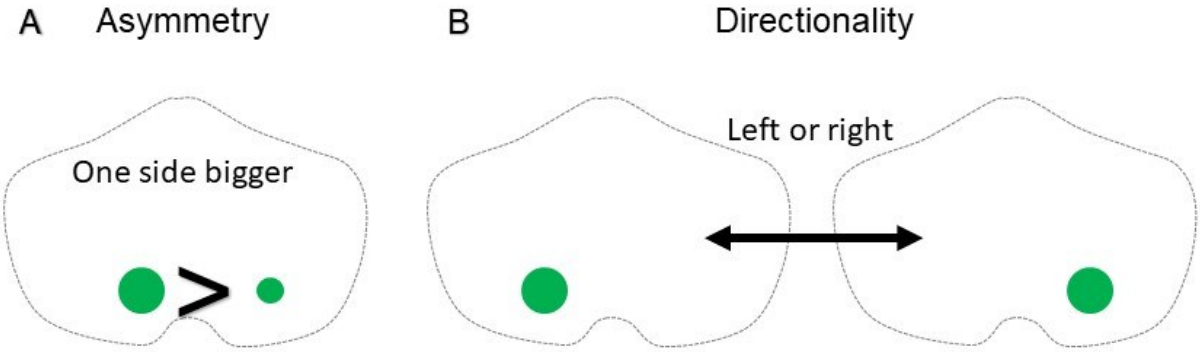

Figure 4: Hypothesis of 2 underlying mechanisms

The hypothesis states that there are 2 underlying mechanisms for the innervation pattern, which is A asymmetry of the innervation with one side bigger than the other and B directionality of the innervation on the left or right hemisphere

4 Methods

4.1 *Drosophila mel.* stock maintenance

4.1.1 *Fly* culturing

Stocks were reared at 25°C temperature and 60% humidity in culture vials with cooked media at the bottom filling 1/4 of every vial.
Stocks were flipped after parent generation laid eggs to counter reproduction with the F1 generation. Crosses were flipped every 3-4 days to have spare vials.
During some experiments the crosses were reared at 29°C temperature.

4.1.2 Fly stocks

In total 52 genes were screened. Transgenic fly stocks and/or plasmids were obtained from the Vienna *Drosophila* Resource Center (VDRC, www.vdrc.at) [24].

Table 1: Fly stocks that were used during the screen

This table contains the fly stocks that were used during the screen with their internal number, gene name, gene ID, their genotype which can contain markers, where x means the location of the UAS-RNAi on the chromosome, the order number, and the stock center from which it was ordered. The following gene names were too long and were shortened in this table: ADAM*=ADAM metallopeptidase with thrombospondin type 1 motif A; Phosphatase*=Phosphatase and tensin homolog; Guanylyl cyclase*=Guanylyl cyclase β-subunit at 100B; Rho*= Rho guanine nucleotide exchange factor 4.

no.	internal no.	gene name	Gene ID	genotype chromosome 1	2	3	order number	stock center
1	1092	Sema-1a	CG18405		x/CyO	x/x	N/A	
2	1096	neural cadherin (NCAD)	CG7100		x/x		N/A	
3	1593	hibris	CG7449		x/x		N/A	
4	2034	flamingo	CG11895		x/x		N/A	
5	3020	artichoke	CG5195		x/x		31044	VDRC
6	3267	Dab	CG9695			x/x	N/A	VDRC
7	3022	peste	CG7228		x/x		33155	VDRC
8	3024	ADAM*	CG14869			x/TM3	33347	VDRC
9	3025	Rap1 GTPase	CG1956			x/TM3	33437	VDRC
10	3027	CASK ortholog	CG6703			x/x	34184	VDRC
11	3028	pebble	CG8114			x/x	34350	VDRC
12	3029	microtubule star	CG7109			x/x	35171	VDRC
13	3030	pebble	CG8114		x/x		35349	VDRC
14	3031	pebble	CG8114			x/x	35350	VDRC
15	3032	Phosphatase*	CG5671			x/TM3	35731	VDRC
16	3033	Pinin	CG8383		x/CyO		35915	VDRC
17	3035	hattifattener	CG14351		x/CyO		36221	VDRC
18	3037	Wnt oncogene analog 4	CG4698		x/x		38010	VDRC
19	3038	Dmel\CG18095	CG18095			x/x	36256	VDRC
20	3039	Nimrod C2	CG18146			x/x	36264	VDRC
21	3040	Dmel\CG17739	CG17739			x/x	36542	VDRC

22	3041	Dmel\CG6867	CG6867			x/x	37416	VDRC
23	3042	center divider	CG6027		x/x		43634	VDRC
24	3043	midline fasciclin	CG3359		x/x		37888	VDRC
25	3044	Neurexin 1	CG7050		x/x		36328	VDRC
26	3045	Dpr-interacting protein ζ	CG31708			x/x	38261	VDRC
27	3046	Rab18	CG3129			x/x	40228	VDRC
28	3047	rhea	CG6831			x/x	40399	VDRC
29	3049	scribbled	CG5462			x/TM3	41440	VDRC
30	3050	Dmel\CG3829	CG3829			x/x	42872	VDRC
31	3051	Dmel\CG6788	CG6788		x/x		42911	VDRC
32	3227	Tetraspanin 66E	CG4999		x/CyO	MKRS/TM 2	37252	VDRC
33	3228	lambik	CG8434		Sp/Cy O	x/TM2	42571	VDRC
34	3229	discs lost	CG32315		Sp/Cy O	x/TM2	41876	VDRC
35	3230	Tetraspanin 96F	CG6120		Sp/Cy O	x/TM2	3422	VDRC
36	3232	friend of echinoid	CG31774		Sp/Cy O	x/TM2	46180	VDRC
37	3233	Tousled-like kinase	CG34412		Sp/Cy O	x/TM2	46425	VDRC
38	3234	kekkon5	CG12199		Sp/Cy O	x/TM2	47768	VDRC
39	3235	muskelin	CG8811		x/CyO	MKRS/TM 2	29774	VDRC
40	3236	Basigin	CG31605		Sp/Cy O	x/TM2	43307	VDRC
41	3237	warts	CG12072			x/x	N/A	
42	3238	klumpfuss	CG12296			x/TM3	N/A	
43	3239	Guanylyl cyclase*	CG1470			x/x	N/A	
44	3240	Cadherin 89D	CG14900		Sp/Cy O	x/x	N/A	
45	3241	Cadherin 89D	CG14900			x/x	N/A	
46	3242	myospheroid	CG1560			x/x	N/A	
47	3243	Mediator complex subunit 30	CG17183			x/x	N/A	
48	3261	Kinesin heavy chain	CG7765			x/x	N/A	
49	3264	Rho*	CG8606			x/x	N/A	
50	3279	CA07338 Plesup(3)?	N/A			x/x	N/A	
51	3281	CA06588 Sema5U	N/A			x/TM3	N/A	
52	4851	mirr RNAi	CG10601			x/x	N/A	

4.1.3 Fly food

The contains of fly food was mixed for 20l with the following ingredients 18l water, 2000g ice, 1400g cornmeal, 1400g malt extract, 700g beet sugar, 350g dry yeast, 300ml nipagin 240g agar, 200g soy meal and 80ml propionic acid.

4.2 Fly dissection

While determining gender or during other search efforts, the flies were first anesthetized via CO_2. If certain flies were not needed in the experiment the firstly anesthetized flies were put into 96% ethanol. For dissections the anesthetized flies were put into 96% ethanol for 3mins, after that the ethanol was washed out 3 times with PBT.
The dissection of the fly was done with forceps under a compound light microscope in PBS on a silicone plate.

4.3 Fly determination

Different genetic makers in balancer chromosomes were used as makers to determine morphological mutants and thus transgenic flies, to further use them in crosses or stocks.

4.4 Staining protocol

The staining protocol for was as follows:
After the dissection of the brains, they were fixed in 2% PFA (PBS + 2g paraformaldehyde) for 1 hour on a rotor. Brains were washed 4 times for 15mins each with PBT (PBS + TritionX-100 0.3%). Brains were blocked with 10% goat serum (10% goat serum in PBT) for 1 hour. The primary antibody solution 1:1000 with brains were incubated overnight on a shaker in a 4°C fridge. Brains were washed 4 times for 15mins each with PBT. The secondary antibody solution 1:1000 with brains was incubated 3 hours on a shaker in room temperature. Brains were washed 4 times for 15mins each with PBT and mounted under a fluorescence microscope using Vectashield®.

4.5 Imaging

All images were made with the Leica SP5 II confocal laser scanning microscope. Laser settings depended on the antibody solution and brains. Brains were always scanned from top to bottom with 400-700 Hz and with a Z-step size of 1.5µm.

4.6 Image processing

All images were processed with Fiji, an image processing program, with the ability to stack images and choose different channels [25]. Images were cropped, stacked and specific light channels chosen to focus the area of interest. Subsequently all processed images were put together on a PowerPoint presentation to have an overview of the phenotypes.

4.7 RNAi screen

I used UAS-RNAi since the lines are already established and thus a wide variety of targeted genes can be tested at reasonable amount of time. It is transgenic, so no injections or other forms of transformation must be made. The insertion of the transgenic RNAi can be visualized by phenotypical markers on the fly, as balancer chromosomes have "curly" etc. and can be thus selected.
The RNAi is executed through the expression of the targeted GAL-4 gene, which mediates the expression of GAL4 in those cells. GAL4 binds to the UAS-site of the transgenic UAS-RNAi, which mediates the expression of the inverted repeats of the targeted gene, being a dsRNA this in turn activates builds the RISC which splices the mRNA of the targeted gene.
We used RNA interference to knock down genes during development in *Drosophila mel.* to determine the function of those genes. Premade *Drosophila mel.* stocks were ordered which contained UAS-RNAi sites of the specific gene. We screened a list of preselected genes

4.8 GAL4/UAS system

The UAS/Gal4 system was used to express the RNAi in specific tissues. This system was used since the lines are already established and modulation can be done, by just crossing two transgenic fly, one with the Driver-line GAL4 and the other with the UAS-RNAi we tested [26]. We used two driver lines, *elav-Gal4*, which were in almost all brain cells, and *442-Gal4*, which were in the glial transient interhemispheric fibrous ring midline Glia cells [27]. The midline glia cells could have an impact on the transhemispheric connection of the brain and thus were selected [28]. Where *ELAV-Gal4* was more or less a negative control, since the expression of ELAV is postmitotic and all neurons are affected [29], *442-Gal4* was used to specifically see if Glia cells and in specific interhemispheric glia cells have an impact on the development of the interhemispheric bridge between those AB cells and their innervations on both hemispheric sides [30].
We used the Gal4/UAS system to target gene expression in targeted neuron populations in such way, that we induced RNAi or to report gene expression via GFP [31].
Targeted Gal4 and UAS was separately expressed in the progenitor lines, which were then crossed, and the offspring reared. The first generation now had the functioning Gal4 and UAS in it, which let the Gal4/UAS system express the UAS-protein or RNAi in our case.

The Gal4 is extrinsic from yeast, which is why this protein is expressed more at higher temperature. To increase the number of phenotypes and also the phenotype strength, we reared the first generation crosses at 29°C.

4.9 Immunofluorescence staining

The Fasciclin II (FasII) positive AB was visualized using FasII antibody. A transgenic FasII::GFP construct was tried to be used, however the expression of FasII::GFP in the AB cells seemed to be very faint or too late in the development of the fly brain, which is why it couldn't be visualized in the adult brain.
As a background to visualize the brain, N-cadherin (Ncad) antibodies were used and to further enhance the GFP signal in some flies GFP antibodies were used. Other attempts to visualize the AB cells as well as the "probably" connecting neurons from the fan-shaped body were conducted, however no clear results could be made.
We used a 2-antibody system, in which the first antibody targeted the specific target, e.g. GFP and the second antibody, which had a fluorescent marker which is excited at either 488nm, 568nm or 647nm wavelength, binds to the first antibody multiple times, which also increases the signal strength.

5 Results

5.1 RNAi-screen

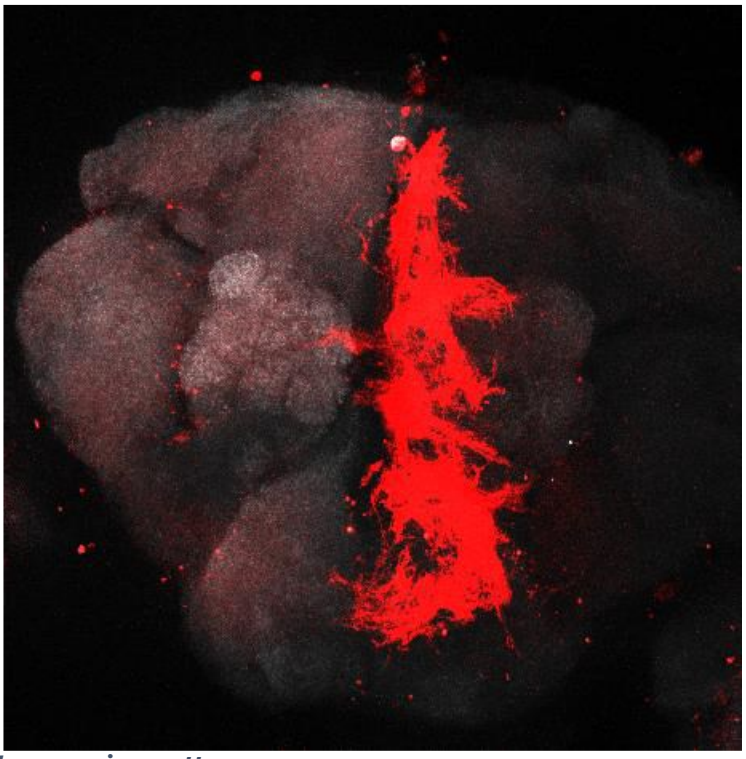

Figure 5: 442-Gal4 expression pattern

A single cell clone of the 442-Gal4 driver was made to show the pattern of its expression. As can be seen the expression is mainly in the midline. This image was made from Charlotte Schönherr.

We used the GAL4/UAS system to knockdown the targeted genes via RNAi in the developing fly. Flies with the two GAL4-drivers, *ELAV-GAL4* and *442-GAL4* (Figure 5), with its genotype in **Table 2**, were crossed with UAS-RNAi reporter lines flies as seen in **Table 1**.

Table 2: GAL4-drivers stocks that were used during the screen

This table contains the GAL4-drivers stocks that were used during the screen, reporter line, their genotype, which can contain markers.

	chromosome		
reporter line	**1**	**2**	**3**
442-GAL4			**442-GAL4/TM2**
ELAV-Gal4	***ELAV-Gal4***	**Sp/CyO**	

In the original study 7,6% of the flies had a symmetrical asymmetrical body (double AB), to control the wildtype occurrence of this phenotype, different wildtype stocks were tested.

This could give us a hint at how high the penetrance of phenotypes must be, to count as a RNAi induced phenotype and not genetical variation.
The RNAi-screen was done with knockdown for several genes, which were pre-picked, either because of their expression region or their associated role in commissure building. Thus many genes already Different AB phenotypes were observed and criteria for those were classified. The RNAi lines, which had the highest phenotype penetrance were then Re-screened with a larger quantity of flies and revalued.
In total 52 lines were screened, which resulted in 104 crosses, 7 of those were lethal the other remaining 4 lines weren't crossed. This in turn were 93 separate crosses, which yielded results with whom 12 lines were determined to be rescreened, 3 of those were screened with both Gal4 driver lines, the remaining with one Gal 4 driver line.
These candidate genes were split up into different gene groups, with different functions and their similar role should give us a hint at their function to the development of the AB.

5.2 Classification of phenotypes

Throughout the screen different AB phenotypes could be observed. Brains in which no signal was observed were not counted, also brains where the overall signal was too weak or too strong were not counted. To make it general and comparable across our self a classification of phenotypes indicated in the in **Figure 6** . The selection criteria for each phenotype were as following: "Wildtype" as in **Figure 6**A was chosen, when only one FasII-positive region (AB innervation) was on the right hemisphere and no innervation was visible on the left hemisphere. "Double AB" as in **Figure 6**B was chosen, when two distinct AB innervation were visible, commonly with one on each hemisphere. "Left" as in **Figure 6**C was chosen, when only one AB innervation was visible on the left hemisphere and no innervation, or a very faint innervation was visible on the right hemisphere. "Middle" as in **Figure 6**D was chosen, when only one big AB innervation was visible in the midline of the brain hemispheres or very close to it.

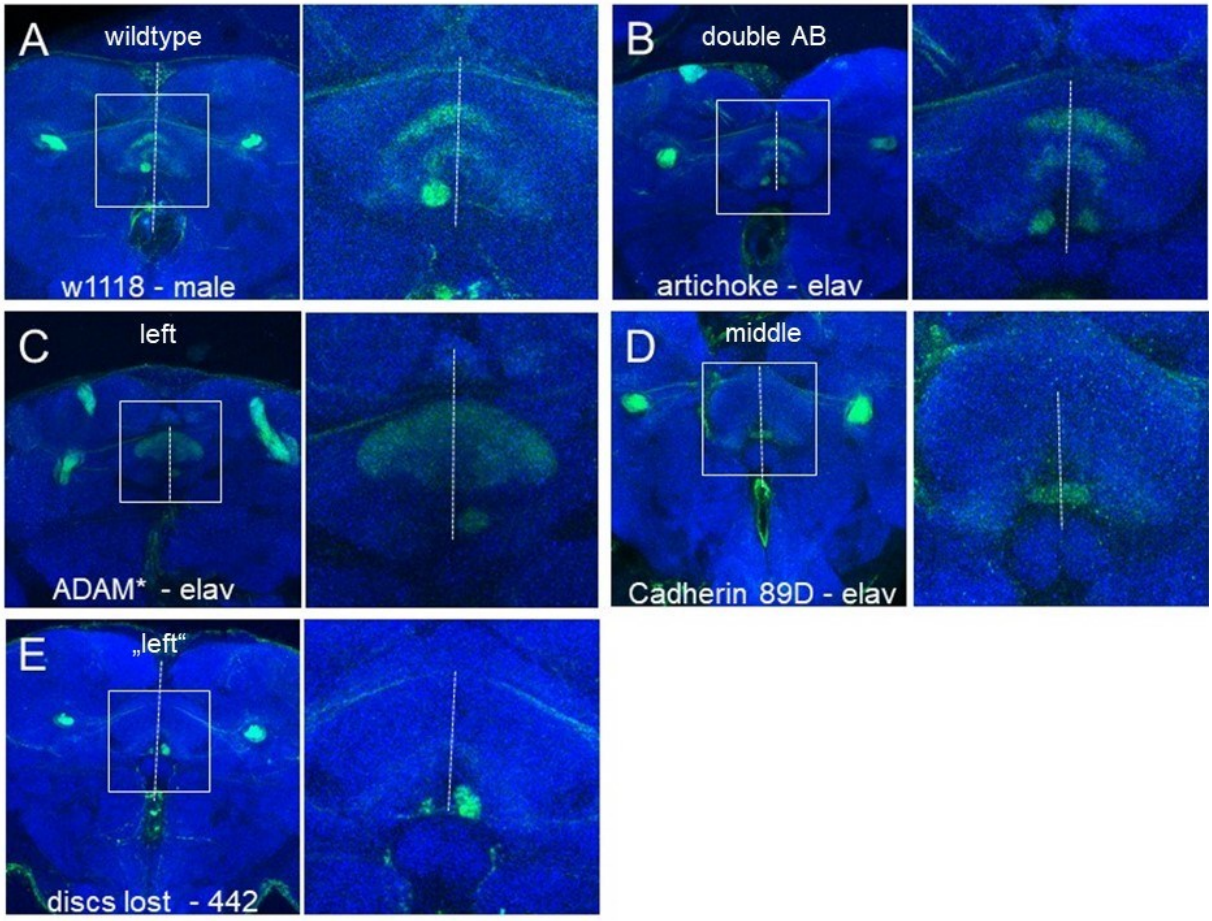

Figure 6: Asymmetrical body phenotypes:

A total of 5 possible phenotypes was observed during the screen, antibody staining with Ncad in blue (647nm) and FasII in green/red (488nm, 568nm) colorization. Compared dots are located left/middle or right on the horizontal axis (dotted line) with zoomed pictures on the right side. A) The most common phenotype seen in flies with one dot on the left hemisphere. B) 2 dots "double AB", where one dot was on the left and the other on the right hemisphere. C) the dot was "left", where the dot was on the right hemisphere of the brain. D) a dot in the "middle" at the horizontal axis. E) Sometimes the exact cuts of phenotypes couldn't be made, and this particular phenotype was counted as "left", because the size of the dots are significantly different.

5.3 Unilateral/bilateral classes of phenotypes

Phenotypes could be classed as bilateral or unilateral phenotypes, for such simplification or generalization the "middle" and "double AB" are phenotypes which are expressed bilateral and "wildtype" and "left" which are asymmetrically are counted towards unilateral phenotypes.

5.4 Phenotype penetrance in wildtype flies

To determine the mean phenotype penetrance of double AB in wildtype flies, we dissected and scanned brains of different wildtype stocks (Table *3*).

The number of phenotypes different from the wildtype phenotype were 0% across all stocks apart from Canton S females, with a 30% double AB penetrance, and Vienna L female, with a 9% double AB penetrance.

Table 3: AB *phenotypes in Drosophila wild type stocks:*

Visible are the tested wildtype stocks with the stock name, the sex, the number of brains, AB phenotype visible and total amount of phenotype visible in numbers and percentage.

Stock name	Sex	No. of brains	Phenotype (n)	penetrance
Canton S	female	15	Double AB (3)	20%
	male	9		0%
Vienna J	female	10		0%
	male	10		0%
Vienna L	female	11	Double AB (1)	9%
	male	9		0%
w1118	female	13		0%
	male	11		0%

5.5 Phenotype penetrance across the RNAi screen

The phenotypes class and the phenotype penetrance of the AB were documented and counted for each Gal4 driver line

Table 4 . The mean AB phenotype penetrance across all ELAV-Driver lines was 30%, with 7 lethal lines. The mean AB phenotype penetrance across all *442-GAL4*-Driver lines was 23%, with 2 lethal lines. The minimum number of scanned brains per line was 5 across all Driver. Lines with no AB phenotype were present. AB phenotype penetrance across all ELAV-Driver lines were highest in "Tousled-like kinase" (100%), "Phosphatase and tensin homolog"(71%), "artichoke"(67%), "Rab18"(67%) and "Pinin"(67%). AB phenotype penetrance across all 442-Driver lines were highest in "muskelin" (80%), "Wnt oncogene analog 4" (71%), "Neurexin 1" (67%), "Tousled-like kinase" (63%) and "Dmel\CG18095" (56%). The most common AB phenotype was double AB. Since the initial screen was done in part with the re-screen preliminary results were taken to determine possible candidate genes.

Candidate-lines which were later used in the re-screen are coloured coded in the table. With orange being elav driver exclusive candidate, blue being a candidate gene for both drivers and green being a midline glia exclusive candidates.

Table 4: *Results of the RNAi-screen at 29°C:*

These are the results during the RNAi-screen at 29°C. Listed is the name of the RNAi gene target (line name) with their number of scanned brains (No. of brains), the phenotype class, the total number of observed AB phenotypes in numbers and percentage of brains in the driver ELAV-GAL4 or 442-GAL4. ADAM = ADAM metallopeptidase with thrombospondin type 1 motif; Guanylyl cyclase*= Guanylyl cyclase β-subunit at 100B; Rho*= Rho guanine nucleotide exchange factor 4*

No.	Line name	*ELAV-GAL4*				*442-GAL4*			
		No. of brains	AB	No. of phenotypes		No. of brains	AB	No. of phenotypes	
1	Sema-1a	12	d	3	25%	5			0%
2	neural cadherin (NCAD)	6	d	1	17%	11			0%
3	hibris	10			0%	7			0%
4	flamingo	6			0%	8	d	1	13%
5	artichoke	6	d	4	67%	5	d	1	20%
6	Dab					7	d	1	14%
7	peste	7	d	2	29%	11	l	2	18%
8	ADAM*	6	l	1	17%	7	d	1	14%
9	Rap1 GTPase	lethal				9	d/l	4	44%
10	CASK ortholog	7	d	3	43%	6	d/l	2	33%
11	pebble	8	d	1	13%	5	d/l	1	20%
12	microtubule star	10	d	5	50%	11	l	1	9%
13	pebble	6	d	3	50%	6	l	1	17%
14	pebble	lethal				7	d	1	14%
15	Phosphatase and tensin homolog	7	d/m	5	71%	5			0%
16	Pinin	9	d	6	67%	lethal			
17	hattifattener	9	d	1	11%	6	d	1	17%
18	Wnt oncogene analog 4	8	d	3	37%	7	d/l	5	71%
19	Dmel\CG18095	5	d	2	40%	9	l/d	5	56%
20	Nimrod C2	5	d	1	20%	7	l	2	29%
21	Dmel\CG17739					5	l	1	20%
22	Dmel\CG6867	10	d	2	20%	6			0%
23	center divider	7			0%	7	d	2	29%
24	midline fasciclin	13	d	7	54%	10	d	1	10%
25	Neurexin 1	7	d	1	14%	6	d/l	4	67%
26	Dpr-interacting protein ζ	lethal				9			0%
27	Rab18	6	d/l	4	67%	10			0%
28	rhea	lethal				8	d	1	13%
29	scribbled	lethal				9	d/l	2	22%
30	Dmel\CG3829	10	d/l	1	10%	8	d	1	12%
31	Dmel\CG6788	7	d/l	4	57%	10	l/d	4	40%
32	Tetraspanin 66E	9	m	1	11%	11	d	2	18%
33	lambik	9	l	1	11%	9	d	2	22%
34	discs lost	6	d/l	3	50%	12	d	6	50%
35	Tetraspanin 96F	6	d	2	33%	9	d	4	44%
36	friend of echinoid	5	d/l	2	40%	9	l	3	33%
37	Tousled-like kinase	5	d/m	5	100%	8	d/l	5	63%
38	kekkon5	10	d	2	20%	6	d/l	2	33%
39	muskelin	5	d	1	20%	5	l/d	4	80%
40	Basigin	13	d/l/m	5	38%	9	d	1	11%
41	warts	7	m/d	2	29%	7	l	1	14%
42	klumpfuss	14	d	1	7%	13			0%

43	Guanylyl cyclase*	5			0%	7	d	2	29%
44	Cadherin 89D	17	d/m	5	29%	8	d	1	13%
45	Cadherin 89D	lethal				7	d	1	14%
46	myospheroid	5			0%	8			0%
47	Mediator complex subunit 30	12	d	6	50%	9	d/l	2	22%
48	Kinesin heavy chain	10	l/d	3	30%				
49	Rho*	5	D	1	20%				
50	CA07338 Plesup(3)?	5	d	1	20%	6	d	2	33%
51	CA06588 Sema?	8	d	1	13%	9	l	1	11%
52	mirr RNAi	10	d	5	50%	10	l/m	2	20%

5.6 Phenotype penetrance in the re-screen

After screening all RNAi lines, specific candidates, which had a high penetrance of phenotype and looked promising, were chosen to be rescreened with a higher number of brains, to eventually cancel out false positive results. The general AB phenotype penetrance was lower during the Rescreen than in the initial screen with both the *ELAV-GAL4* and *442-GAL4* Driver, except for Dmel\CG6788 with just -1% in elav- and *442-GAL4* and Wnt oncogene analog 4 with an increase of +7% in *442-GAL4*. With a mean AB phenotype penetrance of 42% in *ELAV-GAL4*(Table *5*) and 41% in *442-GAL4 (*Table *6*), the mean percentage of AB phenotypes was higher than in the initial screen (*Table 5*). The most common phenotype was “double AB”. The biggest penetrance difference of -80% from initial screen to Rescreen was with the “Tousled-like kinase” RNAi-line with *ELAV-GAL4*. The “wnt4”-line with *442-GAL4* had the highest penetrance with 79% of brain having a AB phenotype.

Table 5*: Results of the RNAi-re-screen with candidate genes with ELAV-GAL4.*

The Rescreen of genes which looked promising was done like the original screen at 29°C. Visible are the initial screen AB phenotype percentage(SCREEN), candidate number and line name, the amount of brain, the observed AB phenotypes, the total amount of observed AB phenotypes numerical and in percentage of those of the total brains.

SCREEN	Candidate no.	Line name	No. of brains	Phenotype	No. of phenotypes	
67%	5	artichoke	15	d/m	6	40%
71%	15	Phosphatase and tensin homolog	15	d/l	7	47%
67%	16	Pinin	13	d/m	6	46%
54%	24	midline fasciclin	15	d	5	33%
57%	31	Dmel\CG6788	16	d	9	56%
33%	35	Tetraspanin 96F	15	d/m	10	67%
100%	37	Tousled-like kinase	10	d/m	2	20%
50%	52	mirr RNAi	17	d	4	24%

Table 6*: Results of the RNAi-re-screen with the candidate genes with 442-GAL4.*

The Rescreen of genes which looked promising was done like the original screen at 29°C. screen phenotype percentage, candidate number and gene name, the amount of brain and the observed phenotype, the total amount of observed non-wildtype phenotype brains numerical and the percentage of those of the total brains.

SCREEN	Candidate no.	Line name	No. of brains	Phenotype	No. of phenotypes	
71%	18	Wnt oncogene analog 4	14	d/m	11	79%
56%	19	Dmel\CG18095	17	m/l	5	29%
67%	25	Neurexin 1	17	d/m	7	41%
40%	31	Dmel\CG6788	18	d/m/l	7	39%
44%	35	Tetraspanin 96F	14	m/l/d	4	29%
63%	37	Tousled-like kinase	17	m/d	5	29%

6 Discussion

6.1 AB is bilateral

As already mentioned by Wolff et al.(2015) the AB neurons are bilateral with only the fascilin2 positive innervation of the post synapse being asymmetric [22]. This makes the study even more promising, since finding the right genes which are responsible for the development from a bilateral organisation to a unilateral could help to understand asymmetric formation in general. However, this already plays upon the assumption that there was a bilateral structure, which in evolution has proven to be more effective asymmetric. On neuronal level a asymmetric structure have been shown to be in zebra fish [10], *C.elegans* [32], ants [33], honey bees [34], bumble bees [35] and many more [36], [37].
This study is pioneering the asymmetric neuron analysis in *Drosophila*.

6.2 RNAi result interpretation

In this screen 52 genes were knocked down in either all neurons or midline glia cells. Most of those knockdowns had an impact on the correct formation of the innervation of the AB. Three phenotype categories were found with “double AB” being the most prominent, followed by “left” and “middle”. Indicating that through knockdown bilateral phenotypes are more prominent.
There is a natural “double AB” phenotype in wildtype flies, which I needed to consider in the RNAi analysis. In wildtype flies the natural “double AB” phenotype penetrance was also lower than in the original paper discussing the AB of Pascual et al., 2004. This could also indicate that the RNAi-Screen in general was effective and specific in gene knockdown, which in turn would suggest that the results can be taken as valid. However, since I looked at wildtype flies which had no transgenes and on the other hand at transgenic flies, due to balancers and other constructs such as GAL4/UAS which were transformed into the genome of those flies, a disruption of the control mechanism could emerge, which normally develops the AB and maintains its integrity. Therefore, the balancer transgenes alone could have had an impact on the higher mean AB phenotype penetrance in the screen and re-screen.
Different temperatures during the experiment leading to different results, with 29°C was the optimal temperature. Flies could reproduce almost normally and the GAL-4 expression was increased leading to a higher UAS-RNAi expression and thus to a stronger knockdown of genes [31].

Since almost every gene had an impact on the AB phenotype, the developmental mechanism, which maintains the natural AB phenotype could be a relatively new evolutionary step, thus many genes affect its maintenance.
This in turn again would suggest that other close relatives to *Drosophila* don't have this FasII positive region or have a different morphology. In general, it could be said that the structure is either sensitive to RNAi and other gene constructs or the RNAi is working as intended.

6.3 RNAi AB phenotypes

The phenotype range was considerable large, with surprising "left" phenotypes, with the potential conclusion being that the AB innervation, which is happening on one hemisphere is "at random" during the development. This hypothesis would suggest that the penetrance maximum of possible "left" phenotypes across all brains is at 50% with a complete knockdown of the "left" inducing gene. This could be the evolutionary pre-step leading to the side preference of the innervation, which in turn could be the cause of evolutionary advantage. Also interesting was the "middle" phenotype, which could lead in part to a mechanism, which is essential for triggering the innervation on either the left or the right hemisphere, through promoting axonal growth across the midline or inhibiting the innervation at the midline. It could also be, that this phenotype is visible due to the absence of genes promoting the innervation at the normal location. "Double AB" was a phenotype which had a high penetrance in the RNAi-Screen, so targeted genes could be responsible for regulating the inhibition of innervation on one hemisphere.
Even though all observable AB phenotypes were observed in *elav-Gal4* as well as in 442-Gal4, no possible severity of phenotypes can be ordered, since a clear null-mutant wasn't visible. However, the higher the AB phenotype penetrance, the more connected to the AB formation mechanism the targeted gene must be. Also, this suggests, that the Midline Glia, plays as role in the development of the ABs as much as the surrounding neurons.
Since there was not a clear null mutant phenotype, a full knockdown of genes developing the AB and its innervation might be an easy target considering the GAL-4 driver line, which is within the FasII positive AB, has been found already.
The amount of scanned brains during the Re-Screen seemed to make an impact on the percentage of phenotypes, this is simply to due to the stochastic reason that in a lower number of samples the chance of having a higher number of brains, which have a AB phenotype, is higher. The Re-Screen validated this by the more general lower phenotype penetrance to the initial screen.

In general unexpected results were observed with “left” phenotype being the most interesting one, since this would suggest that knockdown can make a unilateral phenotype, also the higher the AB phenotype penetrance of a gene knockdown, the more likely it’ll be closely connected to essential AB genes.

6.4 Protein families

6.5 Wnt4

Wnt4 is expressed downstream of homeotic genes, in early embryogenesis in the head and ectodermal segmentation [38]–[40]. It functions in different areas as germ-line stem-cell homeostasis, axon guidance and heart development [41], [42]. Wnt4 and CG6867 have shown to induce repulsive local cues in motoneurons synaptic target specificity [43]. Foremost it is a repressive gene, which would suggest, that in this study wnt4 is necessary to guide axons from innervating and building synapses unilateral. The gene must be expressed presynaptic on surrounding neurons or in glia cells repressing neuronal connections on off targets. This would explain seeing a high penetrance of double and left AB phenotypes, since the repulsion is lost, innervation unilateral and bilateral are seen. Axons are innervating even before they hit their target because the repulsion is lost. Since the penetrance of phenotypes is higher in glia cells, this would suggest, that the either the expression or the guidance of glia cells are crucial for correct formation, demonstrating how important midline glia cells are for guidance of those neurons. The results suggest that wnt4 is both an asymmetry and directional inducing gene as well.

6.6 Tetraspanin 96F

Tetraspanins are scaffold proteins, recruiting other proteins including other Tetraspanins into a region called tetraspanin enriched microdomains [44], [45]. These microdomains function in quite different ways most importantly for intercellular signalling for neurons and cell to cell adhesion. Tetraspanin 96F is expressed in the whole fly during embryogenesis and adult flies, as such it is also expressed in the brain and could recruit other signalling proteins for axon guidance [46]. We observed a high penetrance of phenotypes for the knockdown in neurons, the knockdown in glia cells, seemed to be not as a high impact, suggesting that Tetraspanin96F is mostly expressed in neurons. Middle and double phenotype suggest that it is important for axonal innervation of the AB neurons. It might recruit signalling proteins at the dendritic sites in its microdomains, which act repulsive to off targets. Middle phenotypes suggest that it also induces the

innervation of dendritic sites, via recruiting of other proteins in its microdomain. Since no signal was sent when neurons passed the target, innervation eventually starts at the midline from both growth cones. The results suggests that Tetraspanin 96F is thus a particularly important asymmetry inducing gene.

6.7 AB location formation

As already proposed in the hypothesis, these different AB phenotypes must have an underlying mechanism. This underlying mechanism can only be partially explained, since no AB innervation-absent phenotype was visible, as well as the development of those phenotypes shortly after the pupae formation was not observed. However different models can be made where most commonly genes are activated in a cascade to induce promotion or inhibition of the AB cell innervation. First, a gene which determines the hemisphere on which the AB structure must innervate, is activated. This is due to the fact a “left” phenotype was observable. In normal circumstances this would be the right hemisphere. This gene activates another gene which inhibits the innervation on the opposite hemisphere. This is due to the fact at least one AB innervation was visible across all brains, thus enabling the “double AB” phenotype. Now this gene or the site-preferring gene activates another gene which inhibits the axonal growth across the midline of one AB cell population. This in turn explains the occurrence of the “middle” phenotype, however the same gene is most certainly also important or linked to another gene which segregates the AB innervation from the midline.
In wildtype adult flies the AB cells on the left hemisphere must pass the midline to innervate to become the AB, whereas the cells on the right hemisphere don’t have to pass the midline to innervate.
Considering this wildtype phenotype and the occurrence of “middle” phenotypes, genes could interact with the axonal growth cone of the AB cells, either promoting or inhibiting signal pathway genes, which in turn means that those genes can’t pass the midline or those genes, don’t promote the AB cell growth to the other hemisphere [47]. What we also must take in consideration is that early findings suggest that there are 3 distinct locations in the early development of the fly where AB cells form innervation, one on the left hemisphere, one on the right hemisphere and one in the midline, in the middle of those two innervations. This could suggest that early on the innervations are degenerated on those sites, which have genes promoting the degeneration.
Constructing a thorough model for the AB cell development is difficult with the current results, with the most promising being that different genes act upon each other in a cascade with target location information.

6.8 Characterization of the AB cells (Further studies)

This study had the aim of characterizing the AB cells through RNAi during the development of the fly brain. The results indicate that there is some connection of genes knockdown with the emergence of AB cell phenotypes, thus the initial aim is covered and another result is that there are a variance of phenotypes which are not lethal, meaning there could be a cognitive or behavioural connection to these findings. Further studies must be conducted, while using the AB cell-GAL4 line for RNAi, to discuss the developmental impact of other cells upon the AB cells versus the impact of RNAi in those AB cells. Thus maybe "the" AB cell gene can be found and characterized or the cascade of genes leading up to the formation of the AB can be determined.

A null mutant AB cell could be found through MARCM, or other single cell targeted mutations, which could cover the question if this structure is essential in the fly brain or if it can be somewhat compensated by other parts of the brain. Since this null mutant was not found during the entire screen, the structure is either essential for normal development or the gene promoting the innervation is an essential gene.

Another interesting results would be the behavioural or cognitive change due to having the AB cell innervation on the left Hemisphere. The same approach as in the initial study could be used. Also, upon finding the null-mutant, behaviour of flies with it are even more interesting, will those flies have an impact on short and or long-term memory or is the effect of having a double innervation reverted?

Furthermore, pre- and post-synaptic neurons can be localised using split GFP or GRASP, to further analyse the role of those neurons in the central complex.

Further analysis of candidate genes could be done using other GAL4 lines in more specific parts of the brain, to classify the range of cells, which impact the AB development. Most promising being the gene-knockdown of neurons directly connecting pre or postsynaptic.

To diminish the probability of screening a brain under the microscope, with the false assumption of screening a UAS-RNAi expressing fly, a reporter gene product like UAS-GFP could be used on the same chromosome as the UAS-RNAi. This would enable the segregation of brains which express the UAS RNAi and thus also express the reporter and none UAS-RNAi expressing brains.

To analyse if a 100% knockdown of the gene can be accomplished in the brain a double knockdown using *ELAV-Gal4* with *442-Gal4* can be used, the phenotype resulting of these experiments could be different from what has been observed already.

To further study the axonal growth of AB cells, the developing brain must be screened at different developmental stages, to easier interpret the gene function of those tested in the

RNAi-Screen. Thus, a model of the mechanism underlying the AB cell formation can be made.
The distribution of the RNAi-Screen genes could be different on left to right hemisphere or to left to right AB cells or to left to right glia cells, or to left to right fan-shaped body cells, which in turn could promote or inhibit the growing, the innervation or the migration of the axonal growth cone or the formation of pre-synapses.
This study has shown that several genes are indeed directly or indirectly regulating the innervation of the AB cells, further studies with different attempts to further analyse this structure, such as trying to find the null-mutant, must be made to characterize the function of the AB cells.

7 Bibliography

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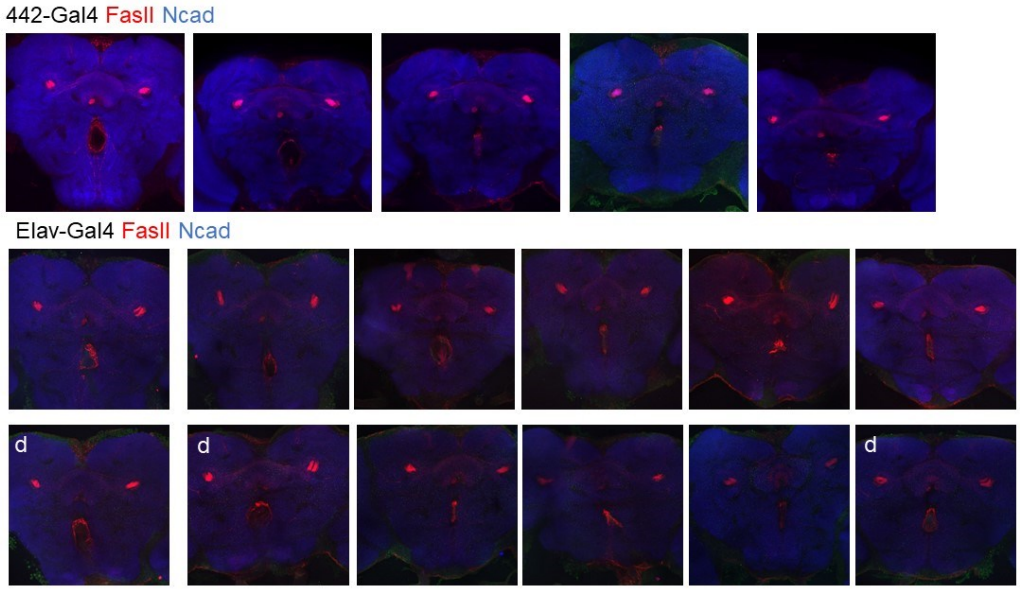
Attachements

Attachment: Figures of the screen

Generally, antibody staining is colour coded into the figure. The antibody staining with Ncad in blue (647nm) and FasII in green/red (488nm, 568nm) colorization. “double AB” phenotype was marked with “d”, middle phenotype with “m” and left phenotype with “l”

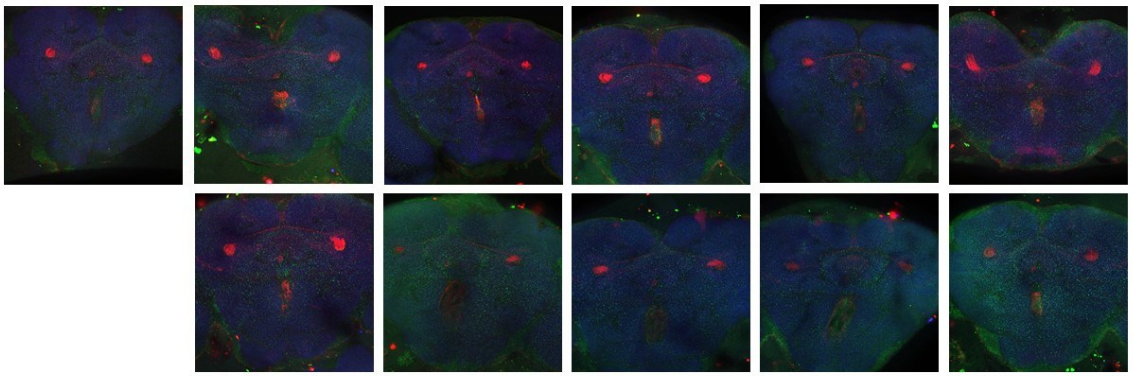
1. Initial screen

1.1. Sema-1a

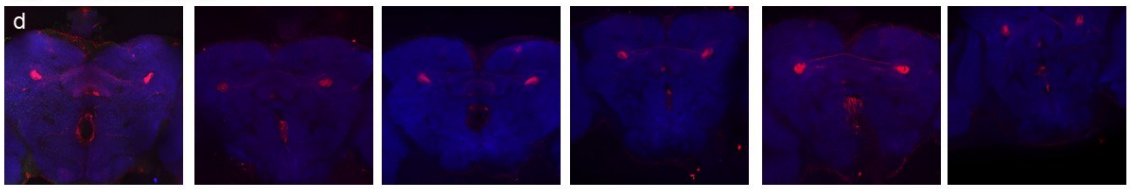


1.2.NCAD

442-Gal4 FasII Ncad

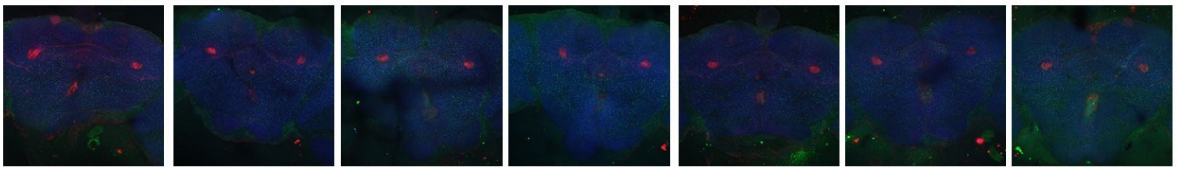


Elav-Gal4 FasII Ncad



1.3. hibris

442-Gal4 FasII Ncad



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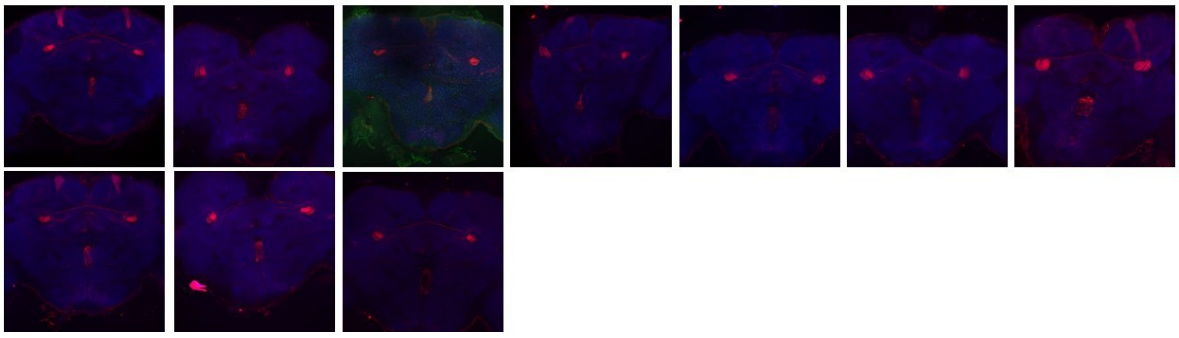
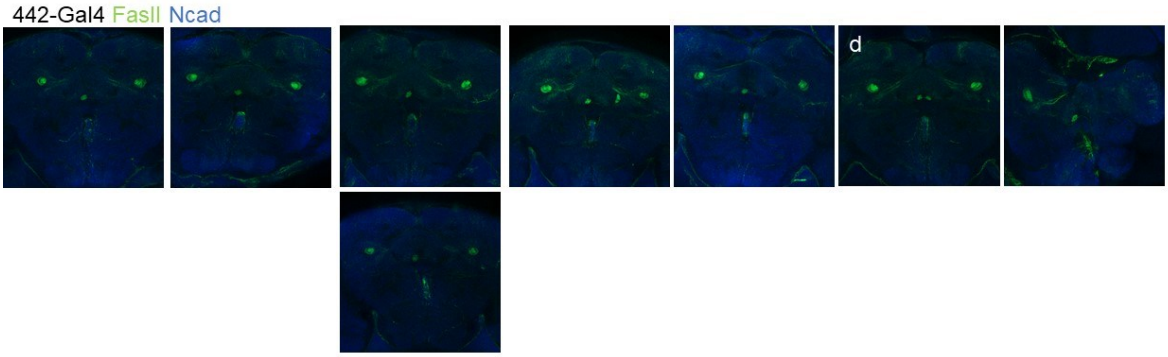
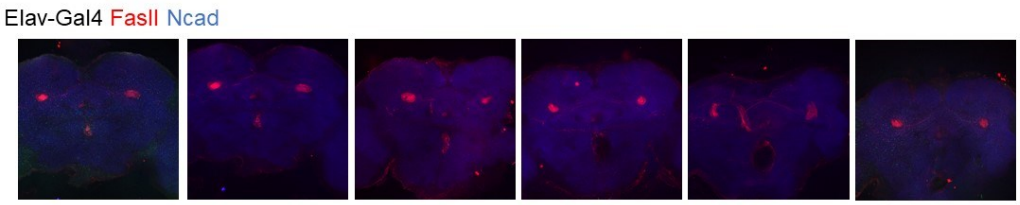


1.4. flamingo





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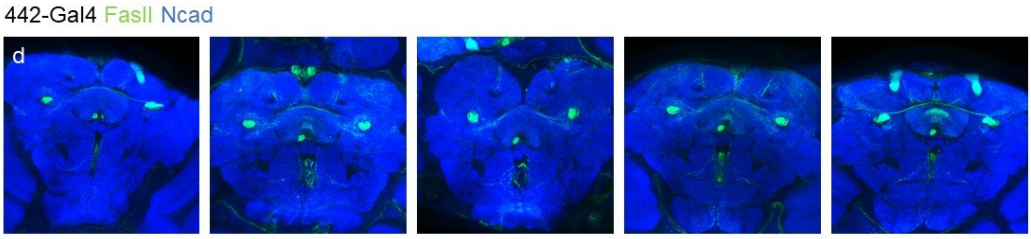
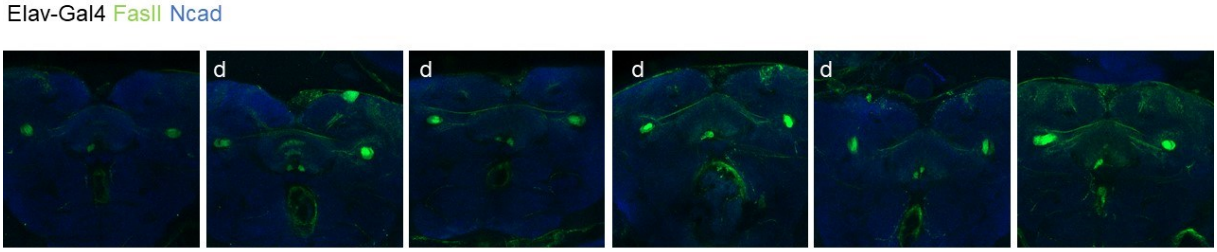
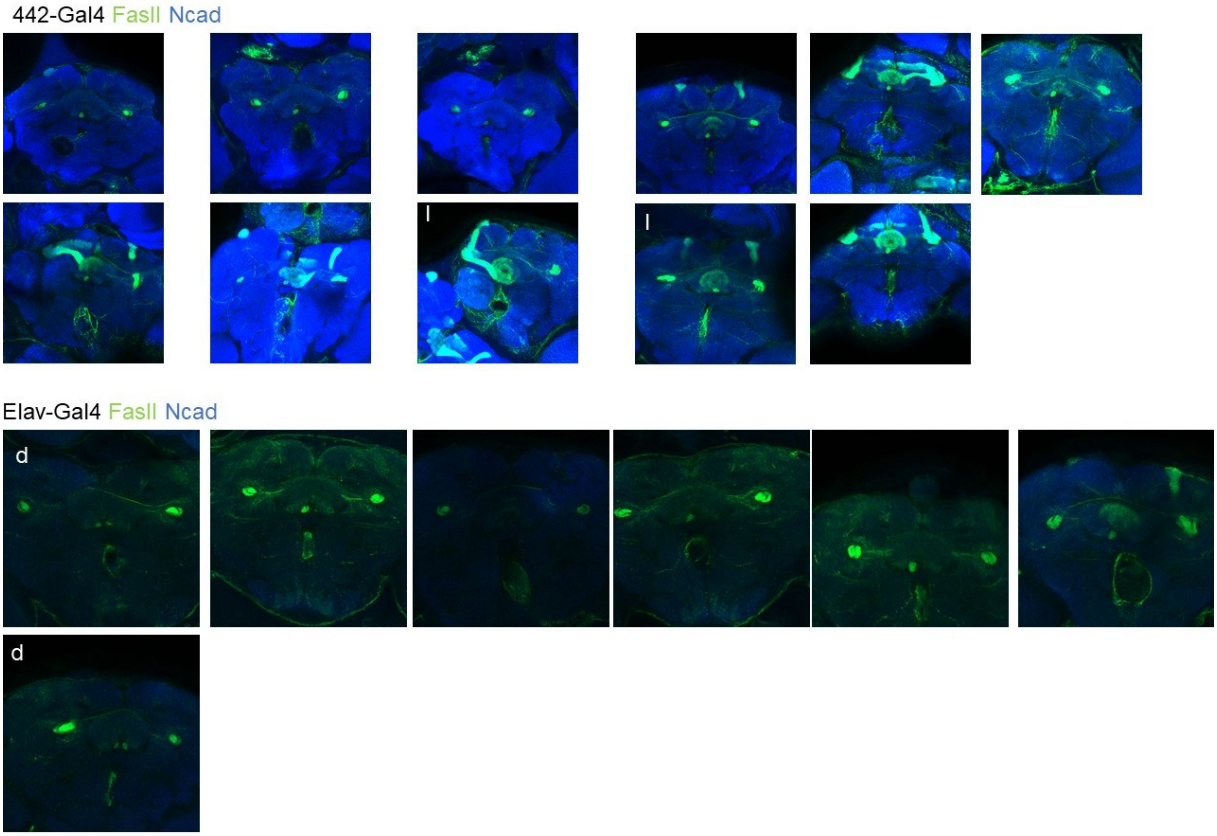
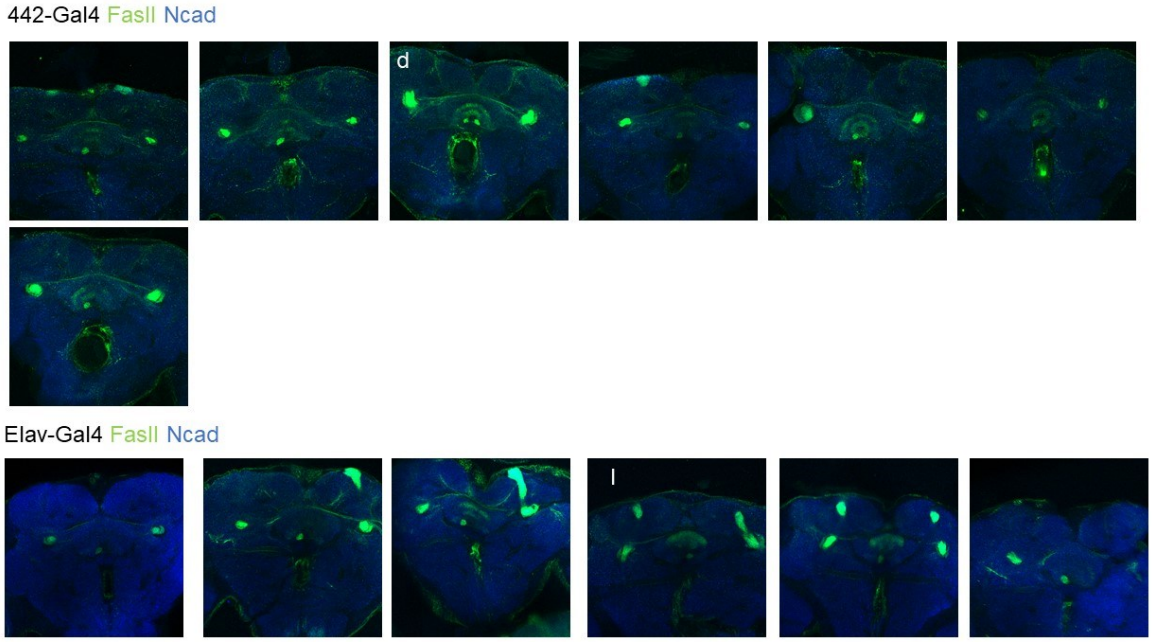




1.6. peste



1.7. ADAM metallopeptidase with thrombospondin type 1 motif A



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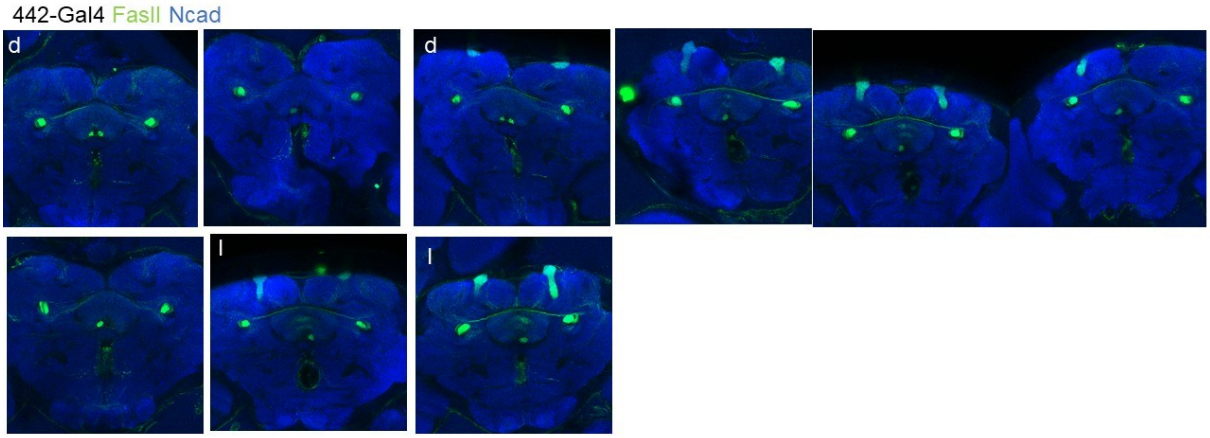
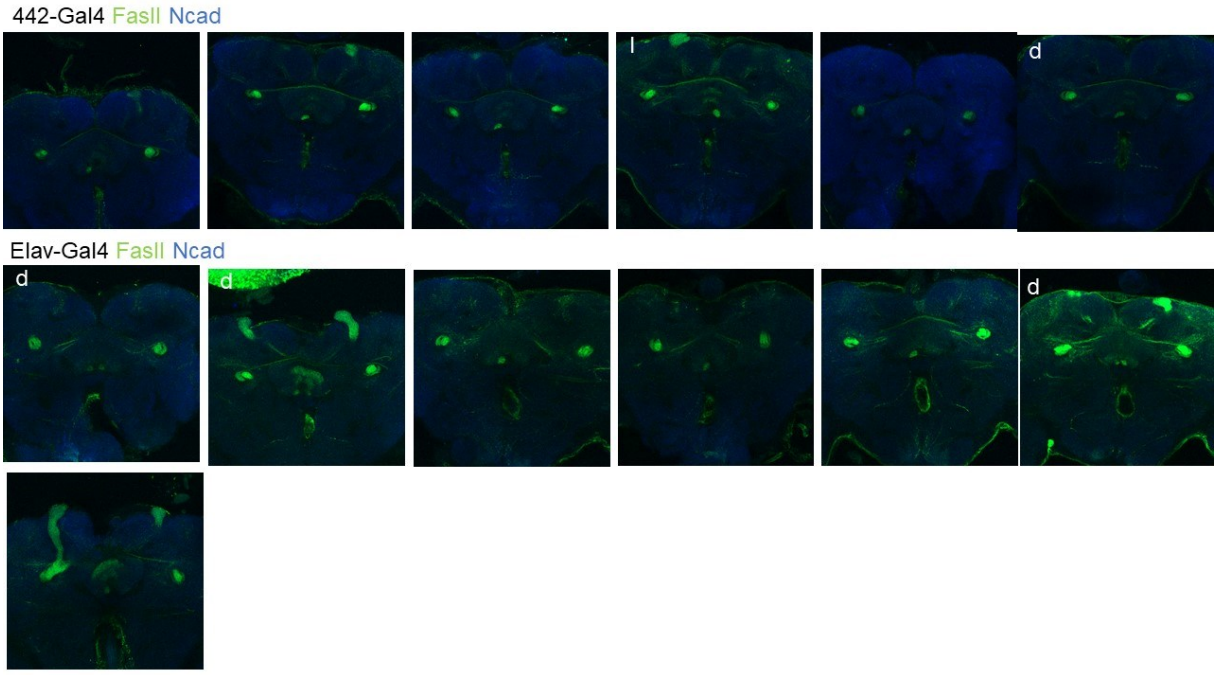
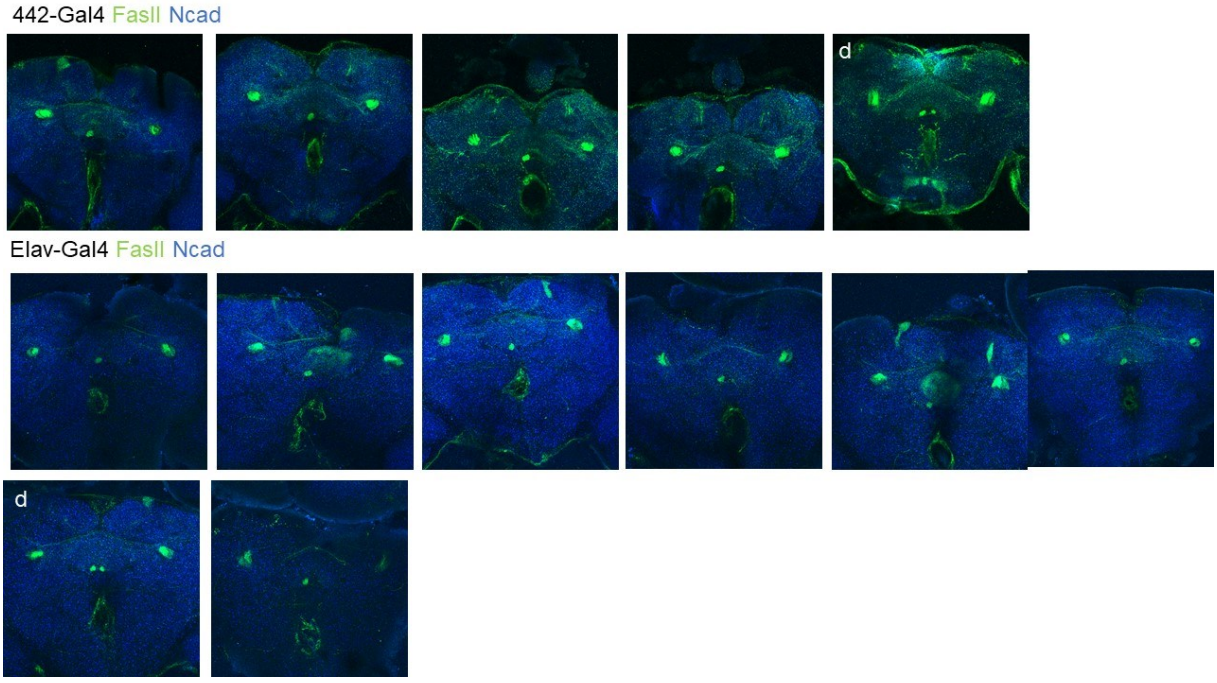


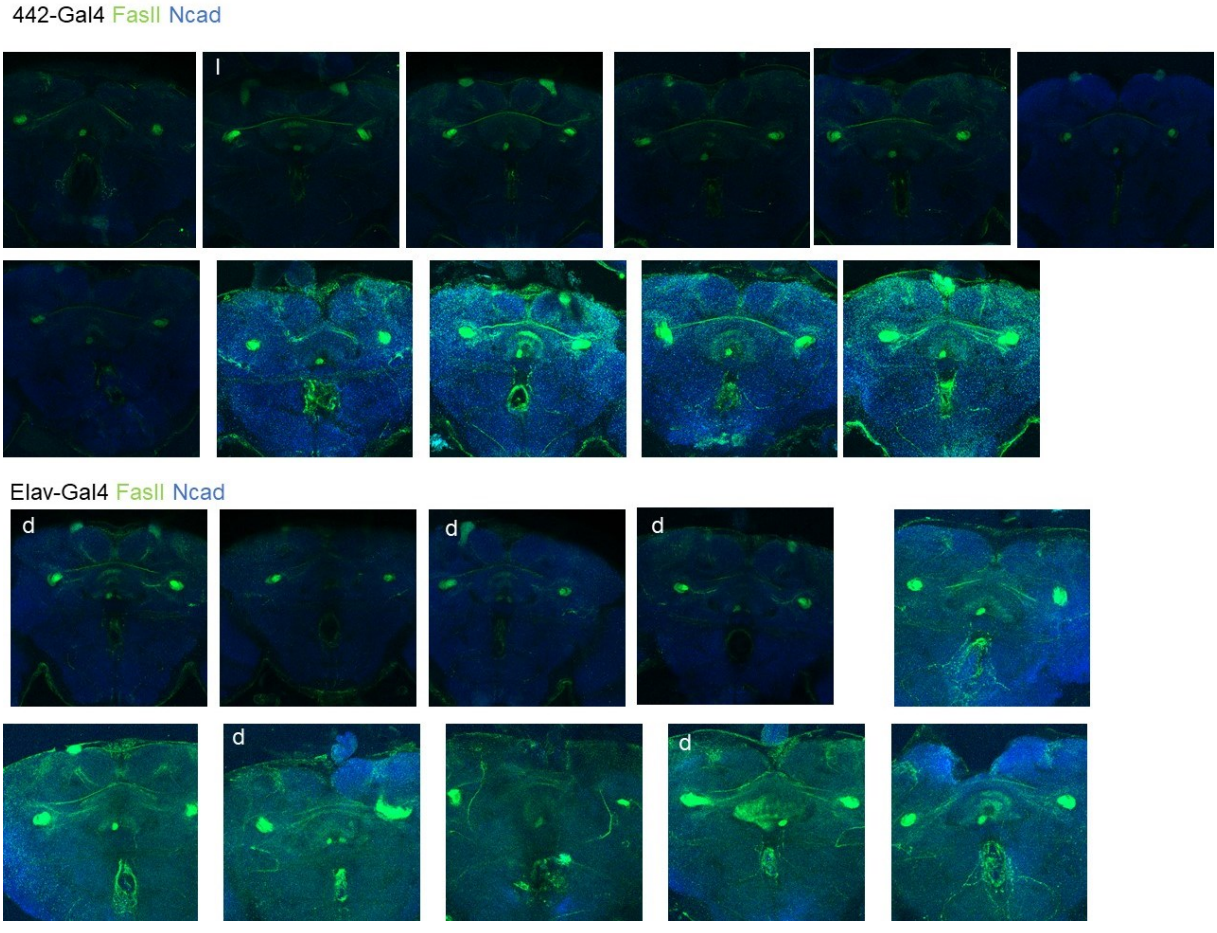
1.9. CASK ortholog



1.10. pebble (11)



1.11. microtubule star



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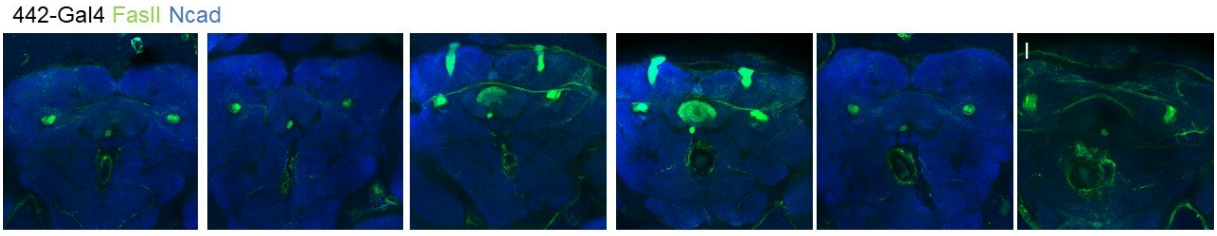

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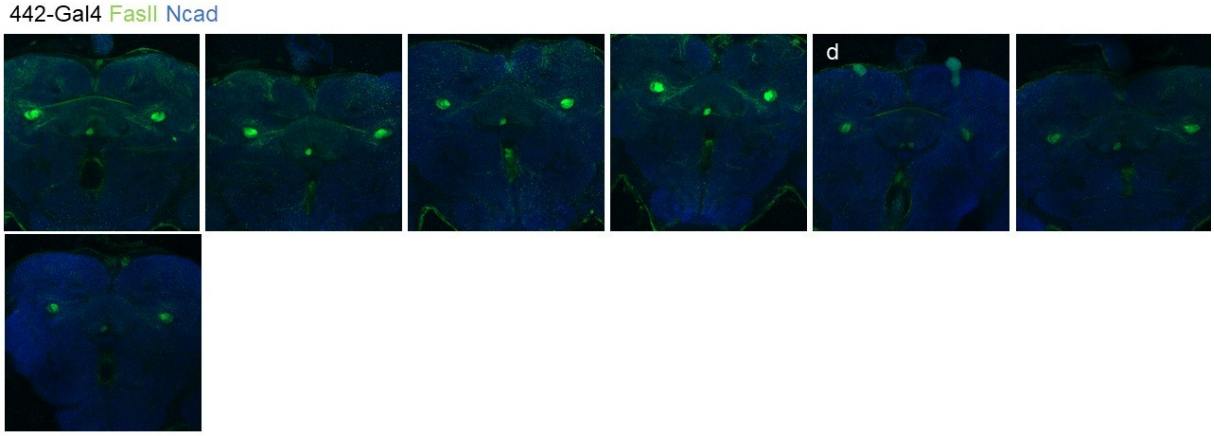
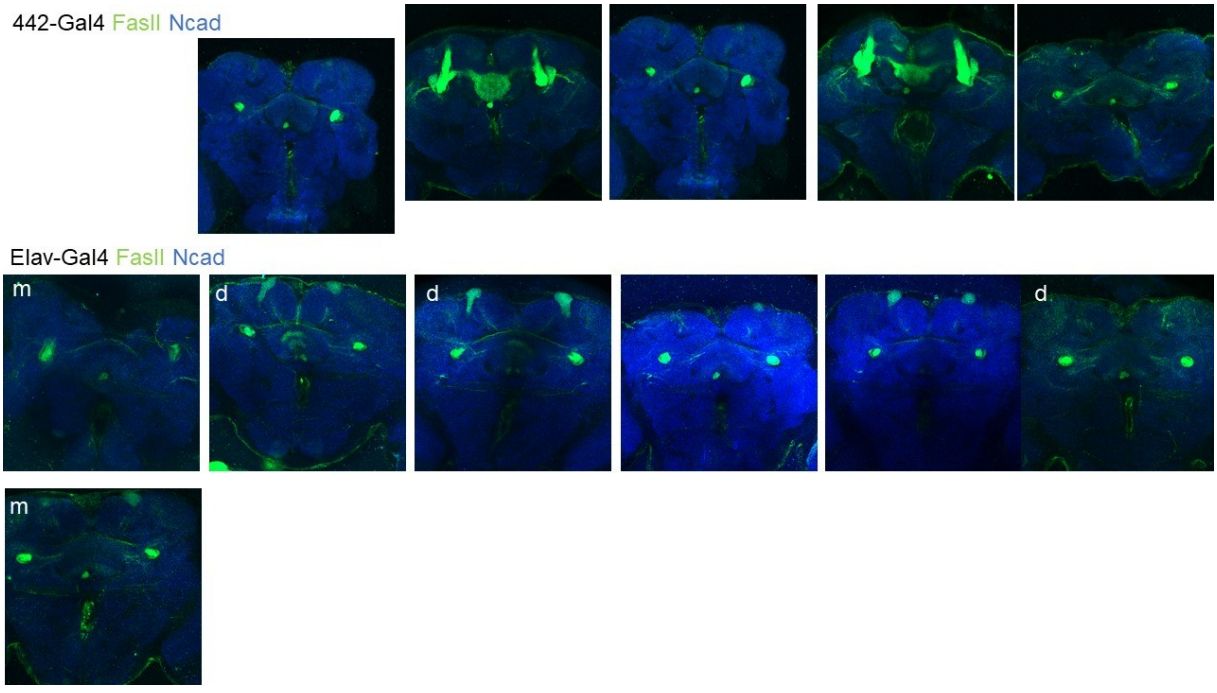


1.14. Phosphatase and tensin homolog



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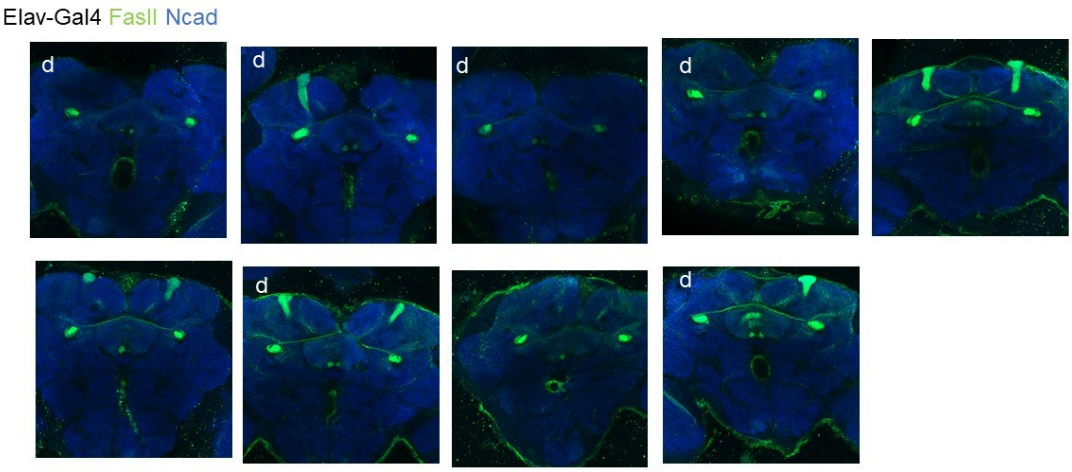

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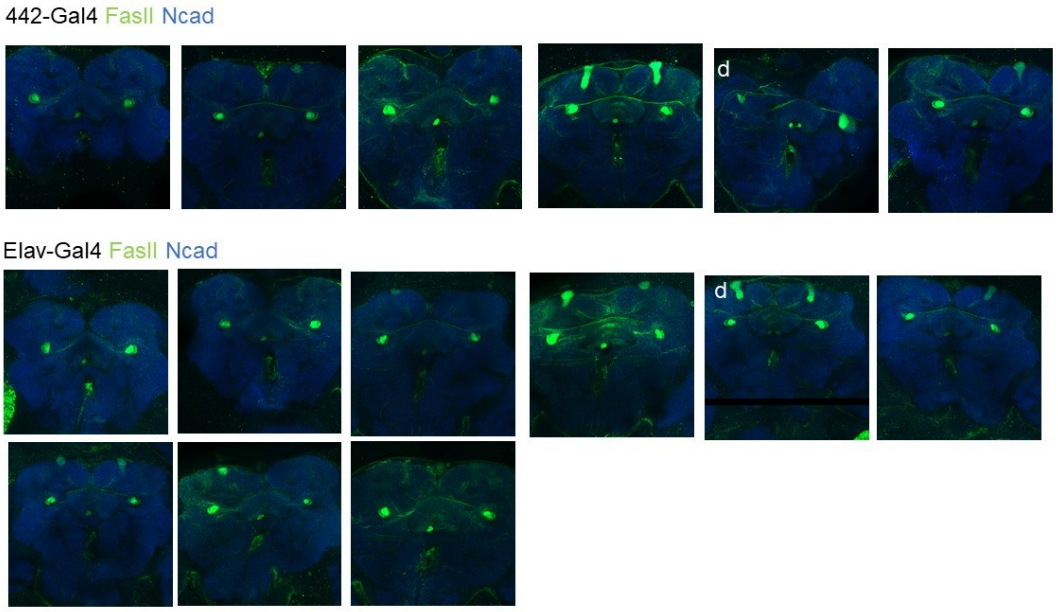
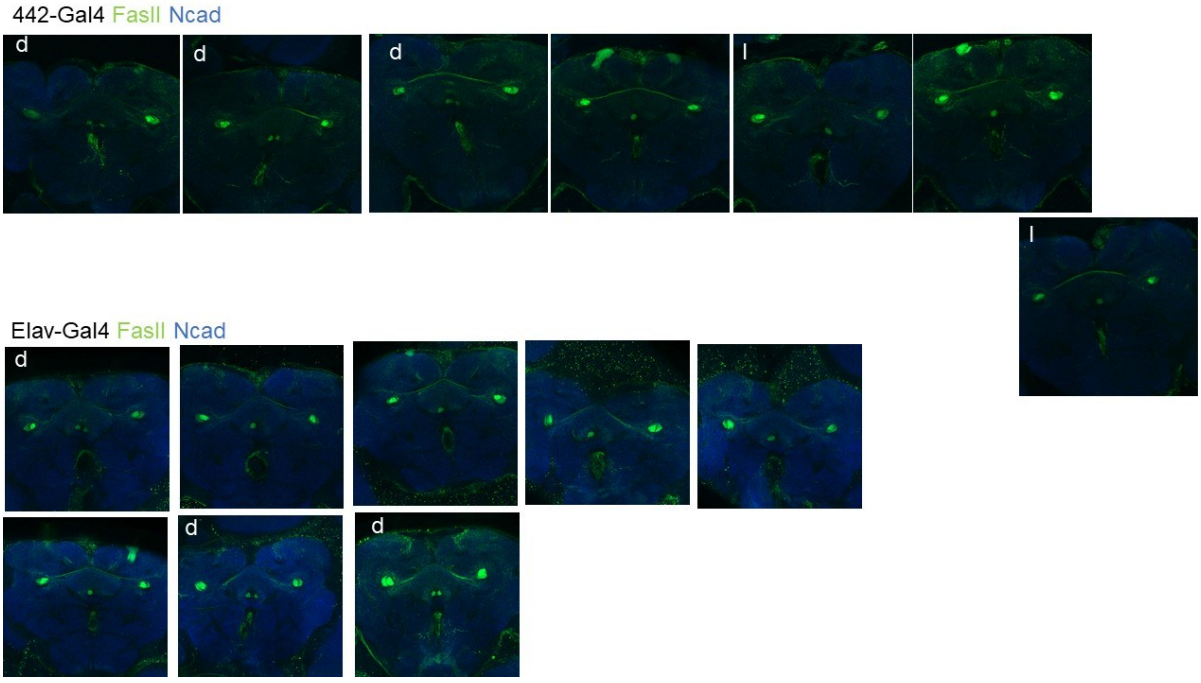
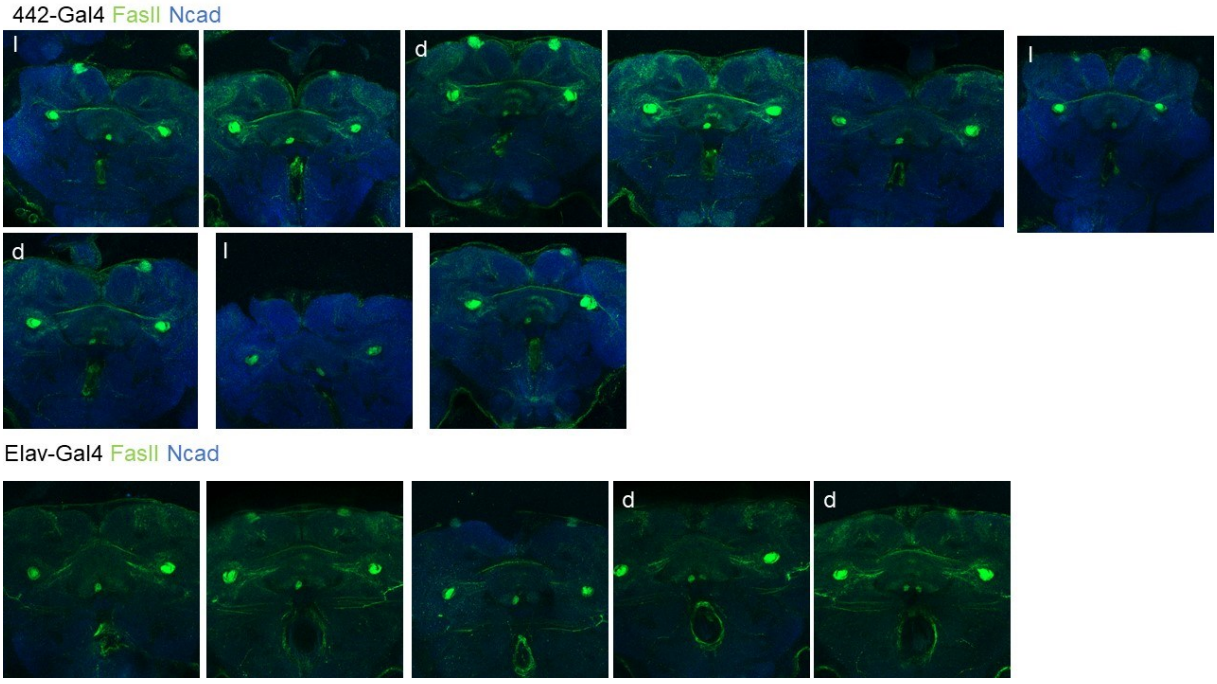


1.17. Wnt oncogene analog 4

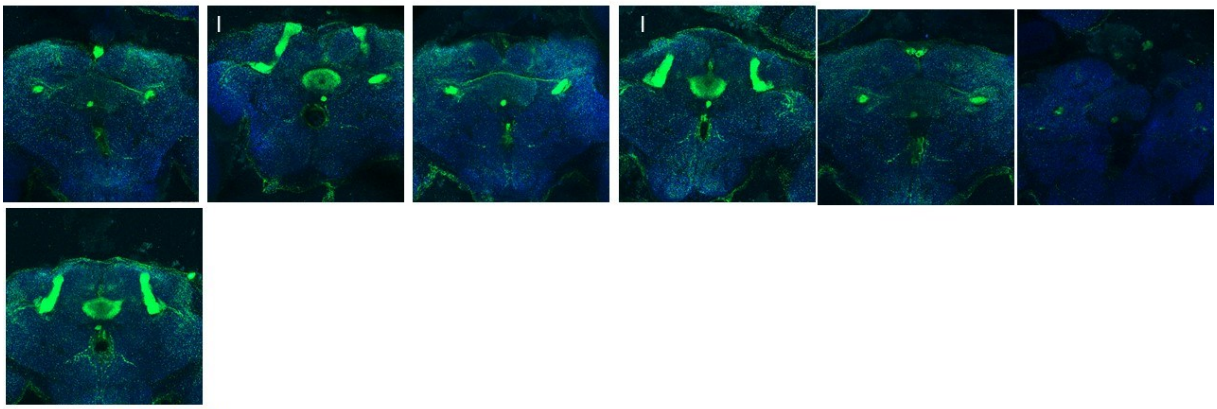


1.18. Dmel\CG18095

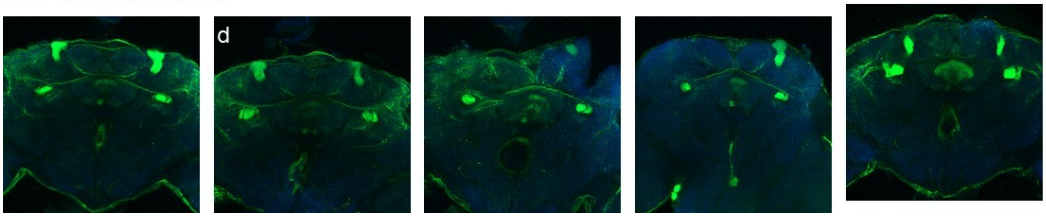


1.19. Nimrod C2

442-Gal4 FasII Ncad

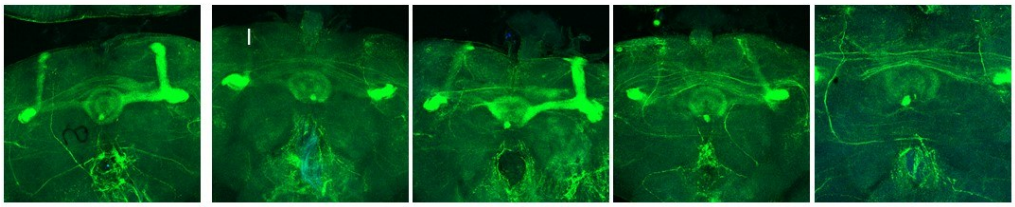


Elav-Gal4 FasII Ncad

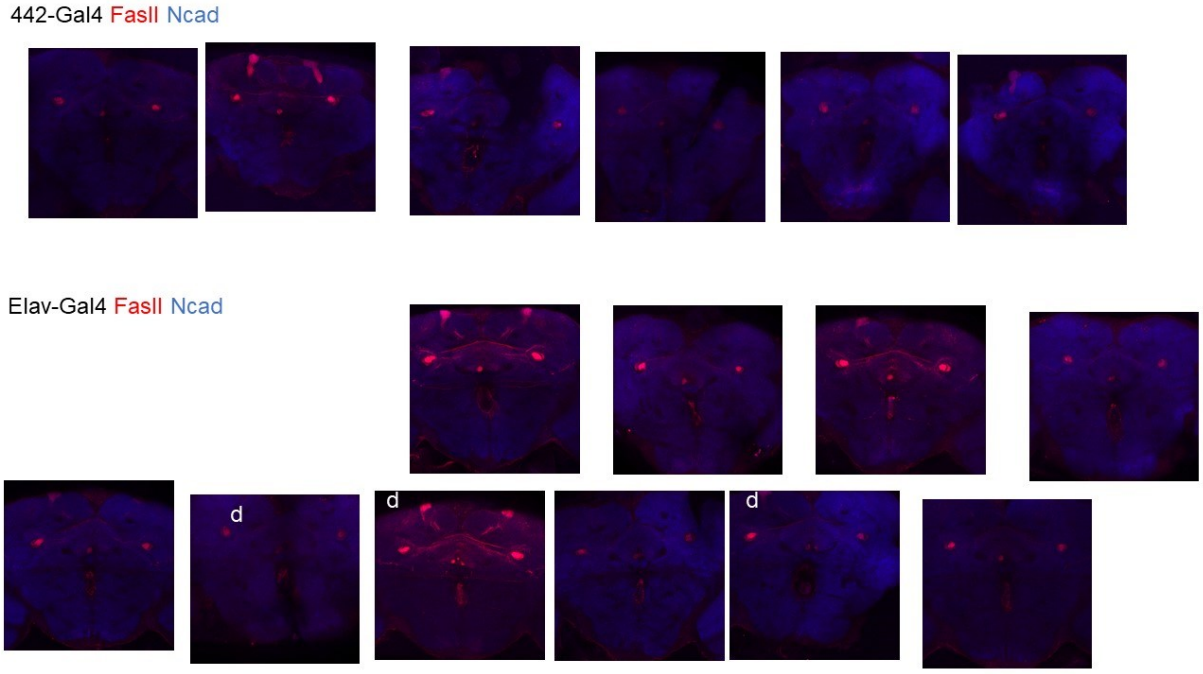


1.20. Dmel\CG17739

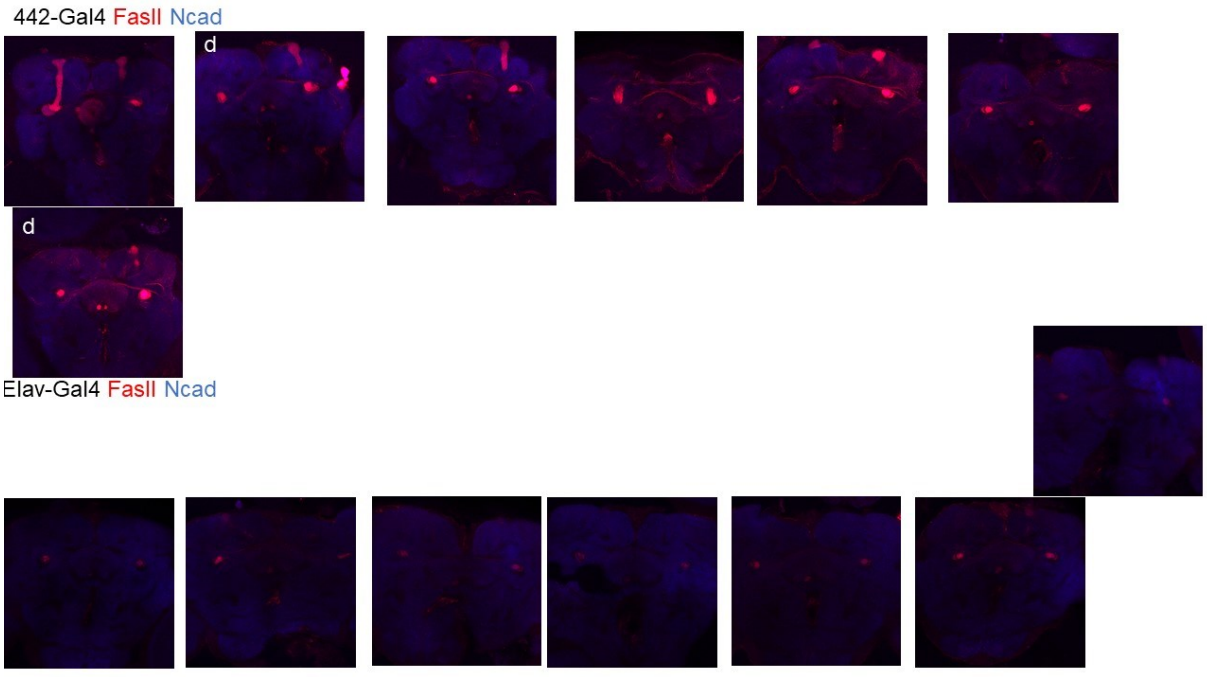
442-Gal4 FasII Ncad



1.21. Dmel\CG6867



1.22. center divider



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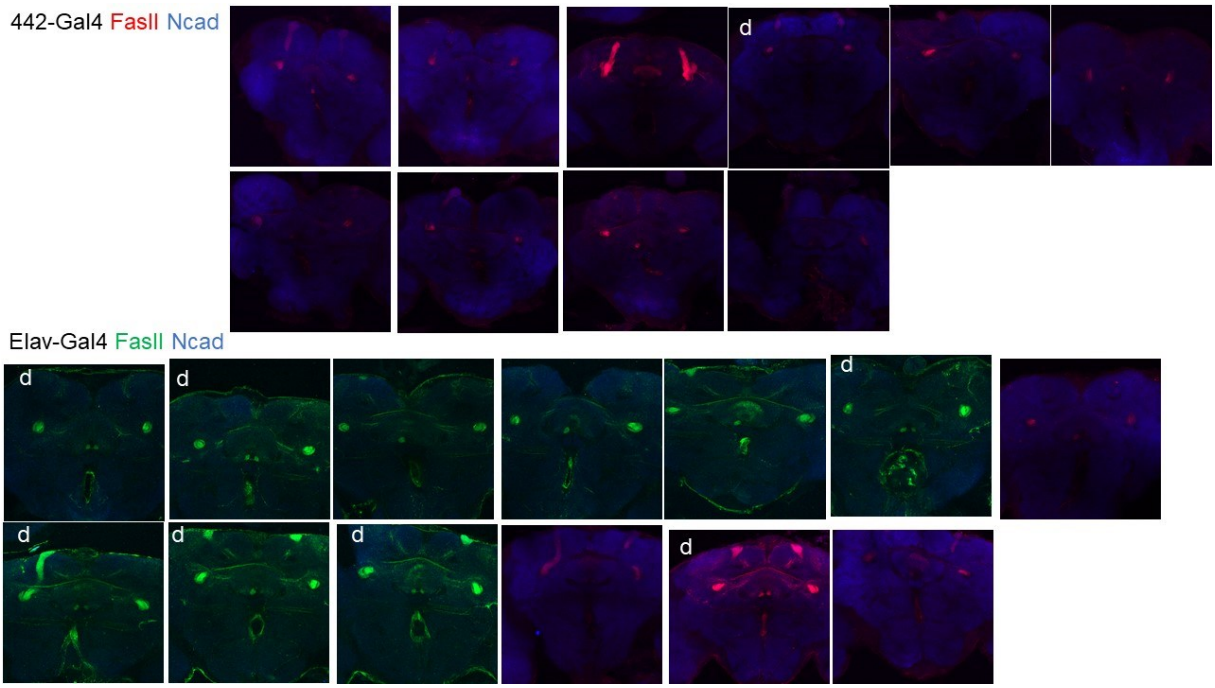

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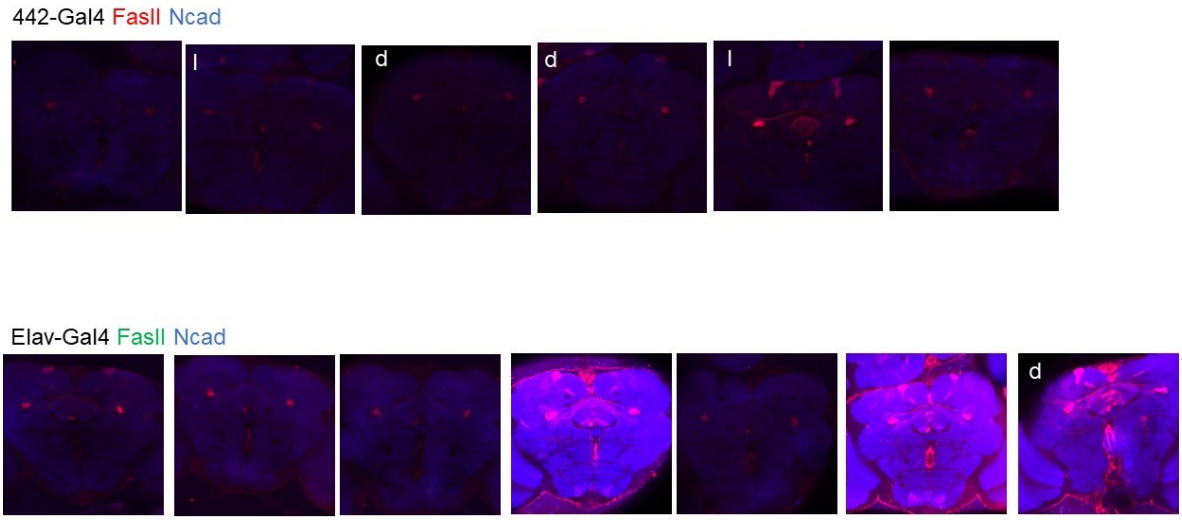

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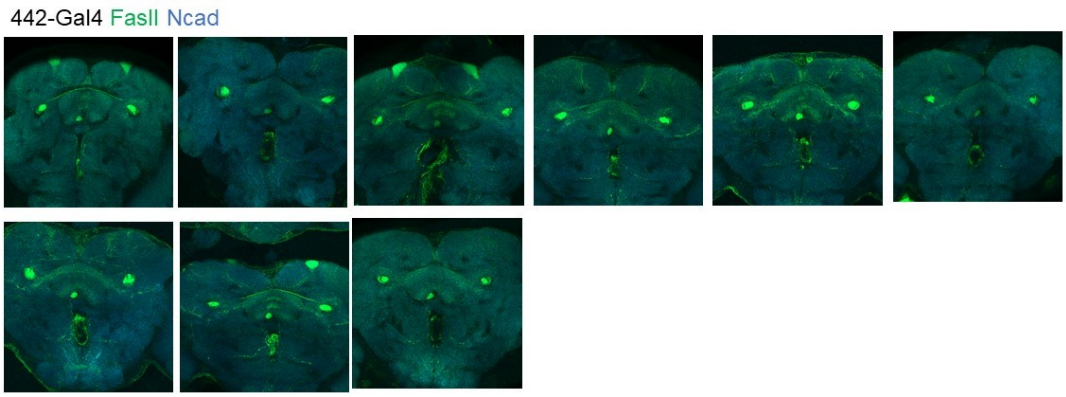
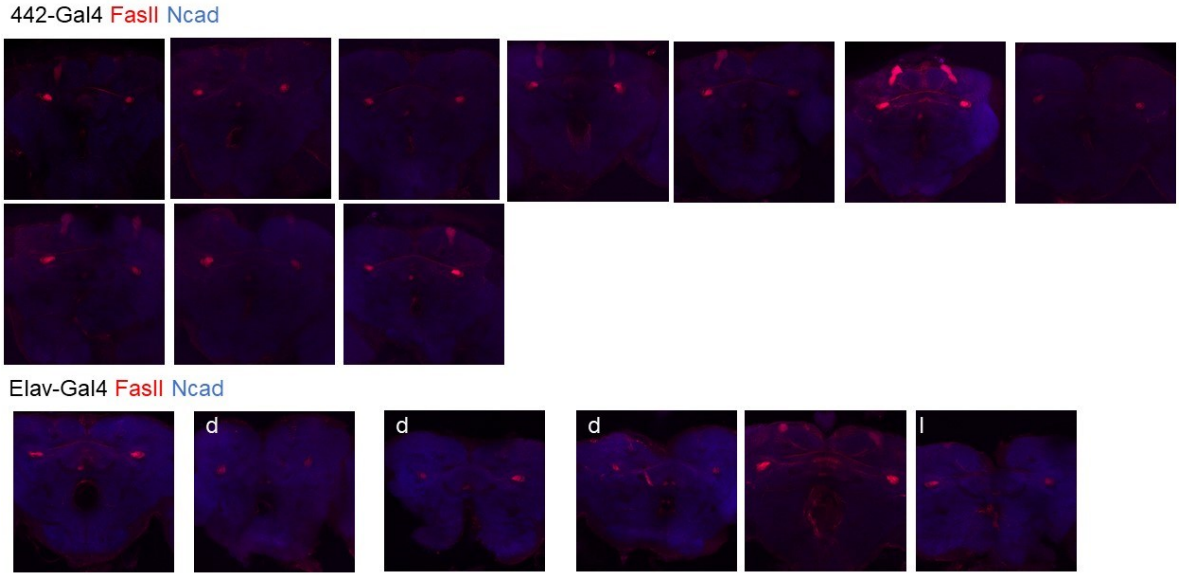


1.26. Rab18



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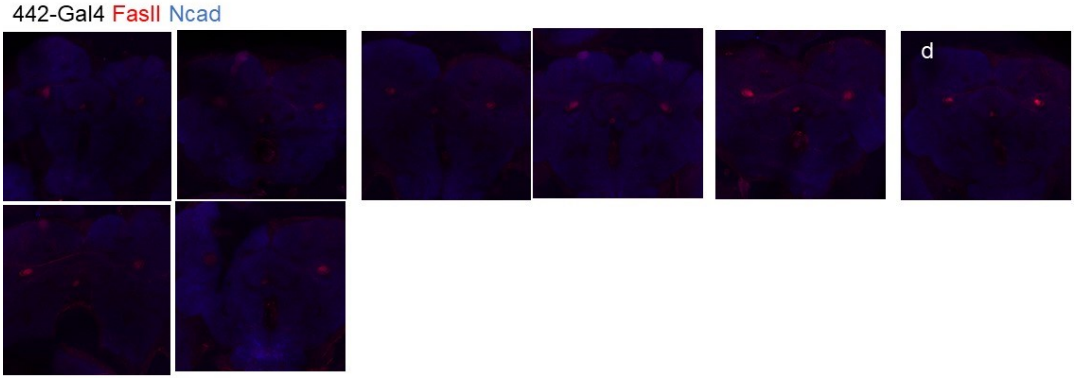

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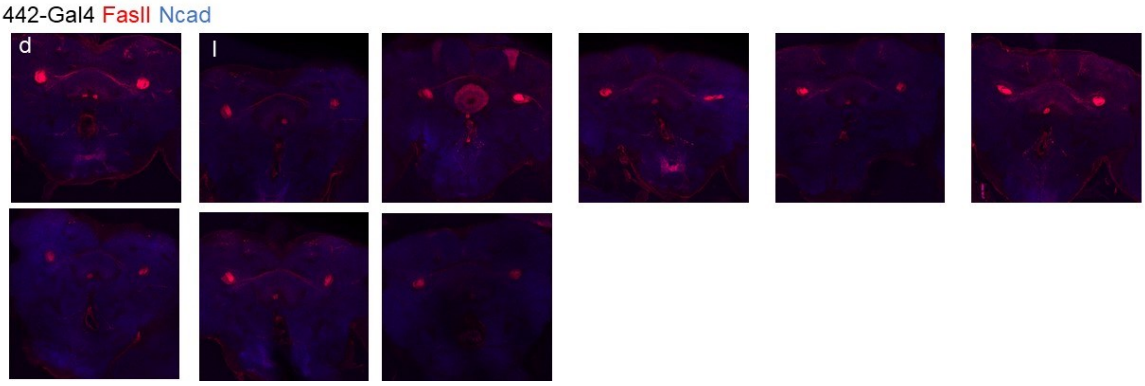

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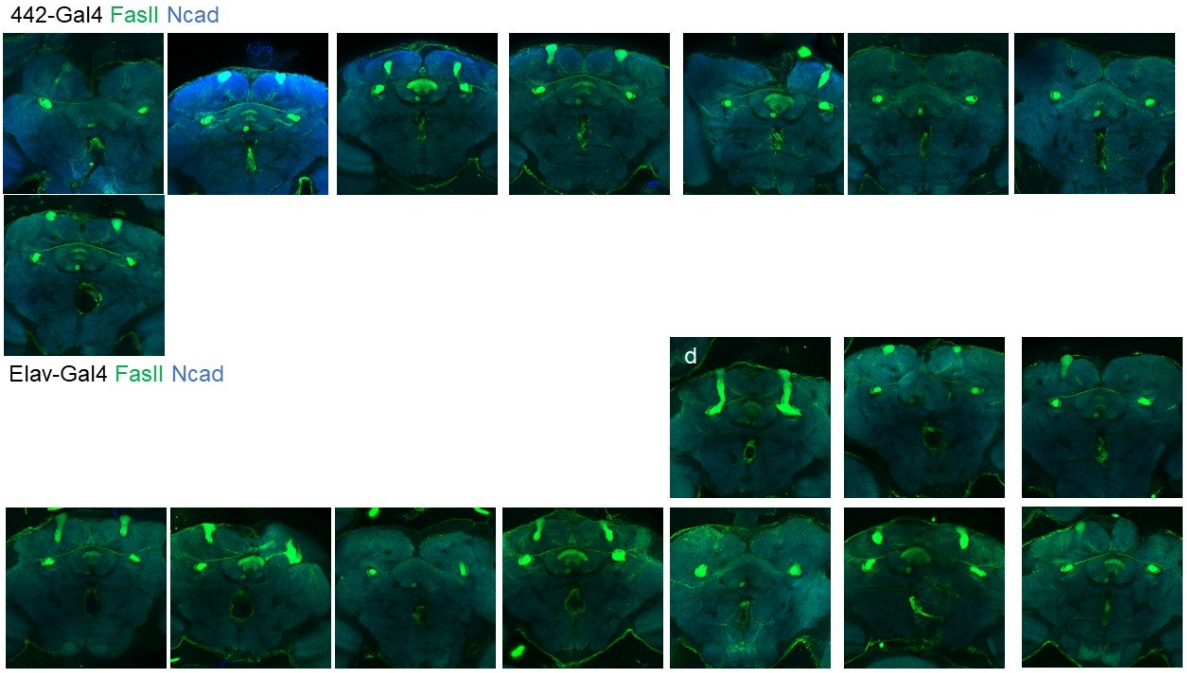
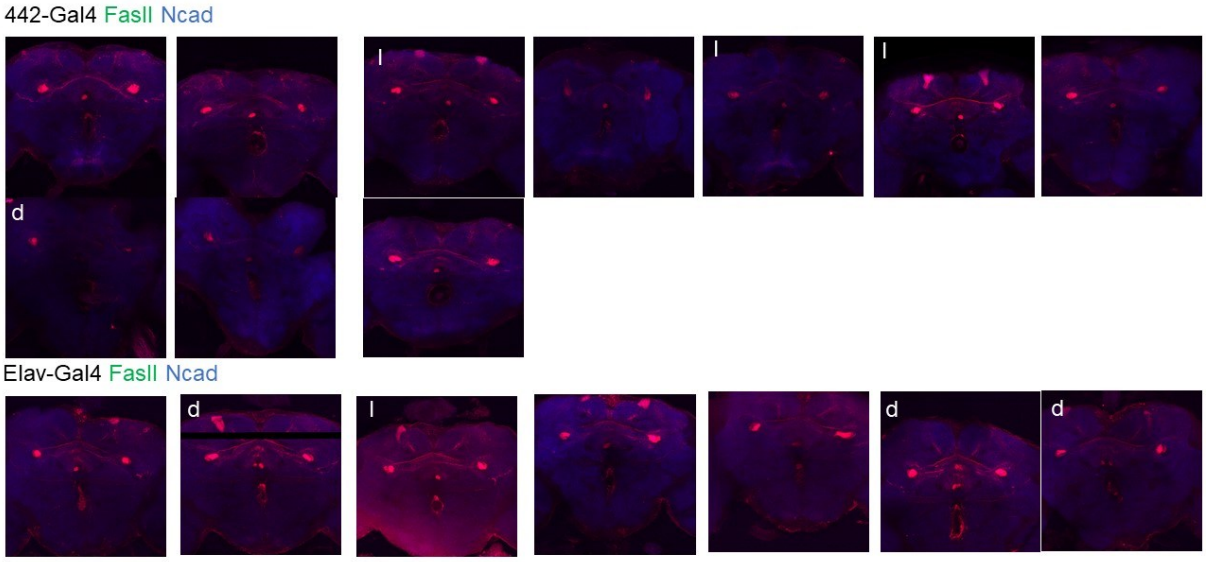
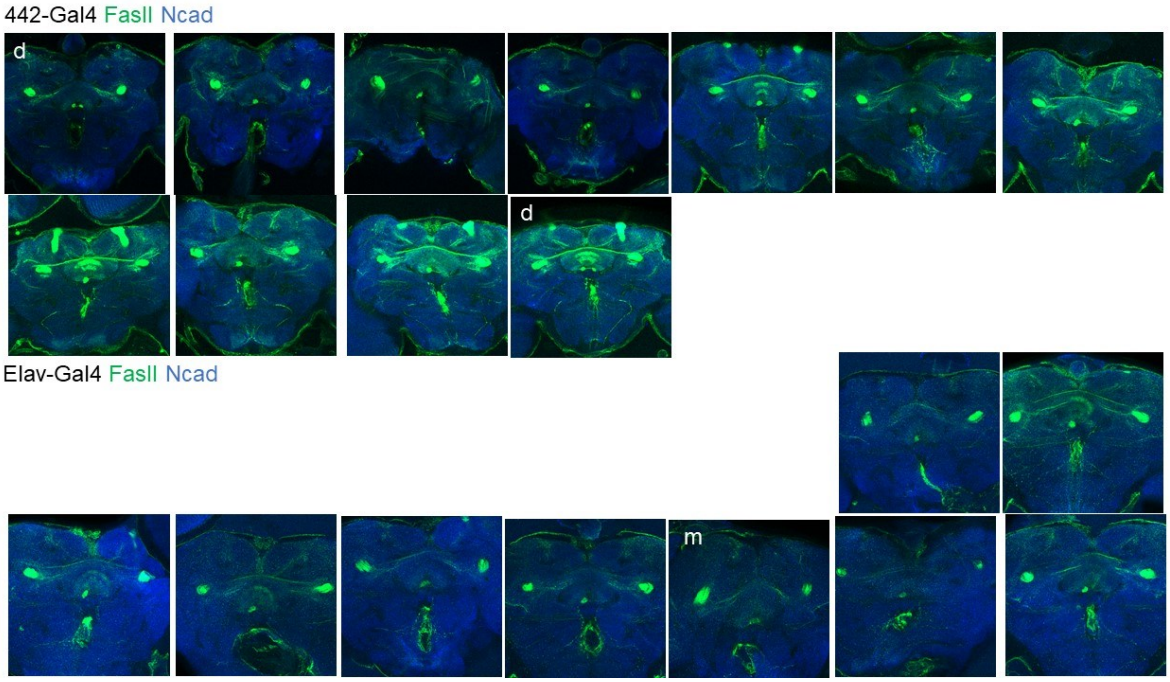


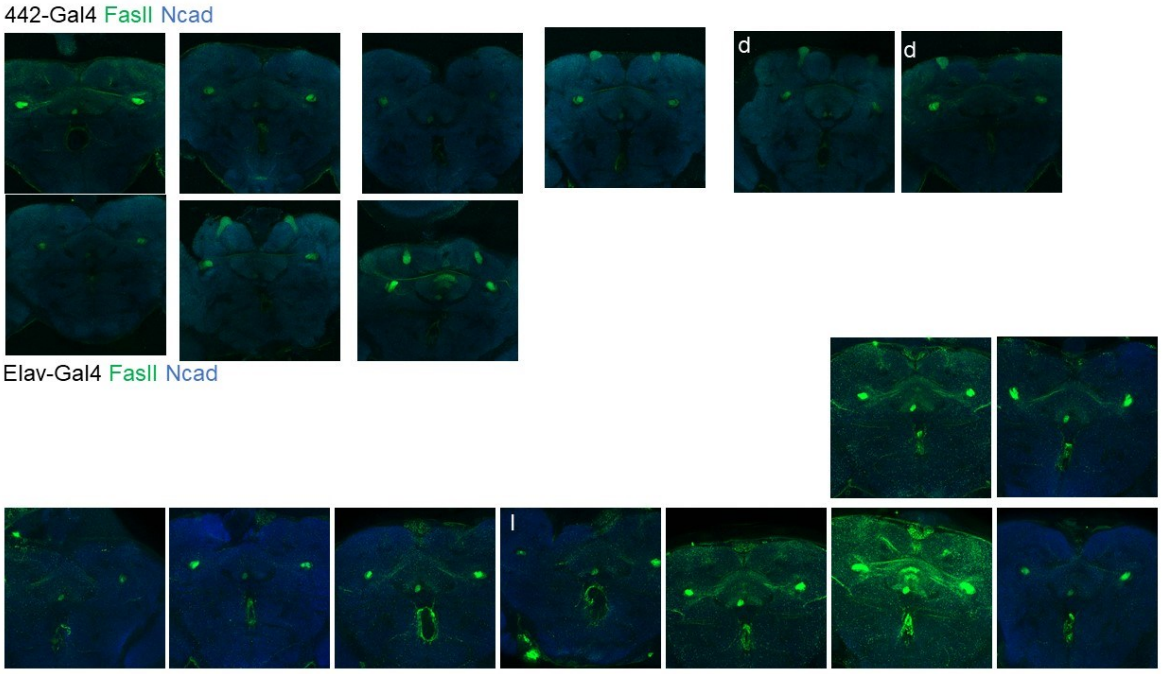
1.30. Dmel\CG6788



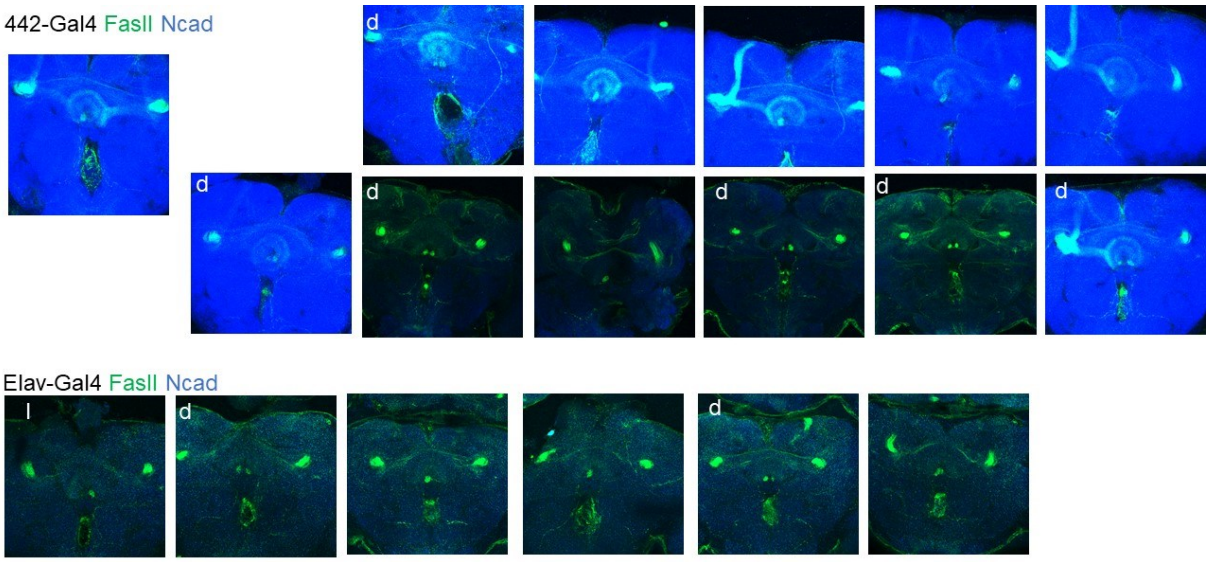
1.31. Tetraspanin 66E



1.32. lambik



1.33. discs lost



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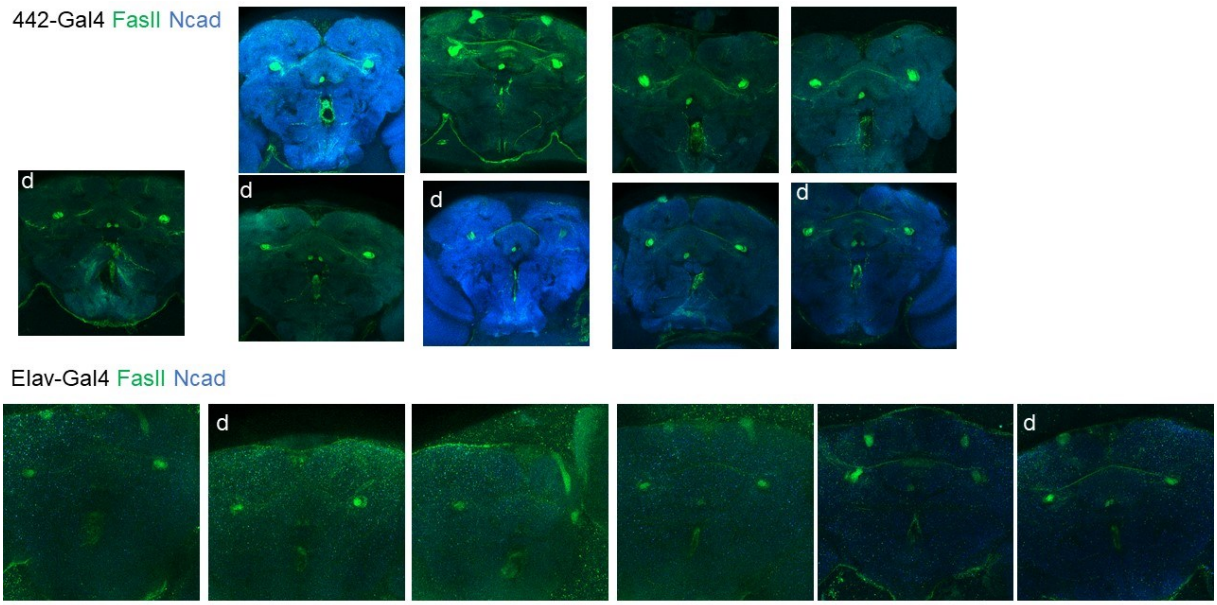
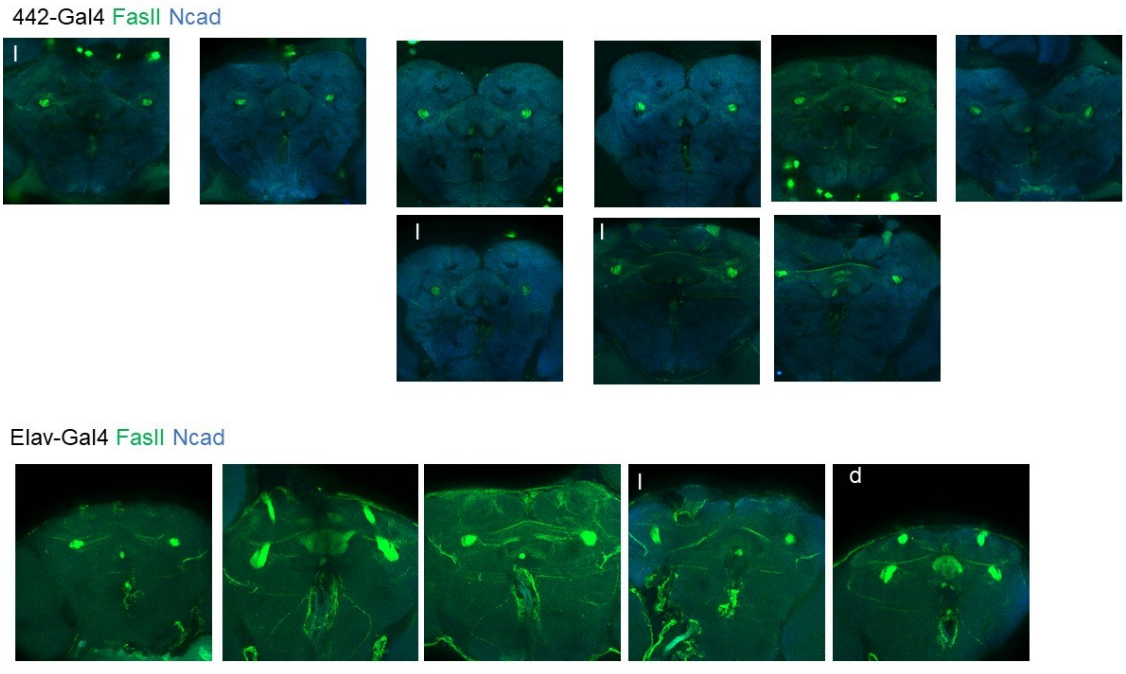
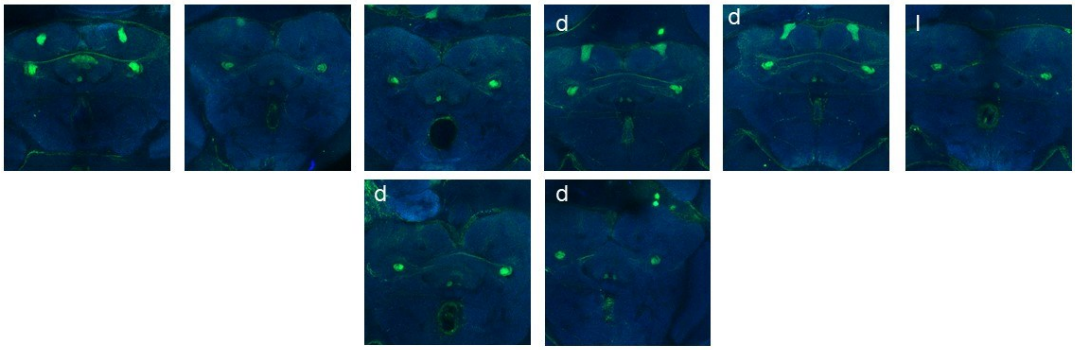


1.35. friend of echinoid



1.36. Tousled-like kinase

442-Gal4 FasII Ncad



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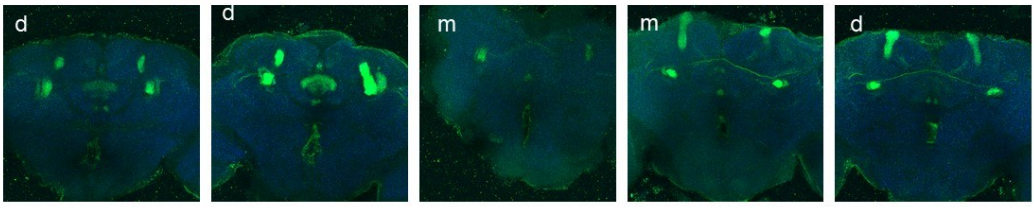
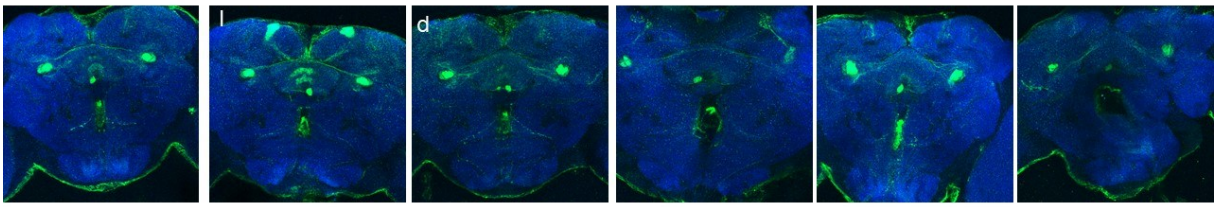


1.37. kekkon5

442-Gal4 FasII Ncad



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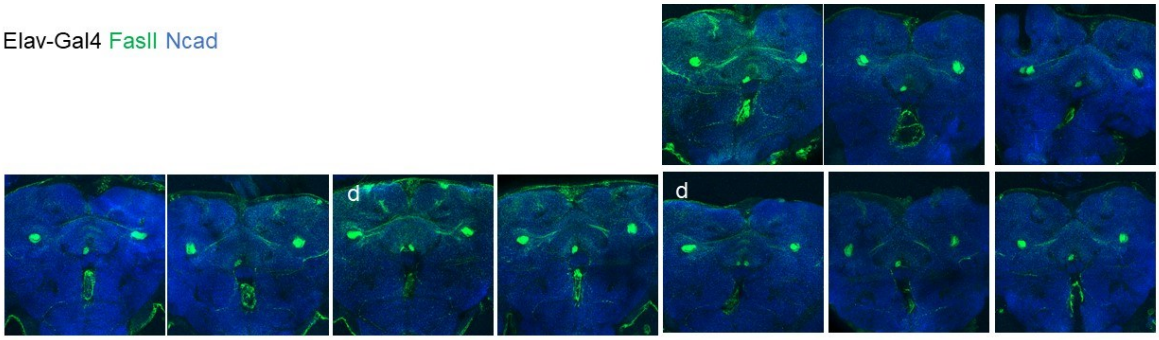
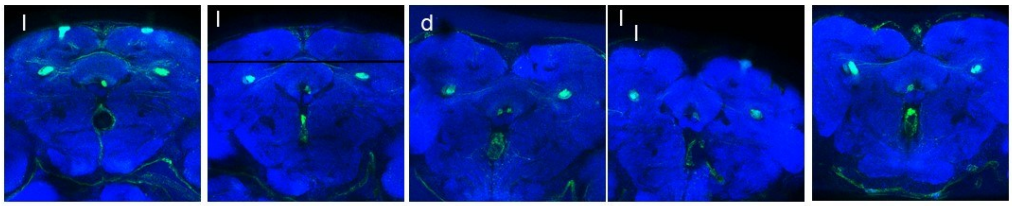
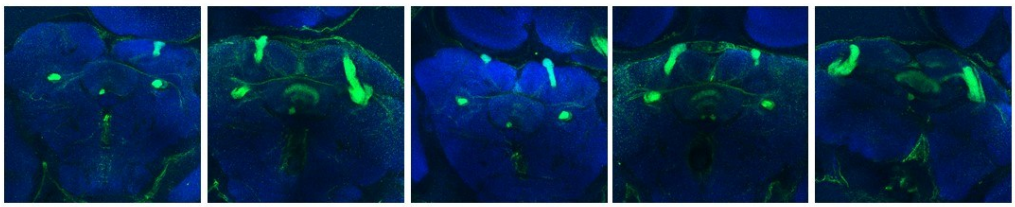


1.38. muskelin

442-Gal4 FasII Ncad

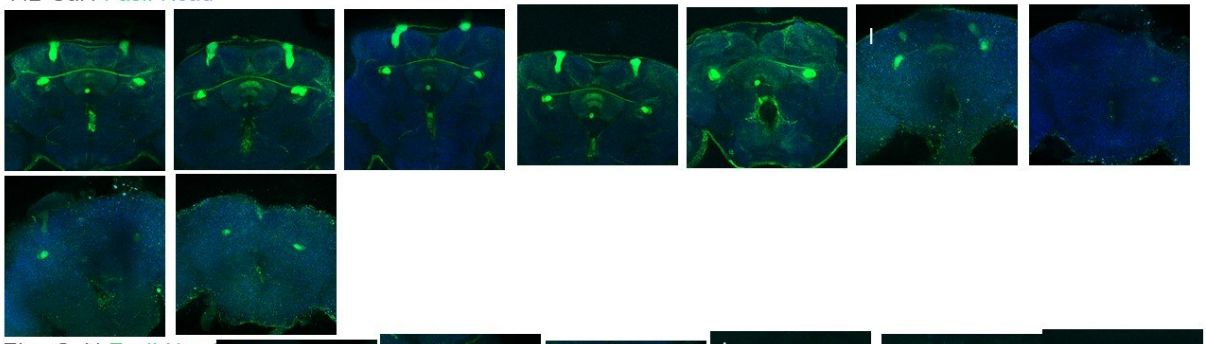


Elav-Gal4 FasII Ncad

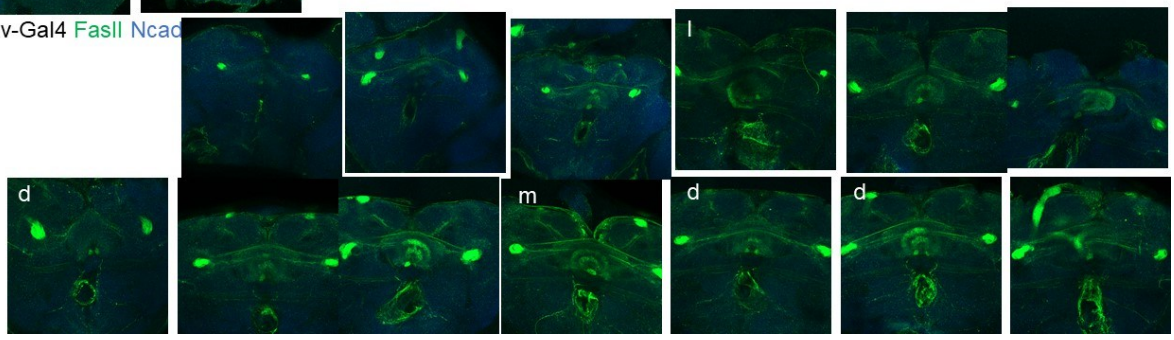


1.39. Basigin

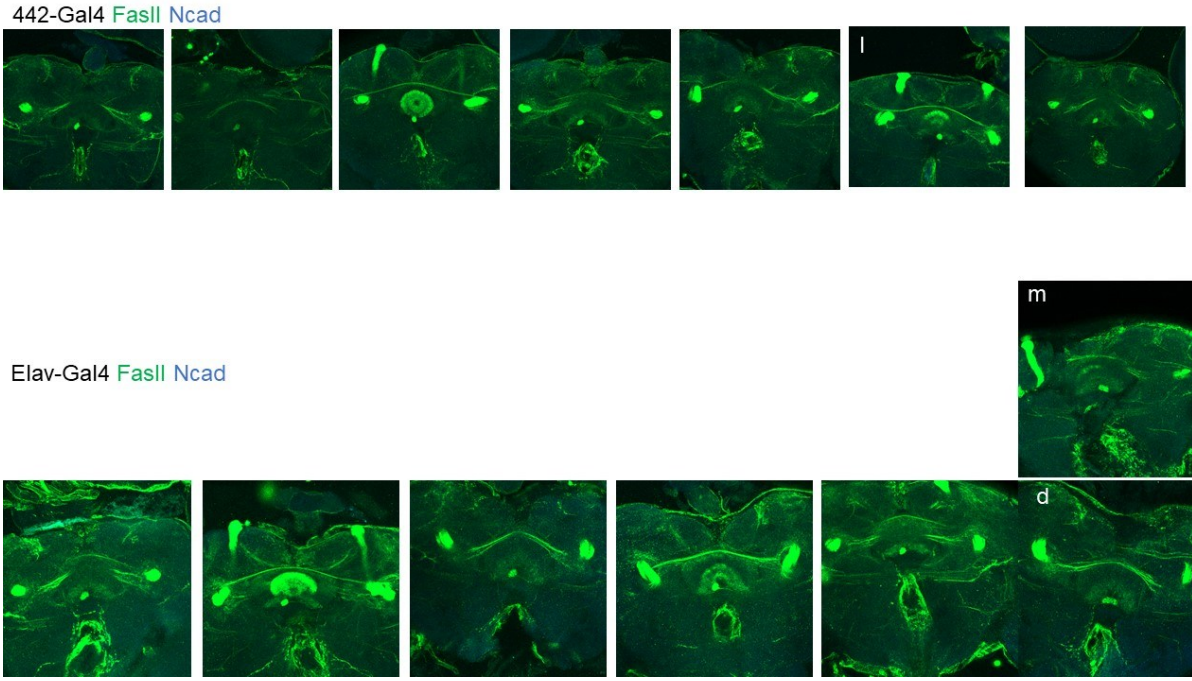
442-Gal4 FasII Ncad



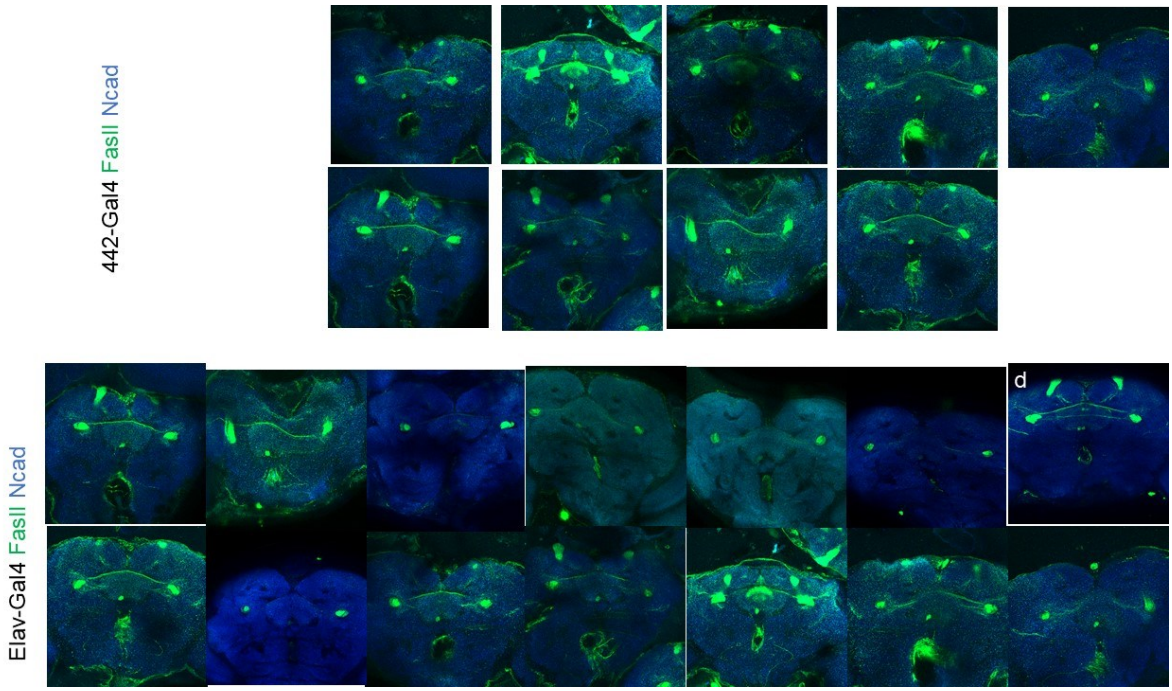
Elav-Gal4 FasII Ncad



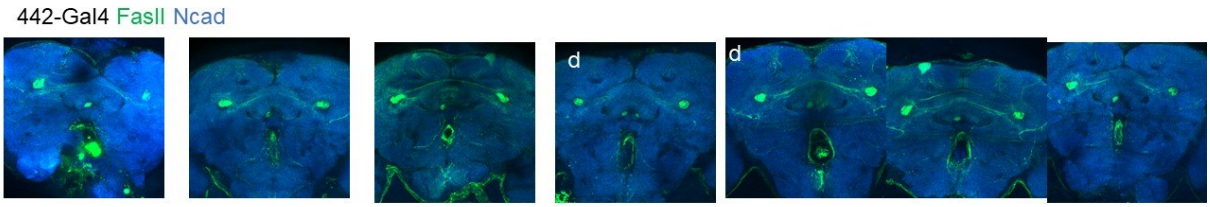
1.40. warts

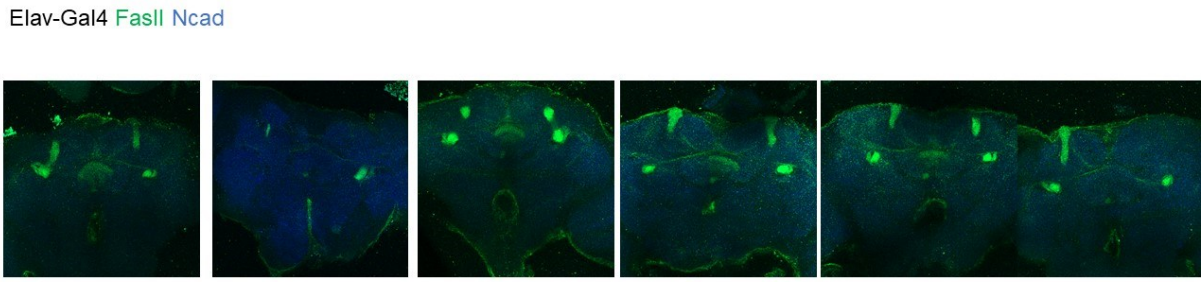


1.41. klumpfuss

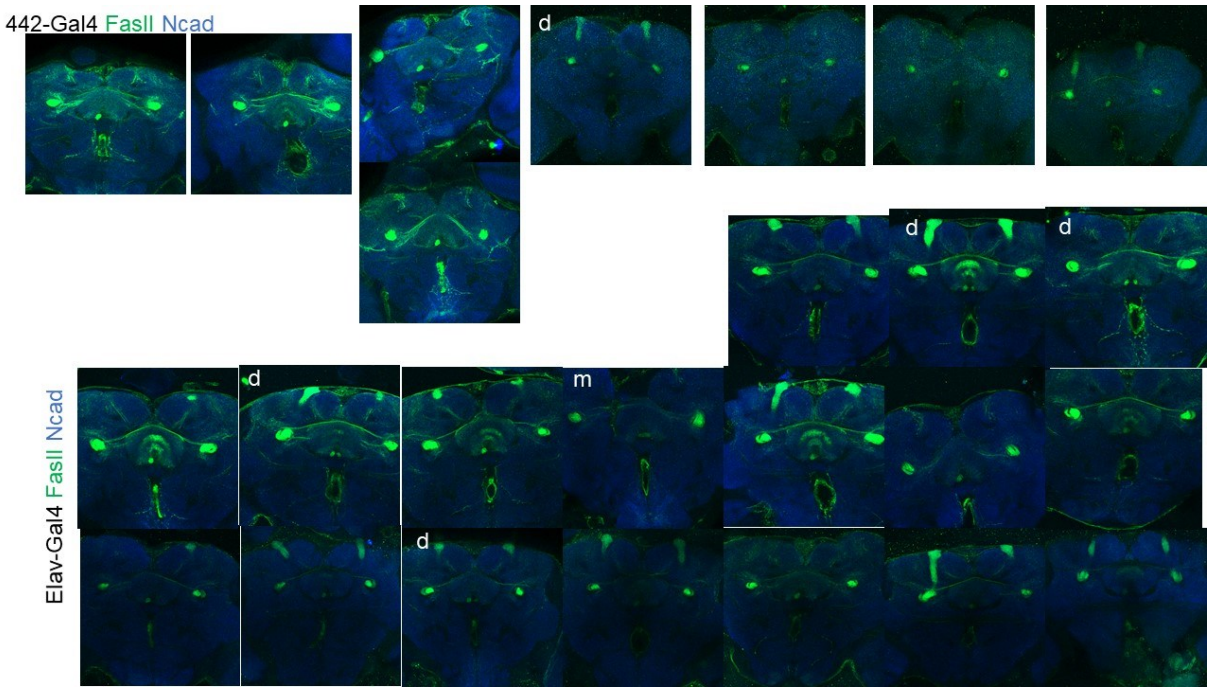


1.42. Guanylyl cyclase β-subunit at 100B

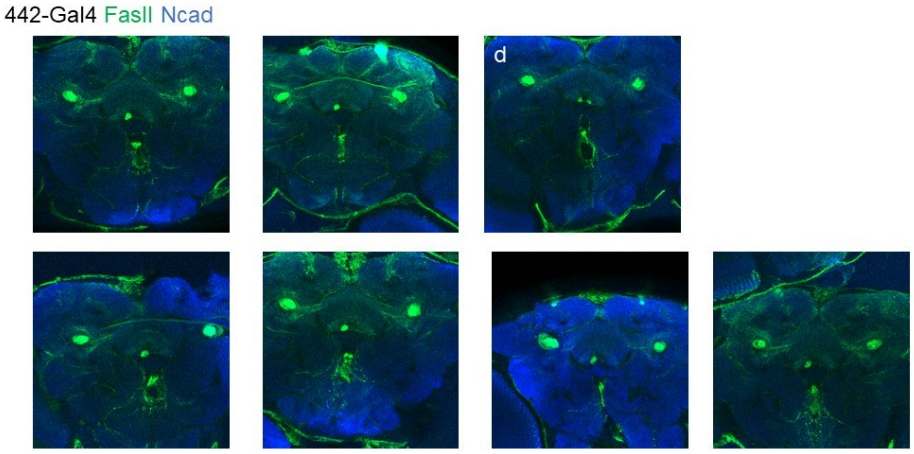




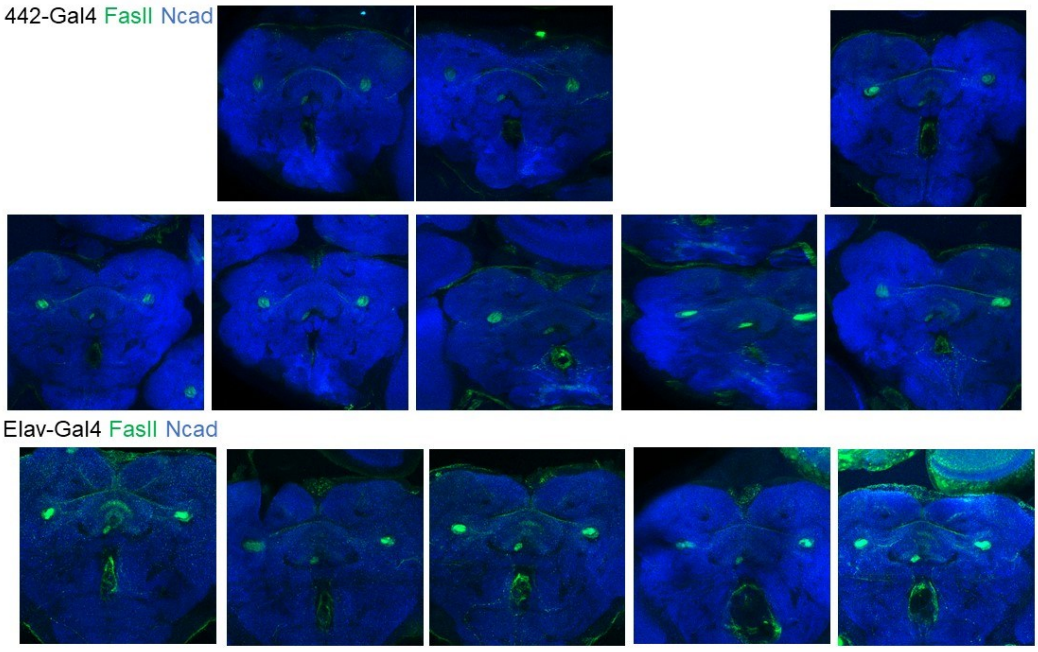
1.43. Cadherin 89D (44)



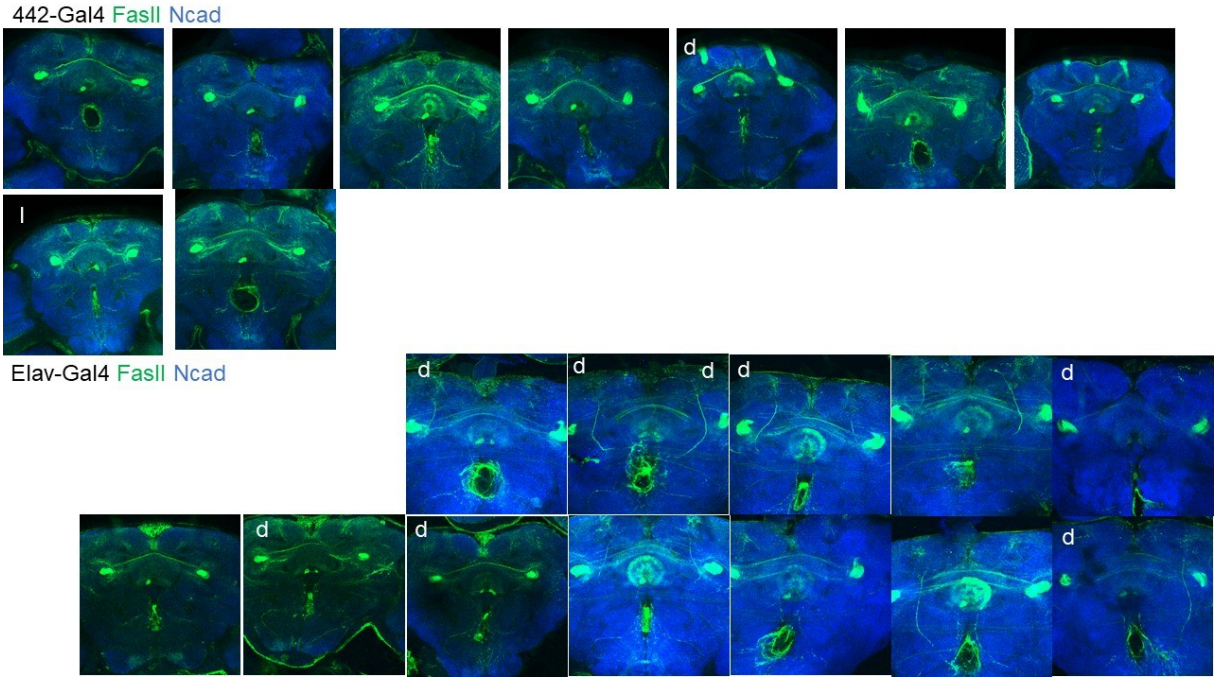
1.44. Cadherin 89D (45)



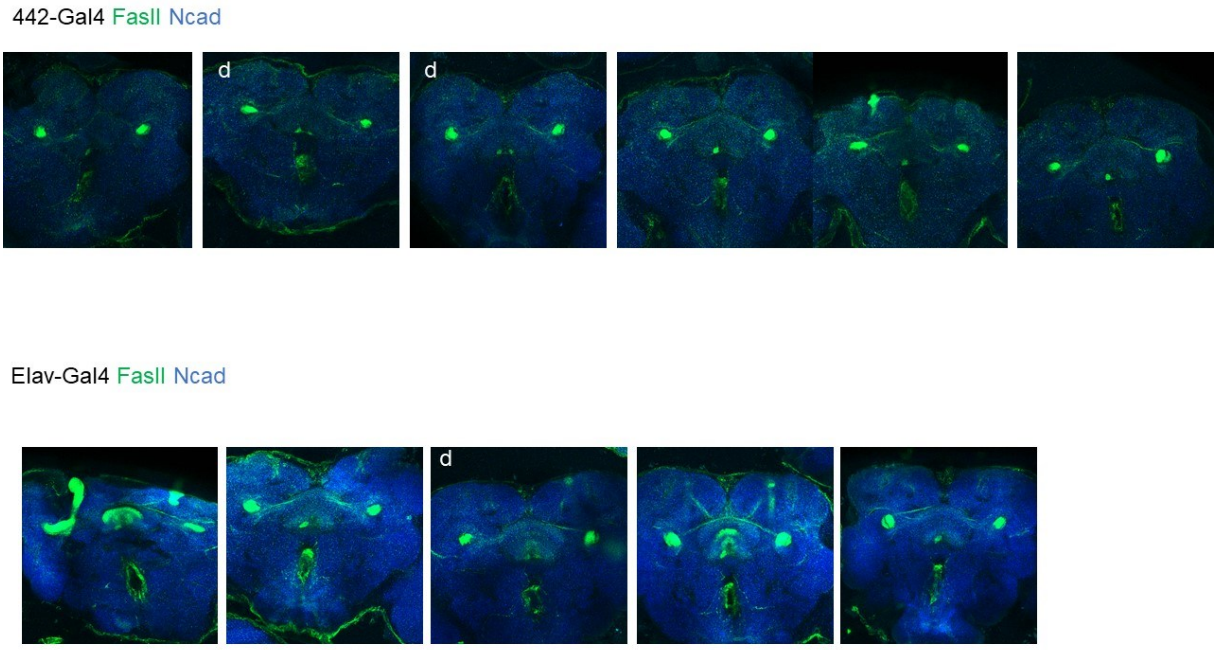
1.45. myospheroid



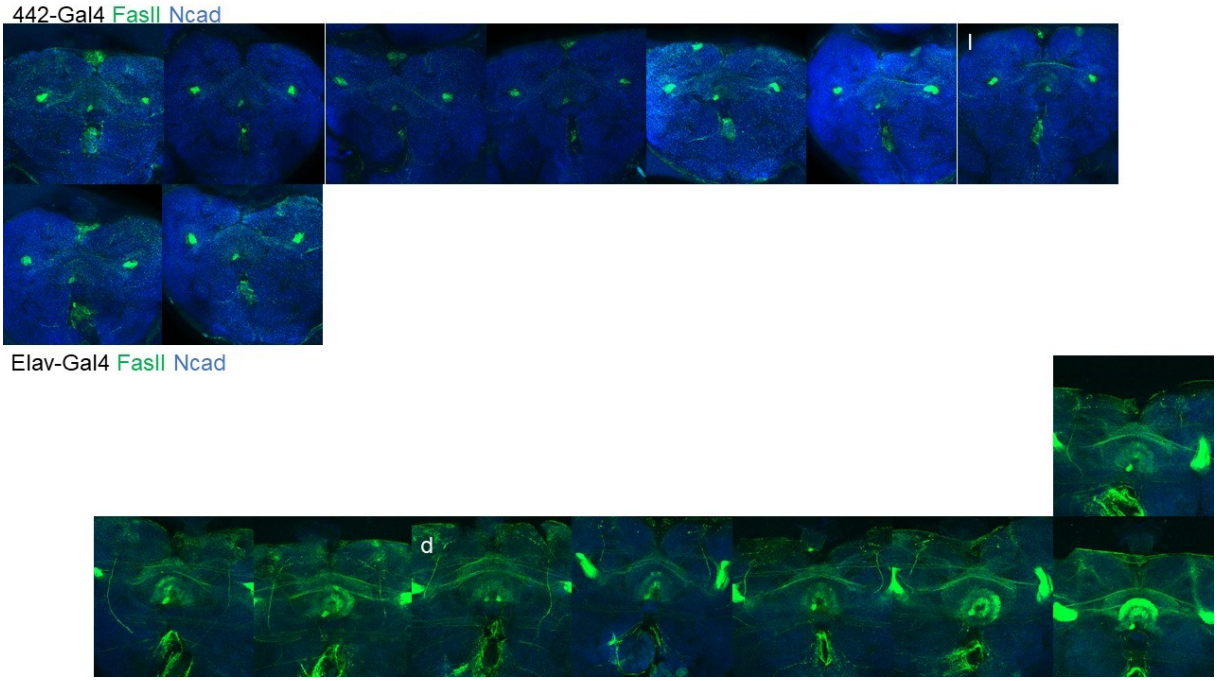
1.46. Mediator complex subunit 30



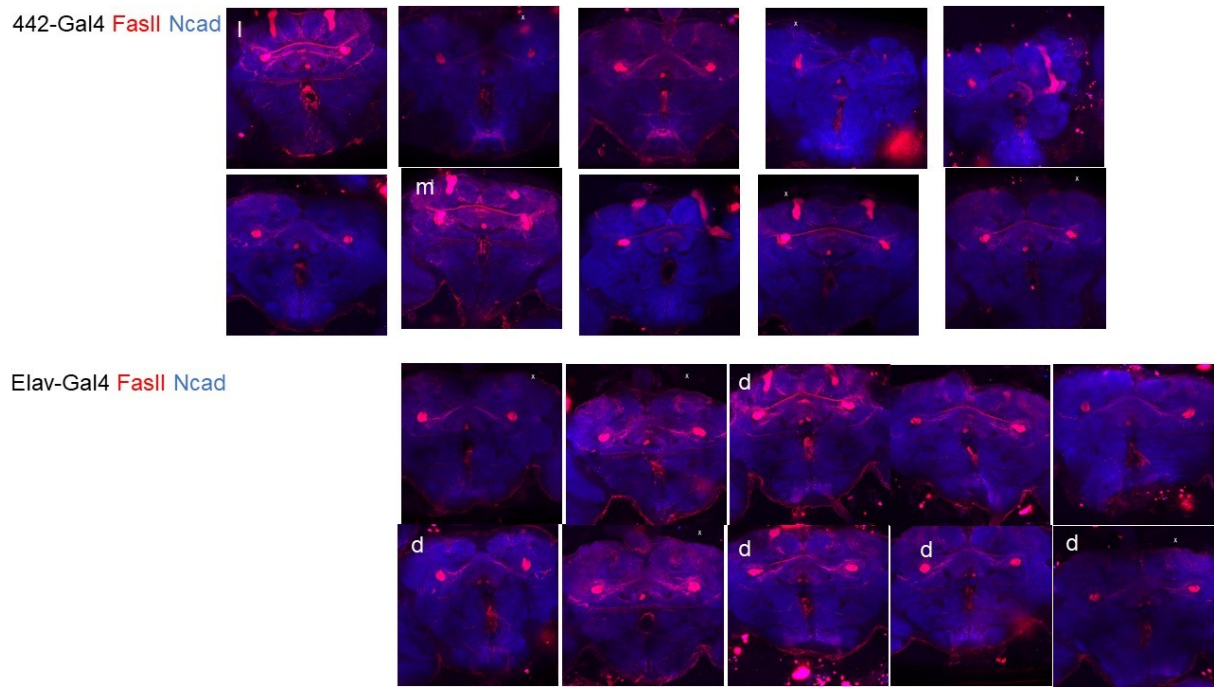
1.47. CA07338 Plesup(3)



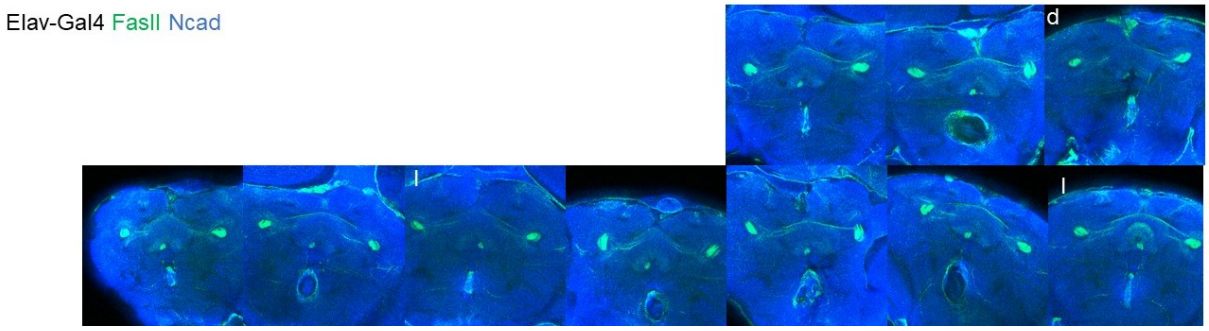
1.48. CA06588 Sema



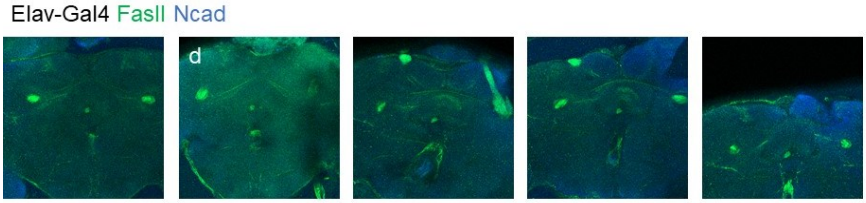
1.49. mirr



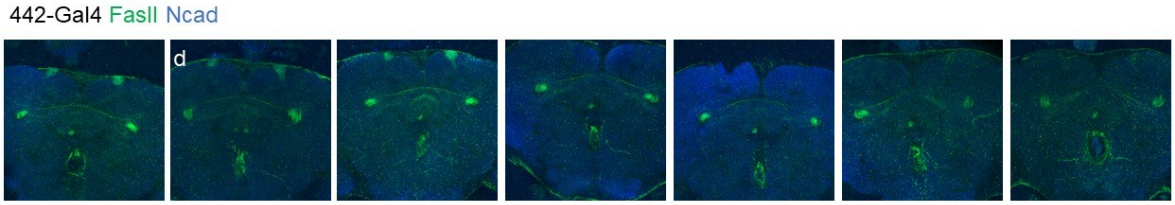
1.50. Kinesin heavy chain



1.51. Rho guanine nucleotide exchange factor 4



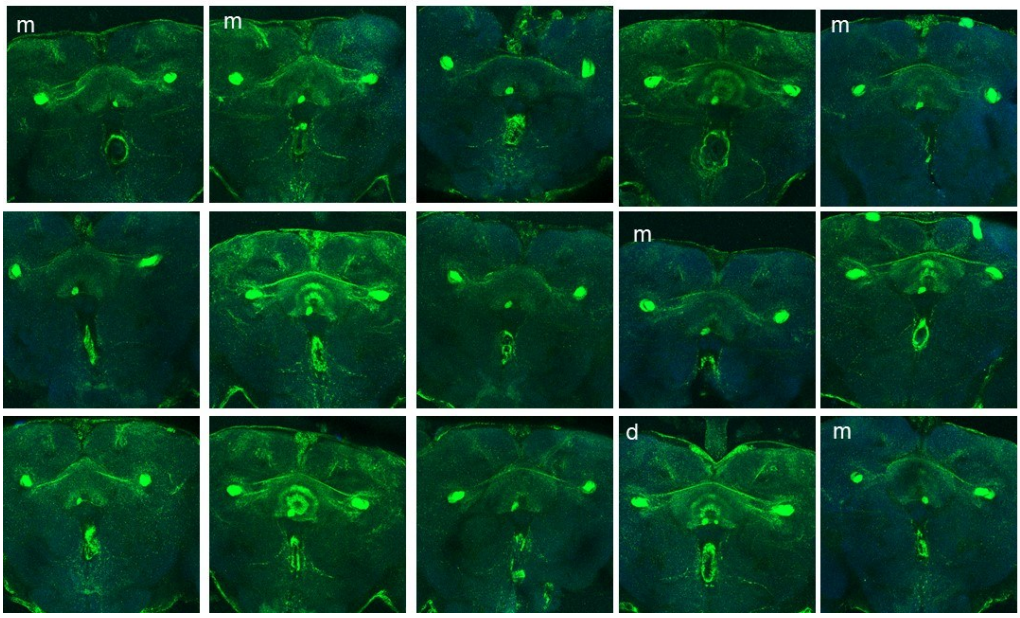
1.52. Dab



2. Re-screen

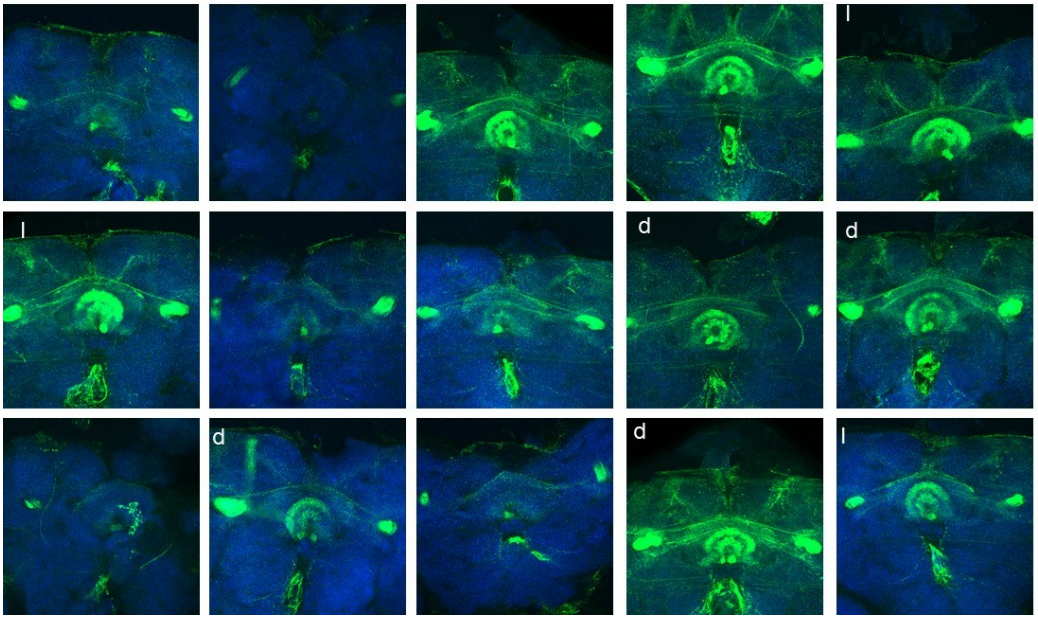
2.1. artichoke

Elav-Gal4 FasII Ncad



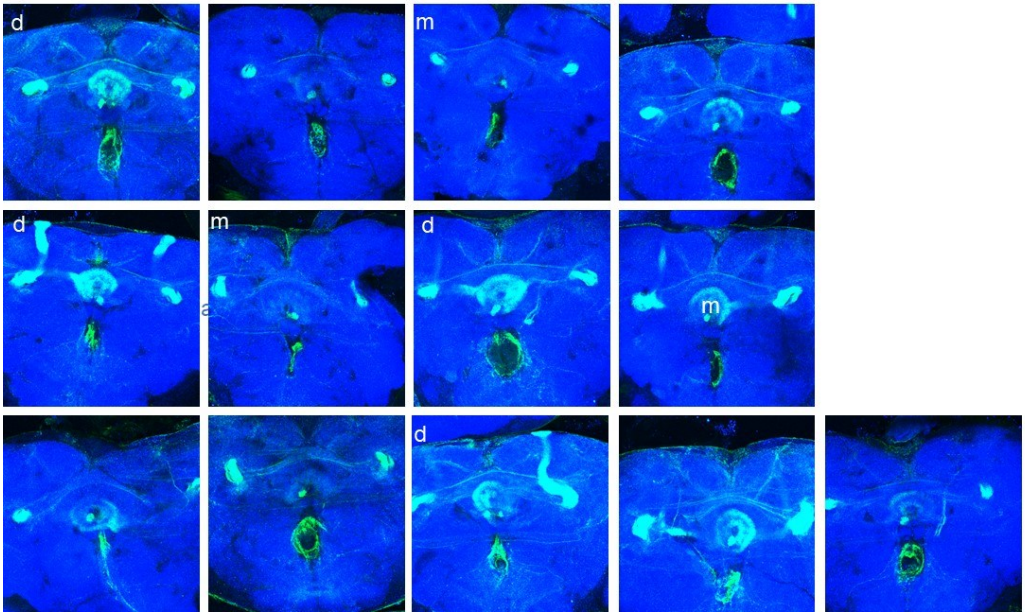
2.2. Phosphatase and tensin homolog

Elav-Gal4 FasII Ncad



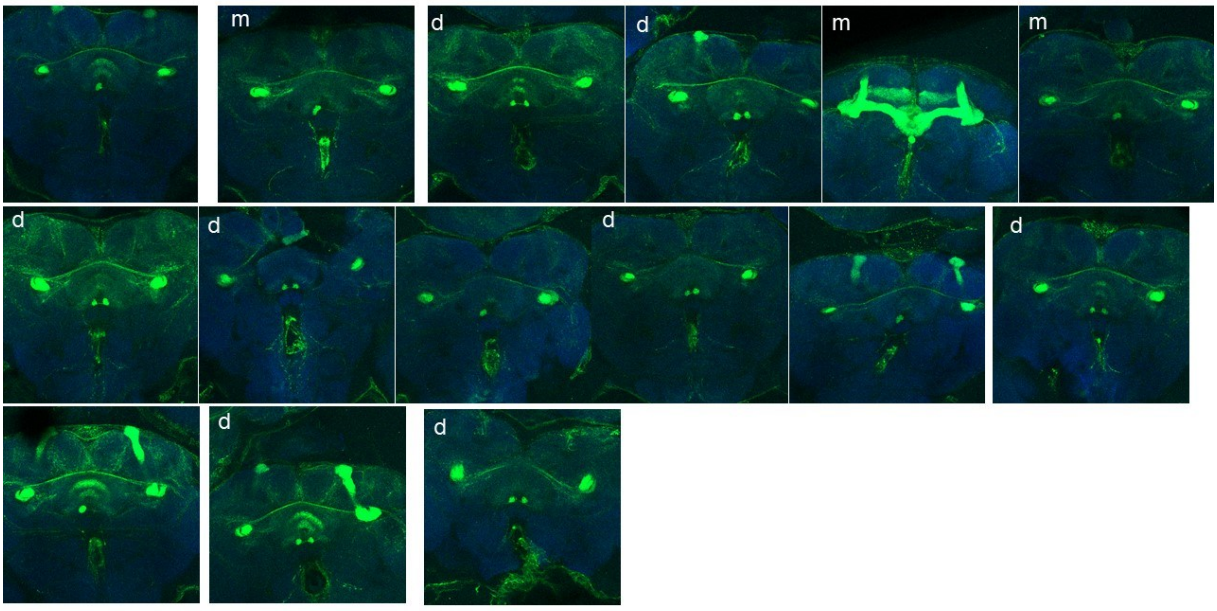
2.3. Pinin

Elav-Gal4 FasII Ncad



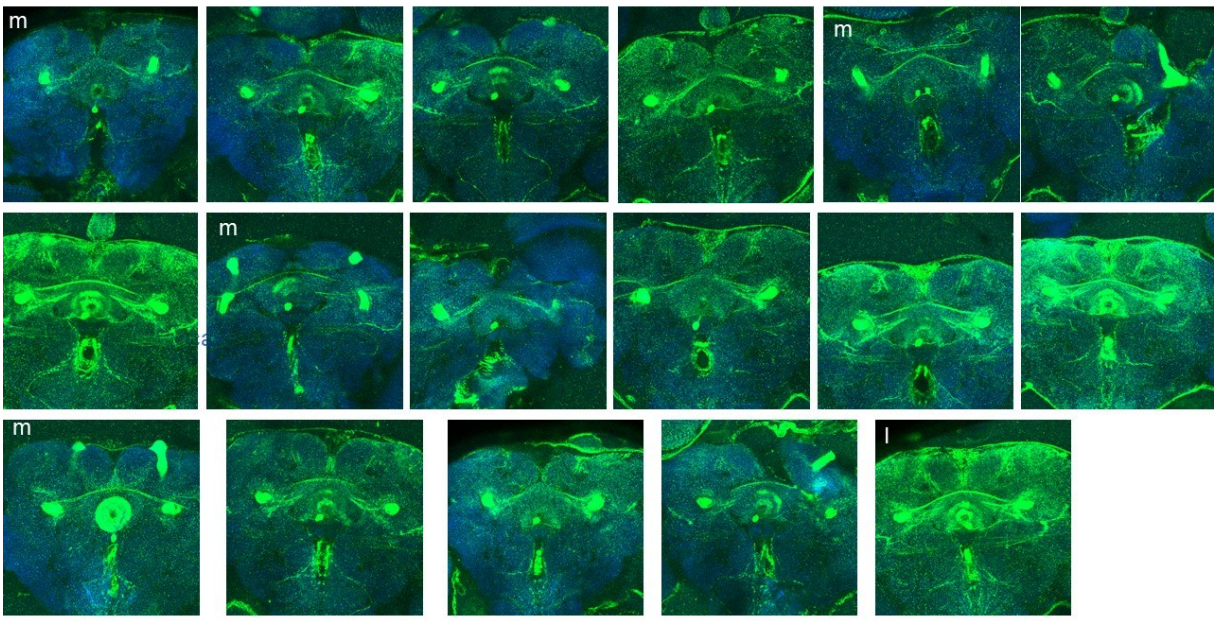
2.4. Wnt oncogene analog 4

442-Gal4 FasII Ncad



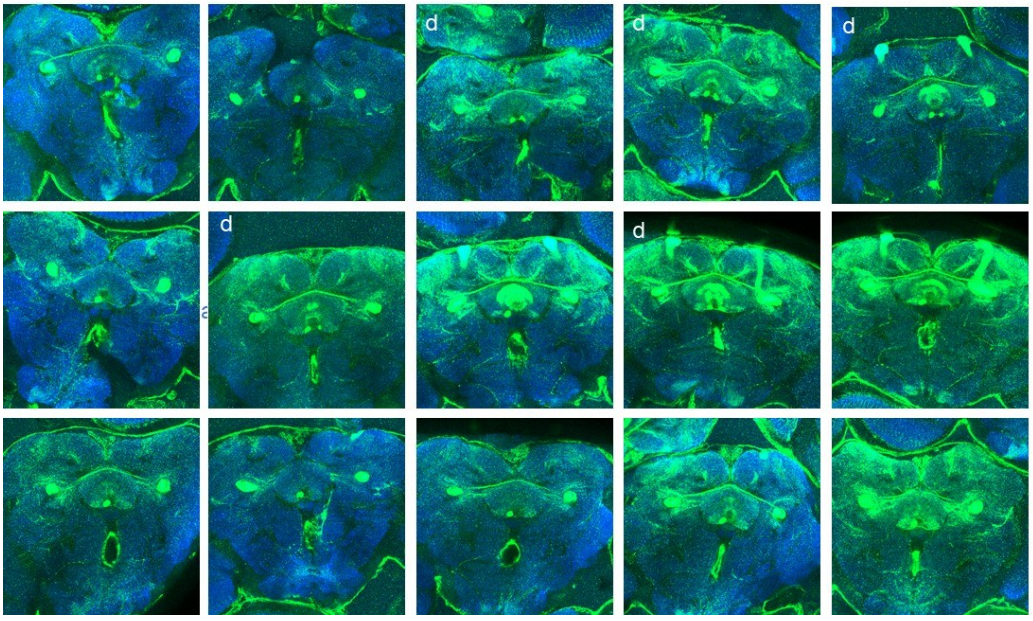
2.5. Dmel\CG18095

442-Gal4 FasII Ncad



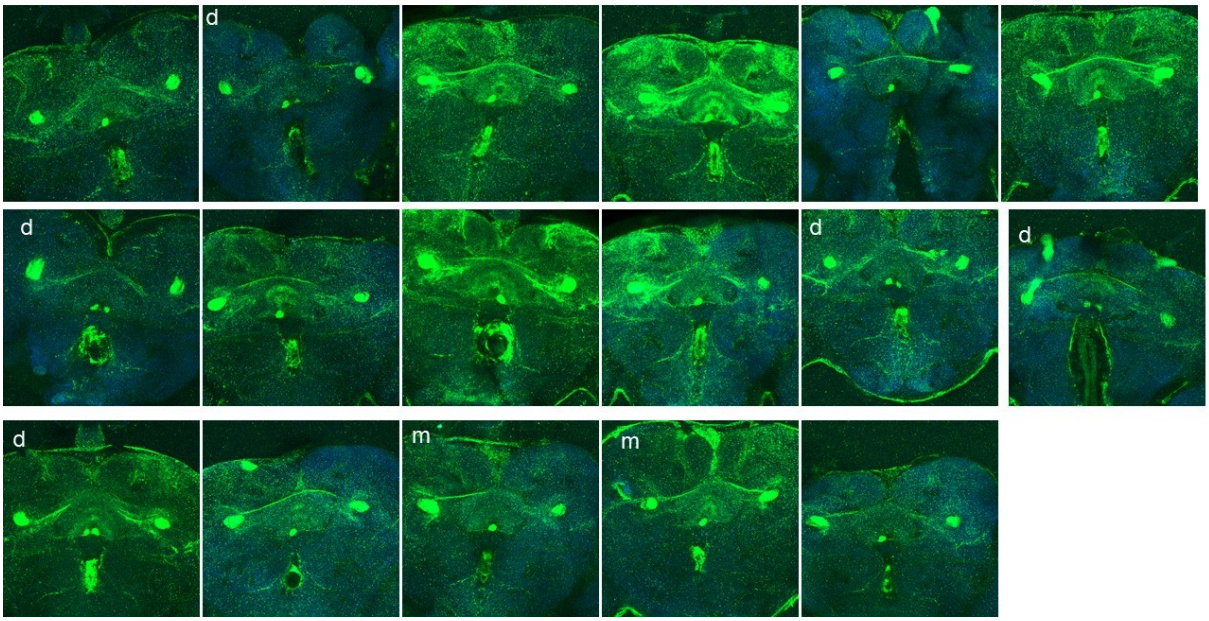
2.6. midline fasciclin

Elav-Gal4 FasII Ncad



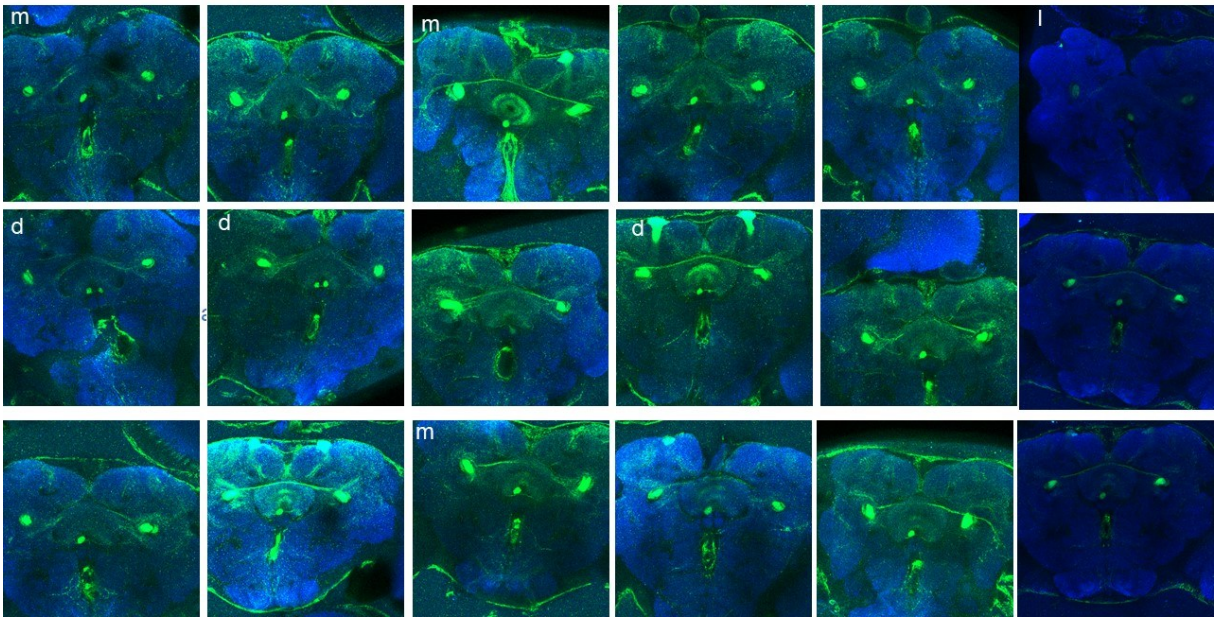
2.7.Neurexin 1

442-Gal4 FasII Ncad

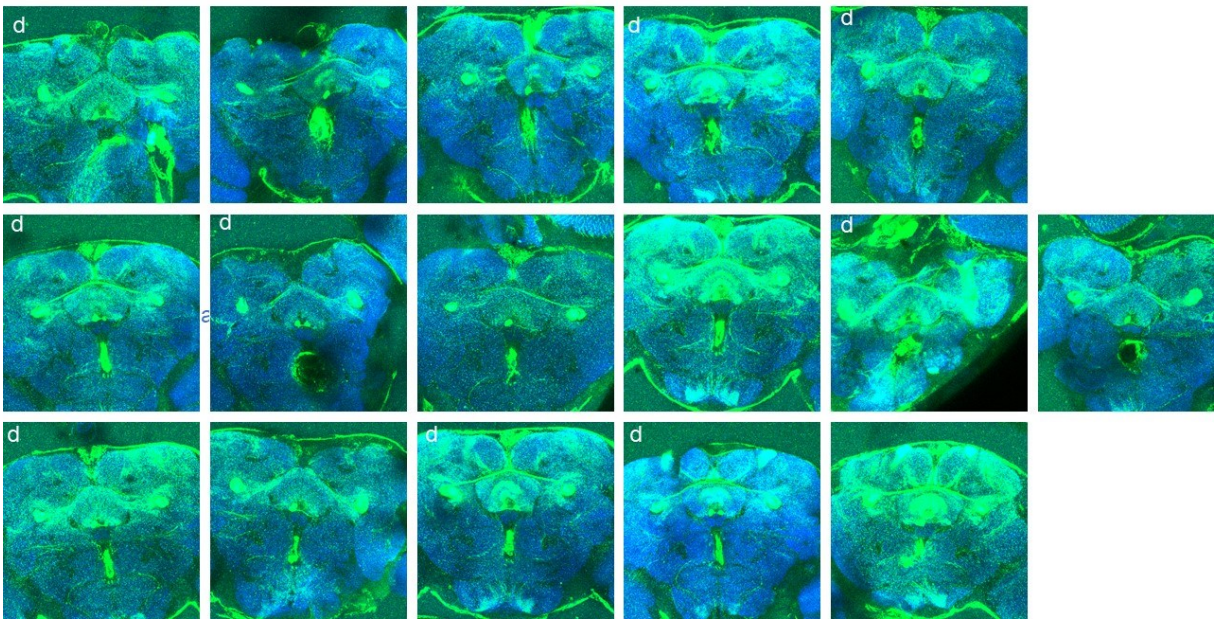


2.8. Dmel\CG6788

442-Gal4 FasII Ncad

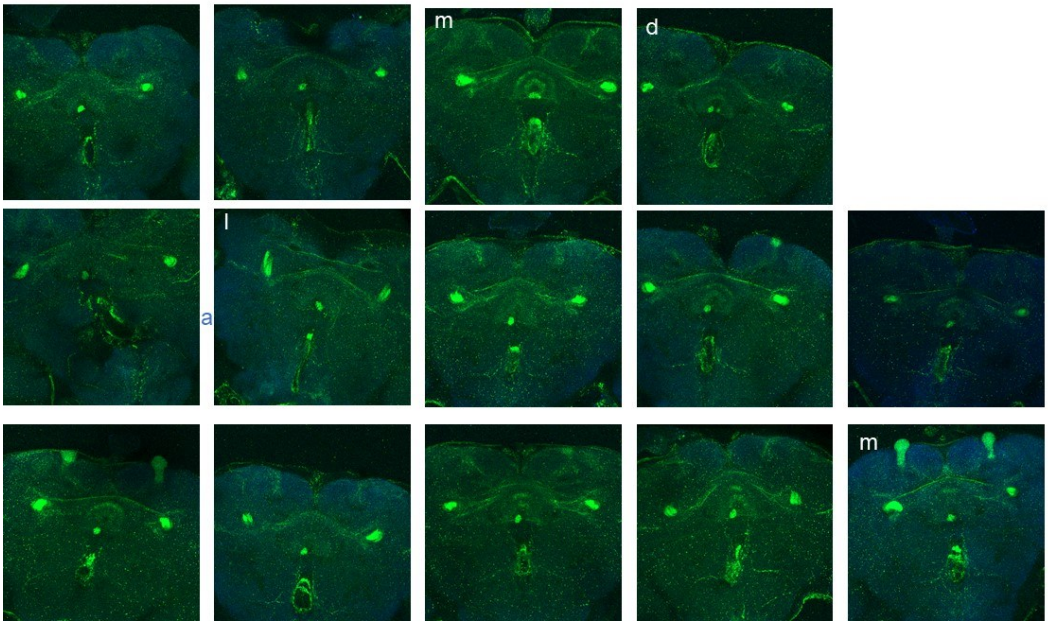


Elav-Gal4 FasII Ncad

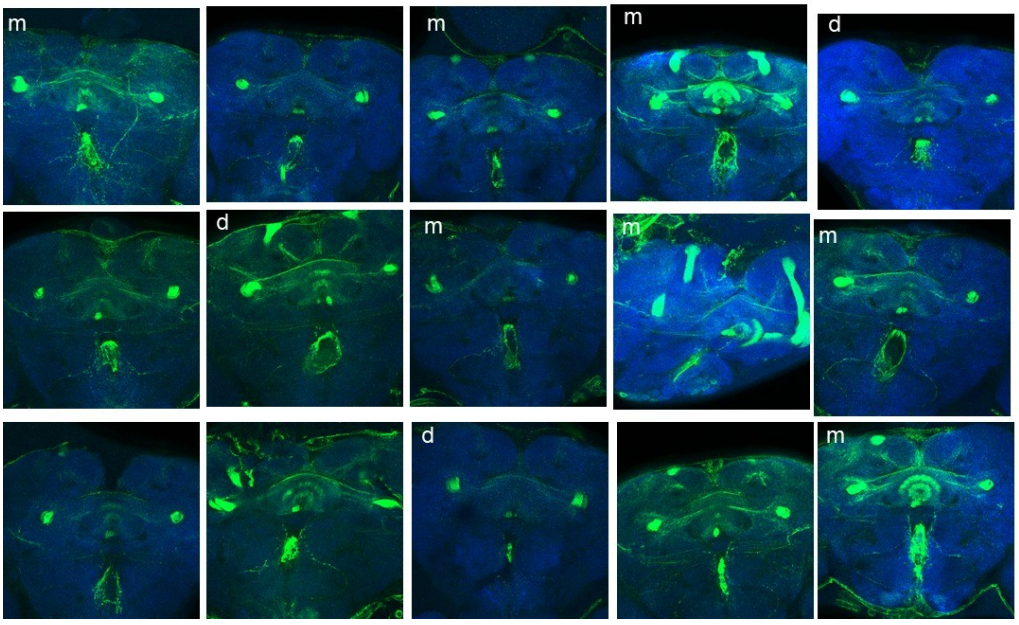


2.9. Tetraspanin 96F

442-Gal4 FasII Ncad

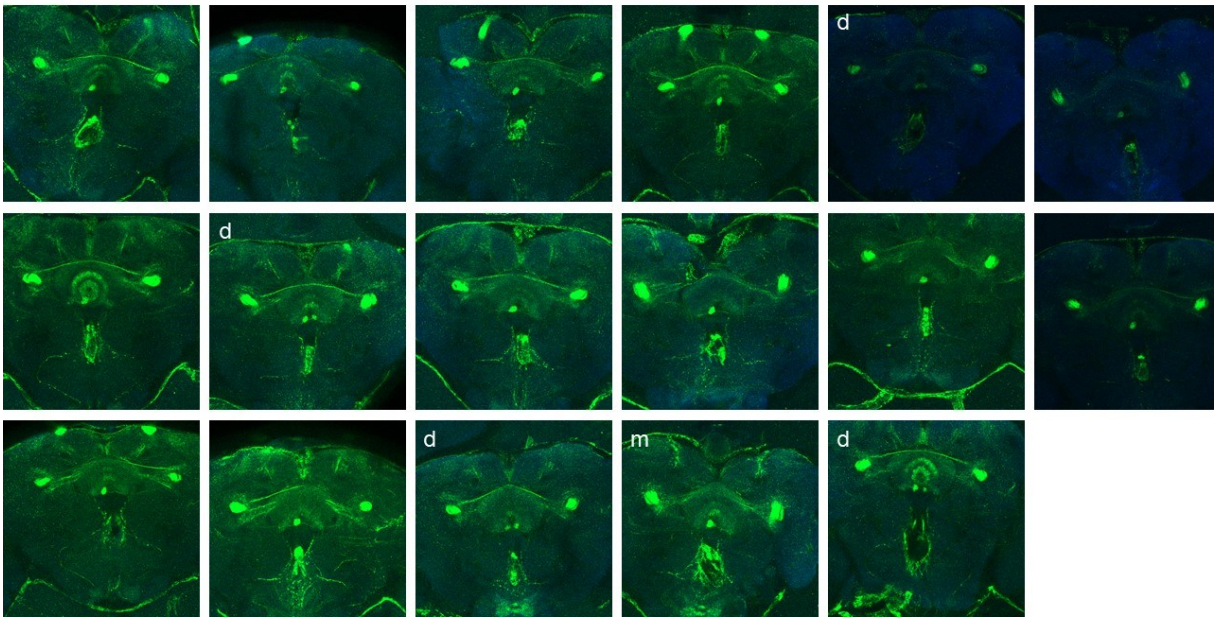


Elav-Gal4 FasII Ncad

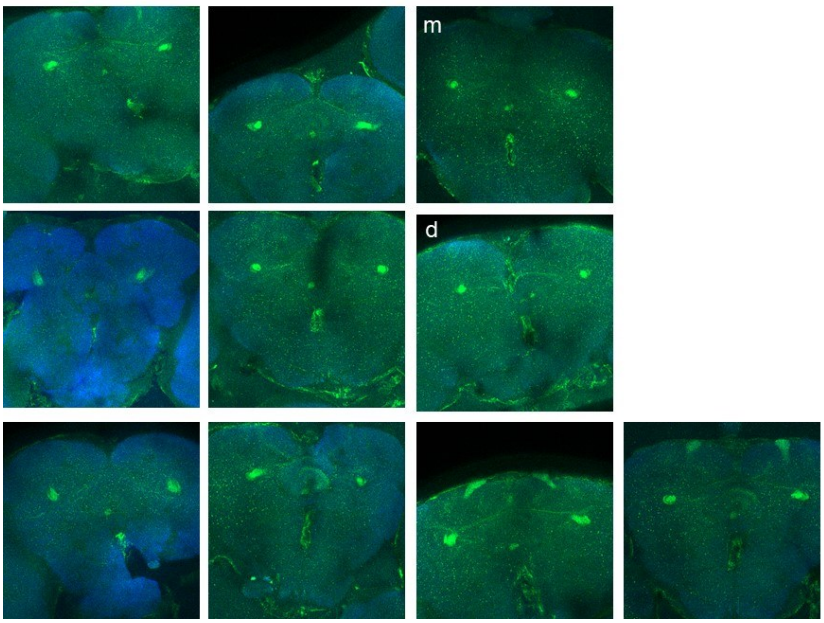


2.10. Tousled-like kinase

442-Gal4 FasII Ncad

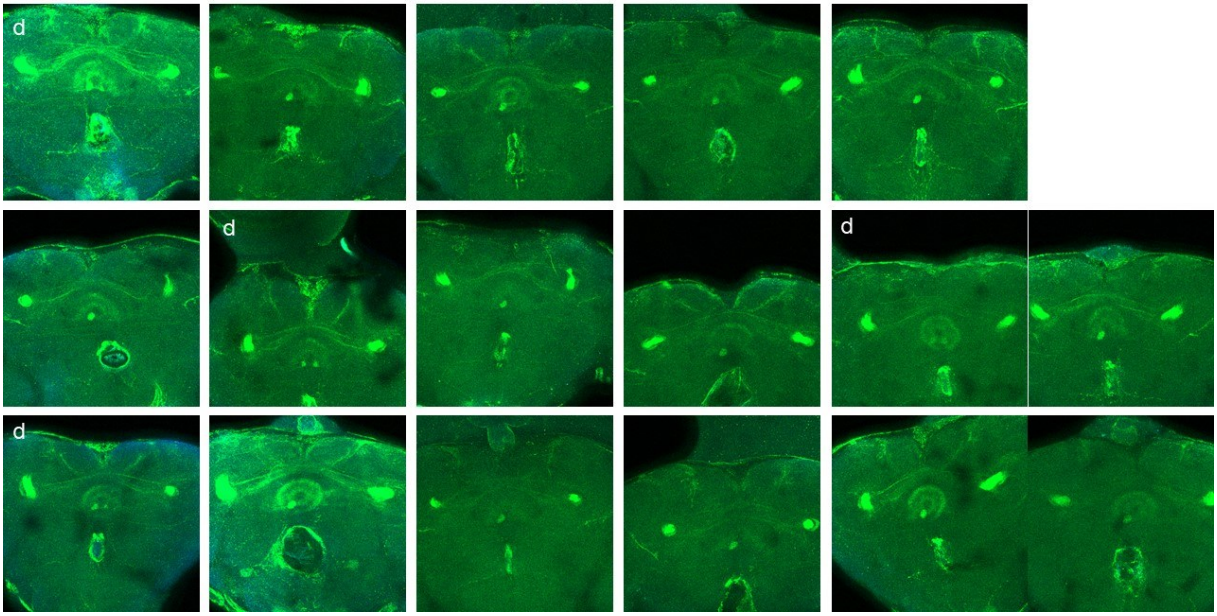


Elav-Gal4 FasII Ncad



2.11. mirr

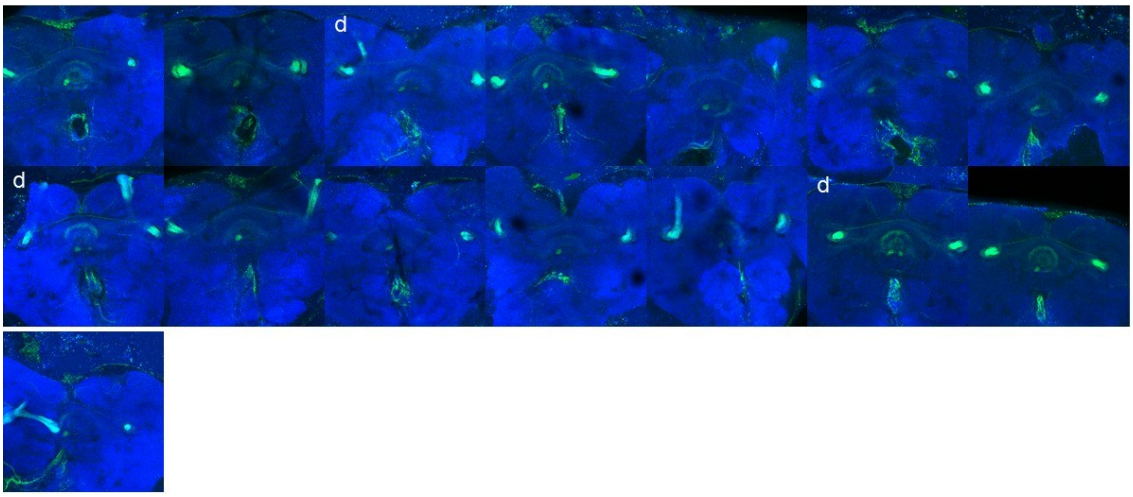
Elav-Gal4 FasII Ncad



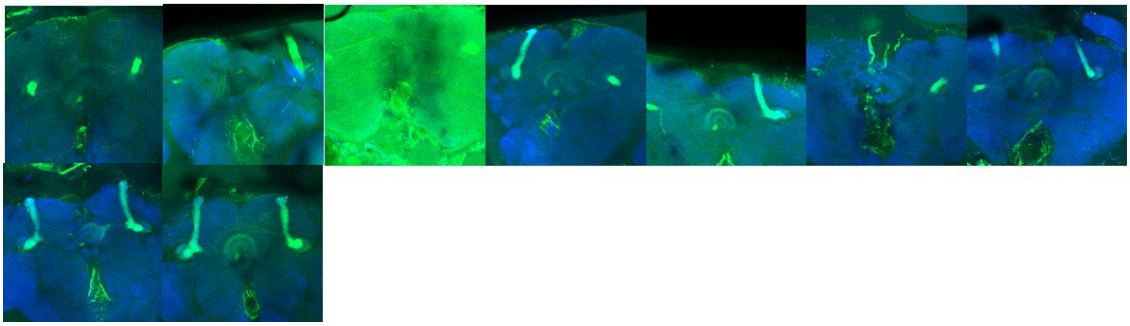
3. wildtype

3.1. Canton S

female FasII Ncad

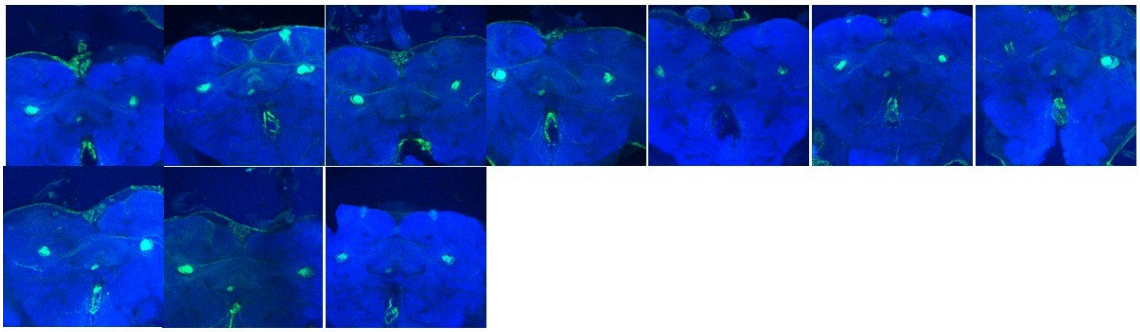


male FasII Ncad

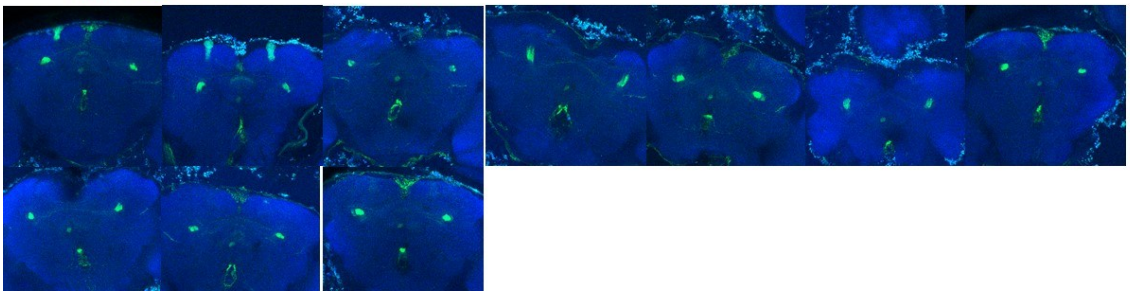


3.2. Vienna-J

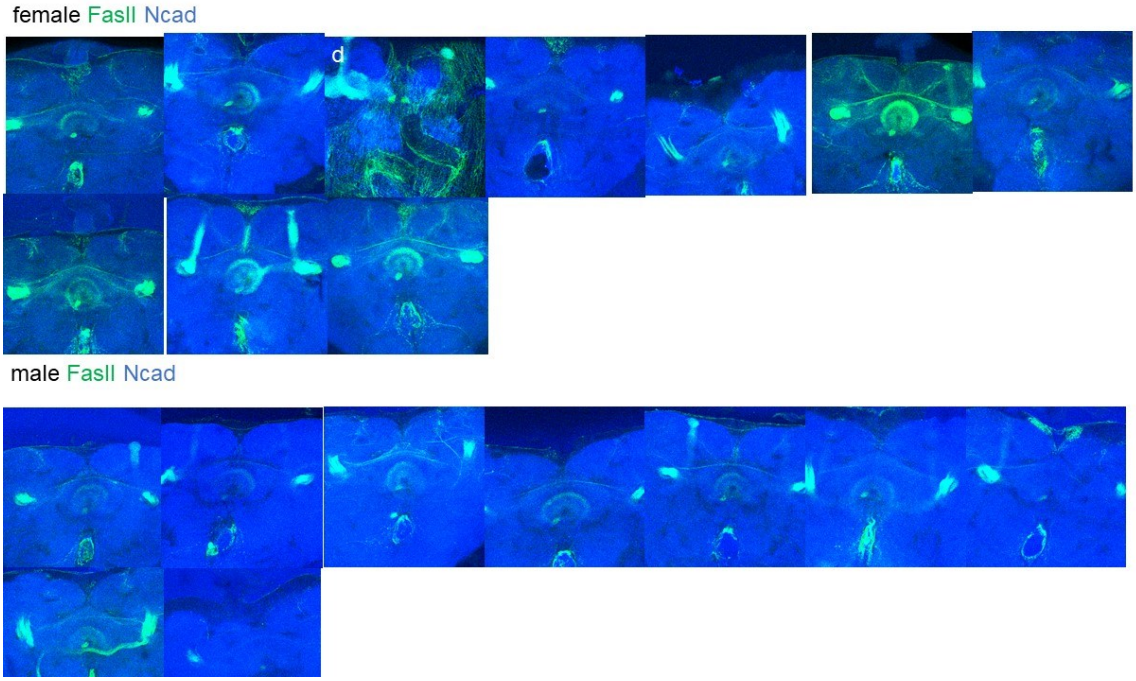
female FasII Ncad



male FasII Ncad



3.3. Vienna-L



3.4. w1118