Supplementary Information

Supplementary Materials and Methods

Brain analysis

Drosophila MBs were analyzed using ZEN 2010 software and a Zeiss LSM700 confocal microscope. The volumes of α and β lobes were quantified individually using the formula $V=\pi r^2 h$ (where $r = \frac{1}{2}$ of lobe width and h = lobe length) and their sum represents α/β lobe volume. The length and the diameter of each lobe were measured from maximum projection images. Ab negative areas were calculated from the analysis of *Δlet-7* and control calyces (72h APF) using the formula $A=\pi r^2$, where A is the Ab negative area and r is ½ of its diameter which crosses the MBN. The Student's t-test was used for statistical analyses.

We used 48h APF brains to quantify Ab positive cells in control and *Δ let-7* mutants. In both cases *let-7*-C expressing cells were labeled with membrane GFP; *let-7-C*^{GK1}*/+; UAS-CD8GFP/+* was used as a control and *let-7-C*^{GK1}*/let-7-C*^{K01}*; UAS-CD8GFP/P{W8, let-7-C*$^{\Delta let\text{-}7}$*}* as a *Δ let-7* mutant. Ab expressing cells and double-labeled cells with GFP and Ab were quantified to find out if the deletion of miRNA *let-7* causes Ab misexpression in *let-7*-C expressing cells. For each sample 11 confocal sections (4 µm steps) through the whole calyx were analyzed; at least 4 calices of each genotype were evaluated. Statistical analysis was done using the Student's t-test.

Associative olfactory learning and electric shock avoidance assays

Groups of about 100 flies each were trained to avoid the odorants 4-Methylcyclohexanol (MCH, Sigma Aldrich) or 3-Octanol (OCT, Sigma Aldrich) as described by Tully and Quinn (Tully & Quinn, 1985). Four experiments were performed simultaneously in a modified learning apparatus described by Schwärzel et al. (Schwaerzel et al, 2002). Olfactory training started one minute after transferring the flies into the learning apparatus. Each odor was presented for one minute with a one-minute break between odor applications. One of the odours (conditioned stimulus +, CS+) was temporally paired with 12 electric shocks of 90V (1.25 s shock and 3.75 s interpulse interval), the second odor was presented without shock (conditioned stimulus -, CS-). After another minute, the flies were transferred to the T-maze part of the apparatus with both odors presented from each side and tested for odor preference for 2 minutes. Subsequently the flies were counted and a preference index was calculated as the number of flies on the side of the CS- minus the number of flies on the side of the CS+ divided by the total number of flies. Each odor was used equally often as CS+ and CS-, and the results of two reciprocal experiments were averaged to calculate a learning index. Normal distribution of the data from several experiments was confirmed using the Lilliefor's test, and

differences in learning indices of different genotypes were tested for significance using the Student's t-test.

Flies were tested for electric shock avoidance to control for the perception of the shock and for a locomotor performance required to exhibit learned behavior. Groups of about 100 flies were transferred into the T-maze. One arm of the T-maze consisted of a tube with an electrifiable grid. 12 electric shocks of 90V were applied during the first minute of a two minute time interval during which the flies could choose one of the arms of the T-maze. The avoidance index was calculated as described above and subjected to the Student's t-test.

EdU labeling

To birth label neurons generated at the L3 stage EdU (5-Ethyl-2'-Deoxyuridine) labeling was performed using Click-iT EdU Alexa Fluor 555 Imaging Kit (Invitrogen/Molecular Probes). The feeding of EdU to *Drosophila* larvae was done following the protocol (Daul et al, 2010).

miRNA target prediction

To identify *let-7* miRNA predicted targets TargetScan Release 5.2 (Ruby et al, 2007) and PicTar (Grun et al, 2005) databases were used.

RNA preparation and real-time quantitative PCR

To determine the effect of the ecdysone synthesis on the miRNA *let-7* expression levels and effect of *let-7* loss on *Fas II* mRNA levels quantitative reverse transcription PCRs (RT-qPCR) were performed on total RNA derived from pupal brains (for *let-7* levels) and 1-3 d old adult heads (for Fas II levels). RNAs were extracted from flies using the TRIzol reagent (Invitrogen), followed by reverse transcription using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) following the manufacturer's protocol. *let-7* levels were tested with 2S rRNA as an endogenous control for q-PCR using TaqMan® MicroRNA Assays and Fas II levels were tested with RpL32 as an endogenous control for q-PCR using Fast SYBR® Green master mix on a Step One Plus 96 well system (Applied Systems). Primers were used as follows: RpL32 forward — AAGATGACCATCCGCCCAGC; RpL32 reverse – GTCGATACCCTTGGGCTTGC; Fas II forward – ACTTGTCGTTGGTCGTGCGG; Fas II reverse – CACCTGGACAAATGCCCCTG. All reactions were run in triplicate with appropriate blank controls. The threshold cycle (CT) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The ΔCT value was determined by subtracting the average RpL32 CT value from the average Fas II CT value or the average 2S rRNA CT value from the average *let-7* CT value. The ΔΔCT value was calculated by subtracting the ΔCT of the control sample (*OregonR*) from the ΔCT of the suspect sample (*Δlet-7* or ecd^{1ts}). The relative amount of mRNA was then determined using the expression $2^{-\Delta\Delta CT}$.

Supplementary Tables

Supplementary Table I. Volume of α/β mushroom body lobes

Genotype	Analyzed MB lobes, n	MB lobes volume[a] relative to control (3-6d old adult brains)			
		Vα (AVE±SD)	Vβ (AVE±SD)	α/β	
				Vα/β (AVE±SD)	Statistics[b] *P*-value
Control (*let-7-C*^{GK1}/+)	n=17	1.00±0.22	1.00±0.23	1.00±0.17	-
Δ *let-7* (*let-7-C*^{GK1}/*let-7-C*^{KO1}; *P{W8, let-7-C*$^{\Delta let-7}$*}*)	n=18	0.60±0.31	0.36±0.26	0.52±0.18	2.06×10^{-9} *** [c]
Rescue with *let-7*-C (*P{W8, let-7-C}; let-7-C*^{GK1}/*let-7-C*^{KO1})	n=8	0.80±0.19	1.06±0.31	0.89±0.18	0.19 [c] 4.92×10^{-5} *** [d]
Apt overexpression (*let-7-C*^{GK1}/*UAS-apt*)	n=8	0.83±0.14	1.23±0.37	0.98±0.16	0.79 [c]
Ab overexpression[e] (*let-7-C*^{GK1}/*UAS-ab*)	n=10	0.10±0.13	0.82±0.31	0.72±0.26	3.22×10^{-3} ** [c]
Rescue with ab (*let-7 miR-125, ab*^{1}/ *let-7 miR-125, ab*^{1D})	n=12	0.84±0.19	1.38±0.51	1.04±0.27	0.63 [c] 7.10×10^{-7}*** [d]
Ab downregulation (*ab*^{1}/*ab*^{1})	n=12	0.82±0.18	0.90±0.18	0.85±0.12	0.02* [c]
Ab downregulation (*ab*^{1}/*ab*^{1D})	n=22	1.03±0.36	1.15±0.49	1.07±0.37	0.46
Fas II downregulation (*let-7-C*^{GK1}/*Fas2*^{RNAi})	n=12	0.86±0.43	1.06±0.52	0.93±0.43	0.57
DE-Cadherin downregulation (*let-7-C*^{GK1}/*shotgun*^{RNAi})	n=10	0.73±0.14	1.01±0.18	0.87±0.11	0.055

[a] lobe volumes were quantified separately for α and β lobes (Vα and Vβ) using the formula $V=\pi r^2 h$ and α/β lobes volume (Vα/β) was calculated as Vα+Vβ;
[b]statistics were calculated using a two-tailed Student's t-test and the *P*-value is presented; * $P \leq 0.05$; ** $P \leq 0.01$; ***$P \leq 0.001$
[c]in comparison to control;
[d]in comparison to rescue;
[e]analyzed at the stage of 90h APF because of lethality

Supplementary Table II. Olfactory learning in *let-7* mutants

Learning index for reciprocal experiment[1]		
Control (*Oregon R*)	**Δ*let-7*** (*let-7-C*^{GK1}/*let-7-C*^{KO1}; *P{W8, let-7-C*$^{\Delta let\text{-}7}$*}*)	**Rescue** (*P{W8, let-7-C}; let-7-C*^{GK1}/*let-7-C*^{KO1})
0.71661	0.4549	0.36066
0.47032	0.45644	0.40745
0.57601	0.14326	0.52605
0.75296	0.39116	0.41800
0.48264	0.46275	0.61824
0.41282	0.37500	0.45877
0.46759	0.37616	0.44683
0.42620	0.26126	0.46036
0.66006	0.32403	0.49842
0.63541	0.37606	0.36941
0.48465	0.25714	0.61998
0.52577	0.32148	0.51515
0.51171	0.29355	0.61906
0.62003	0.31851	0.58498
0.51050	0.29963	0.44859
0.64921	0.24848	0.75565

[1] represents an avoidance of the shocked odor

It has been previously shown that mutants that had all three (*miR-100*, *let-7* and *miR-125*) miRNAs or just *let-7* miRNA deleted had severe locomotion defects measured by the spontaneous locomotion assay (Sokol et al, 2008). Therefore, we first tested if ***Δ****let-7-C* and ***Δ****let-7* animals can be assayed in the olfactory conditioning assay. For this we analyzed if mutants can respond to electric shock stimulation (see experimental procedures) and observed that ***Δ****let-7-C* mutants could not respond to electric shock and therefore could not be assayed for the learning test. Mutants deficient for miRNA *let-7* had only a slightly decreased electric shock avoidance index in comparison to control.

Avoidance indices: *OregonR* 0.71314 (SEM ±0.06975) n=11
Δ*let-7* 0.54743 (SEM ±0.08489) n=7, p=0.15

Supplementary Table III. miRNA *let-7* levels in *ecd*^{1ts} mutants*

Genotypes and conditions	*let-7* Average C_T	*2S rRNA* Average C_T	ΔC_T[1]	$\Delta\Delta C_T$[2]	Average miRNA *let-7* levels relative to control[3]
***Oregon R**,* 18°C	24.59±0.15	5.03±0.24	19.56±0.31	0.00±0.31	1.00±0.23
***Oregon R**,* 29°C	22.54±0.15	4.78±0.23	17.76±0.11	-1.89±0.11	3.70±0.27
ecd^{1ts}, 18°C	26.02±0.48	12.18±1.01	13.85±1.25	0.00±1.25	1.00±0.86
ecd^{1ts}, 29°C	23.76±1.03	9.36±1.74	14.40±0.71	0.56±0.71	0.68±0.31

*- measurments of *let-7* mRNA levels at 18°C in comparison to 29°C suggests that *let-7* expression is temperature-dependent

[1] the ΔC_T value is determined by subtracting the average *2S* C_T value from the average *let-7* C_T value. Mean (3 independent experiments done in triplicate) ± SD (standard deviation). SD is calculated from the SDs of the *2S rRNA* and *let-7* values using the following formula: $SD=\sqrt{SD1^2+SD2^2}$;

[2] the calculation of the $\Delta\Delta C_T$ involves subtraction by the ΔC_T calibrator value. $\Delta\Delta C_T = \Delta C_T - \Delta C_T$ (control at 18°C).

[3] the range given for *let-7* levels at 29°C relative to control (18°C) was calculated as $2^{-\Delta\Delta CT}$

Table IV. Volume of α/β mushroom body lobes in the mutants with the perturbed ecdysone signaling pathway

Genotype	Stages when ecdysone synthesis was disrupted				α/β lobe defects in adult MBs
	L3 larva	Prepupa	Pupa	Pharate	
					-
		2 days[1] (from mid L3 larva to mid Pupa)			+
$ecd1^{ts}$		2 days (Prepupa to Pupa)			+
			1 day		+
				2 days	-

Genotype	Analyzed MB lobes, n	MB lobes volume[a] relative to control (3-6d old adult brains)			
		Vα (AVE±SD)	Vβ (AVE±SD)	Vα/β (AVE±SD)	Statistics[b] *P*-value
Control *ecd^{1ts}* (*18°C*)	n=7	1.00±0.24	1±0.39	1±0.23	-
ecd^{1ts} (29°C for 2 days from Prepupa to Pupa)[1]	n=10	0.78±0.58	0.47±0.33	0.58±0.40	0.01** [c]
ecd^{1ts} (29°C for 2 days from Pupa to Pharate)[2]	n=12	1.14±0.85	1.38±0.49	1.26±0.45	0.18
ecd^{1ts} (29°C for 1 day at pupal stage)	n=14	0.72±0.33	0.72±0.34	0.72±0.29	0.04*[c]
ecd^{1ts} (29°C for 2 days from Pharate)	n=4	1.15±0.31	0.93±0.28	1.05±0.19	0.83
$hsGal4\text{-}EcR^{-L.B.D.}/TM6$[3]	n=6	0.43±0.13	0.76±0.29	0.56±0.17	$2.7x10^{-5}$ *** [c]
$hsGal4\text{-}EcR^{-L.B.D.}/UAS\text{-}let\text{-}7$[3]	n=16	1.17±0.29	1.18±0.39	1.14±0.23	0.059 [c] $1.9x10^{-5}$ *** [d]
$hsGal4\text{-}usp^{L.B.D.}/TM6$[3]	n=16	0.62±0.28	0.84±0.30	0.70±0.24	$3.2x10^{-5}$ *** [c]
$hsGal4\text{-}usp^{L.B.D.}/UAS\text{-}let\text{-}7$[3]	n=22	0.85±0.26	1.2±0.39	0.97±0.24	0.77 [c] $1.2x10^{-3}$ *** [d]

[a]lobe volumes were quantified separately for α and β lobes (Vα and Vβ) using the formula $V=\pi r^2 h$ and α/β lobes volume (Vα/β) was calculated as Vα+Vβ;
[b]statistics were calculated using a two-tailed Student's t-test and the *P*-value is presented; * $P\leq0.05$; ** $P\leq0.01$; ***$P\leq0.001$
[c]in comparison to control;
[d]in comparison to mutant;
[1]brains analyzed at the stage of 90h APF because of lethality;
[2] a large range of the phenotypes and high pupal lethality were observed in adult escapers; therefore 48h 29°C treatment was reduced to 24h in the next experiment
[3]animals were heat shocked for 2 days at 12hAPF stage: 3 times per day for 1 h in 37°C water bath

Table V. Morphological changes in α/β mushroom body lobes

Genotype	n (number of analyzed α/β lobes)[1]	Normally developed α/β lobes	α/β lobes with morphological changes (%)			
			Slim	Misguided	β-fused	Underdeveloped
Oregon R	26	100.00%	0.00%	0.00%	0.00%	0.00%
Δ let-7	32	28.12%	31.25%	3.13%	9.38%	28.13%
ecd^{1ts}*	18	33.33%	50.00%	0.00%	16.67%	0.00%
hsGal4-EcR^{LBD} **	27	11.11%	40.74%	11.11%	18.52%	18.52%
hsGal4-usp^{LBD} **	36	33.33%	44.44%	5.56%	13.89%	2.78%
let-7^{GK1}*; EcR*^{RNAi}	23	21.74%	65.22%	0.00%	4.35%	8.69%
c739Gal4; EcR^{RNAi}	13	26.16%	50.77%	0.00%	7.69%	15.38%
hs EcR.A **	33	45.45%	24.24%	0.00%	12.12%	18.19%
hs EcR.B1 **	20	80.00%	20.00%	0.00%	0.00%	0.00%
let-7-C^{K01}*/EcR*^{Q50}*;* *P{W8, let-7-C*$^{\Delta let\text{-}7}$*}/+*	30	43.33%	40.00%	3.33%	0.00%	13.34%
let-7-C^{K01}*/EcR*^{M554fs}*;* *P{W8, let-7-C*$^{\Delta let\text{-}7}$*}/+*	36	41.67%	41.67%	2.78%	5.56%	8.33%
let-7-C^{K01}*/usp*^{4}*;* *P{W8, let-7-C*$^{\Delta let\text{-}7}$*}/+*	6	50.00%	50.00%	0.00%	0.00%	0.00%
usp^{4}*/+; EcR*^{Q50}*/+*	29	72.41%	10.34%	0.00%	17.24%	0.00%
let-7-C^{K01}*/Fas II*^{G0081}*;* *P{W8, let-7-C*$^{\Delta let\text{-}7}$*}/+*	18	16.66%	61.11%	0.00%	0.00%	22.22%

[1]MB morphology was analysed in Fas II stained 1-3d old adult brains

*animals were transferred from 18°C to 29°C environment for 24h at 12hAPF stage

**animals were heat-shocked for 1h in 37°C water bath 3 times per day at 12hAPF stage

Supplementary Table VI. Predicted *let-7* targets

let-7 target[1]	Con-served sites[2]	Molecular function	Biological process
A3-3	1	transcription factor	nervous system development
ab	3	transcription factor	dendrite morphogenesis; neuron development
apt	1	transcription factor	PNS development
Arr1	1	metarhodopsin binding	photoreceptor cell maintenance; endocytosis
bin	1	DNA binding	mesodermal cell fate commitment
CG11050	1	metal ion binding; phosphoric diester hydrolase activity	unknown
CG12130	1	peptidylamidoglycolate lyase activity	peptide metabolic process
CG12701	1	DNA binding	mitotic cell cycle; cellularization
CG14614	1	unknown	unknown
CG15887	1	unknown	unknown
CG17090	1	protein serine/threonine kinase activity	positive regulation of Wnt receptor signaling pathway; positive regulation of Notch signaling pathway
CG17100	1	DNA binding	regulation of circadian rhythm
CG18265	2	zinc ion binding	unknown
CG2093	1	unknown	protein targeting to vacuole
CG31176	1	unknown	unknown
CG33203	1	unknown	unknown
CG5026	1	protein tyrosine/serine/threonine phosphatase activity	dephosphorylation
CG6014	1	unknown	tissue regeneration
CG6490	1	unknown	unknown
CG8494	1	ubiquitin thiolesterase activity	nervous system development
CG9098	1	SH3/SH2 adaptor activity	small GTPase mediated signal transduction
CG9514	1	choline dehydrogenase activity	unknown
Dh	1	neuropeptide hormone activity	body fluid secretion
Eip93F	1	transcription factor	induction of apoptosis; cellular response to hypoxia; autophagic cell death
hairy	1	protein binding	response to hypoxia; tube morphogenesis; cell morphogenesis; tracheal system development
hkb	1	transcription factor	CNS development; glial cell differentiation; germ cell migration
ifc	1	sphingolipid delta-4 desaturase activity	spermatogenesis
iHog	1	heparin binding; hedgehog receptor; protein homodimerization	smoothened signaling pathway; cuticle pattern formation; wing disc pattern formation
IM2	1	unknown	unknown
Notum	1	unknown	Wnt receptor signaling pathway
rib	1	transcription factor	tracheal system development; embryonic development via the syncytial blastoderm; digestive tract development; cell polarity; gland morphogenesis
Sac1	1	polyphosphatidylinositol phosphatase activity	negative regulation of JNK cascade
sqd	1	mRNA binding	RNA splicing; ovarian follicle cell development; RNA metabolic process; oocyte anterior/posterior axis specification
stet	1	serine-type peptidase activity	epidermal growth factor receptor signaling pathway
ytr	1	mRNA binding	hemocyte differentiation

[1]targets predicted by TargetScan (Ruby et al, 2007) and PicTar (Grun et al, 2005) databases;
[2]conserved binding sites predicted by TargetScan

Supplementary Table VII. The frequency of Ab positive cells is increased in *Δ let-7* mutants

Genotype	Number of Ab-positive cells per calyx AVE±AVEDEV	GFP and Ab double labeled cells per calyx		*P*-Value[1]
		Number AVE±AVEDEV	Percentage AVE±AVEDEV	
Control *let-7-C^{GK1}/+; UAS-CD8GFP/+*	63.25±13.63	553.50±30.75	11.37±2.33	-
Δlet-7 *let-7-C^{GK1}/ let-7-C^{K01}; UAS-CD8GFP/P{W8, let-7-C$^{\Delta let\text{-}7}$}*	158.75±31.63	621.25±38.75	25.46±4.45	6.7×10^{-3} **

[1]statistics were calculated using a two-tailed Student's t-test and the *P*-value is presented; ** $P \leq 0.01$

Supplementary Table VIII. Chronological lineage analysis of MB neurons using the MARCM technique shows that miRNA *let-7* and the nuclear factor Ab are required cell autonomously for establishment of α/ß and α'/ß' neuronal cell fate respectively

Stage of clonal induction[a]	Genotype	Analyzed MB lobes (n)	Lobes with MBN-derived clones	Lobes with GMC/neuron-derived clones	*P*-value[b]	Single/double cell clones within γ lobe[c]	Single/double cell clones within α'/β' lobe[c]	Single/double cell clones within α/β lobe[c]
L1	***Parental control***[d]	38	60.53%	39.47%		100% n=15	-	-
	let-7	54	75.93%	24,07%	0.11	100% n=13	-	-
	ab^{k02807}	30	73.33%	26.67%	0.28	100% n=8	-	-
L3	***Parental control***	42	33.33%	66.67%	-	-	100% n=28	-
	let-7	29	55.17%	44.83%	0.07	-	100% n=13	-
	ab^{k02807}	50	52.00%	48.00%	0.07	53.85%*** n=21	5.13%*** n=2	41.03%*** n=16
P	***Parental control***	19	68.42%	31.58%	-	-	-	100% n=6
	let-7	56	58.93%	41.07%	0.15	-	68.00%*** n=17	32.00%*** n=8
	ab^{k02807}	25	28.00%	72.00%	0.54	-	-	100% n=18

[a] for the possible outcome of clonal induction at different developmental stages please refer to Supplementary Figure 2A

[b] significance between MBN-derived versus single/double cell clones was calculated using a two-tailed Student's t-test and the *P*-value is presented; 3-5 independent experiments were performed for each stage of heat shock induction and genotype

[c] In order to analyze the significance between the frequencies of cell identities (γ, α'/β' or α/β) acquired by single/double cell MARCM clonal neurons induced at different developmental stages (L1, L3, P) in different genetic backgrounds (*Parental control, let-7,* and *ab*^{k02807}) two-way tables and chi-squared test with 4 degrees of freedom were used

[d] due to the leakiness of heat-shock promoter even without heat pulse, MARCM clones can be generated with the frequency of 3.20±1.68 clonal cells per brain; however, only 0.10±0.18 clones per MB cell cluster, which is not significantly different from the null. After heat-shock induction every brain had at least one or more single/double-cell MB clone. See also data below:

hsFlp, UAS-CD8GFP/+; tubGal80 FRT 40A/FRT 40A; tubGal4/+	**Single cell clones per brain (Mean±AveDev)**	**NB derived clones per brain (Mean±AveDev)**	**Single cell clones per MB (Mean±AveDev)**	**MBN derived clones (Mean±AveDev)**	**Number of analyzed brains (MBs)**
	3.20±1.68***	0.10±0.18	0.10±0.18	0.00±0.00	10 (20)
p-Value (*compared to 0*)	1.72×10^{-4}	0.15	0.15	ND	-

***p≤0.001

Supplementary Table IX. ***Fas II*** **mRNA levels**

Genotype	***Fas II*** Average C_T	***RpL32*** Average C_T	ΔC_T[1]	$\Delta\Delta C_T$[2]	Average ***Fas II*** mRNA levels relative to control[3]
Control[a] (*Oregon R*)	33.04±1.20	28.77±1.17	4.27±0.35	0.00±0.35	1.02±0.23
Δ *let-7* (*let-7-C^{GK1}/let-7-C^{KO1}; P{W8, let-7-C$^{\Delta let\text{-}7}$}*)	32.79±0.42	26.62±0.12	6.17±0.38	1.90±0.38	0.27±0.08
Δ *let-7*, ab (*let-7 miR-125, ab^1/ let-7 miR-125, ab^{1D}*)	32.62±0.12	27.67±0.12	4.95±0.17	0.68±0.17	0.63±0.07
Fas II (*Fas2^{RNAi}/let-7-C^{GK1}*)	31.81±0.28	26.21±0.28	5.6±0.56	1.32±0.56	0.42±0.15
Control[b] (*ab^1/CyO*)	19.65±0.09	14.09±0.33	5.57±0.09	0.00±0.09	1.00±0.06
ab (*ab^1/ab^{1D}*)	20.40±0.02	15.37±0.07	5.03±0.03	-0.53±0.03	1.44±0.03

[1] the ΔC_T value is determined by subtracting the average *RpL32* C_T value from the average *Fas II* C_T value. Mean (2 independent experiments done in triplicate) ± SD (standard deviation). SD is calculated from the SDs of the *RpL32* and *Fas II* values using the following formula: $SD=\sqrt{SD1^2+SD2^2}$;

[2] the calculation of the $\Delta\Delta C_T$ involves subtraction by the ΔC_T calibrator value. $\Delta\Delta C_T = \Delta C_T - \Delta C_T$ (Control).

[3] the range given for *Fas II* levels in *Δ let-7* mutant relative to Control[a] (*Oregon R*) or in *ab* mutant relative to Control[b] (*ab^1/CyO*) was calculated as $2^{-\Delta\Delta CT}$

Supplementary Table X. Viability and MB phenotypes of studied mutants

Supplementary Figure Legends

***Gal4* driver**	***UAS*-transgene**	**Lethality stage**	**α/β lobe phenotype**
let-7-C^{GK1} x	*UAS-ab*	Pharate	underdeveloped, slim α/β lobes
	UAS-apt	viable	no visible phenotype
	UAS-fas2^{RNAi}	viable	fused β lobes (50%), vacuolated α/β lobes
	UAS-shotgun^{RNAi}	viable	no visible phenotype
	UAS-EcR^{RNAi}	viable	underdeveloped, slim α/β lobes, fused β lobes
	UAS-Fas II	viable	no visible phenotype
c309Gal4 x	*UAS-let-7*	pharate	mislocalized Fas II
	UAS-mut-let-7	viable	no visible phenotype
c305aGal4 x	*UAS-Fas II*	viable	Trio-positive cells project into α/β lobes
	UAS-ab	pupa	-
c739Gal4 x	*UAS-ab*	viable	weak Fas II staining
	UAS-EcR^{RNAi}	viable	underdeveloped, slim α/β lobes

Supplementary Figure Legends

Figure S1. *let-7-C* expression in adult *Drosophila* brain

(**A**) *let-7-C* is expressed in the lobula (*lo*) and lobula plate (*lo p*), (**B**) antennal lobes and (**C-D**) central complex ellipsoid body (*eb*), fun-shaped body (*fb*), superior arch (*sa*), and mushroom bodies (*mb*). *let-7-C* expression is marked with membrane GFP – green and nuclear LacZ – red *(let-7C^{GK1};UAS-CD8-GFP,UAS-nLacZ).* MicroRNA *let-7* (**E-F**) has a broad expression signal in the control (*Oregon R,* ***E***), but not *let-7* mutant (*let-7-C^{GK1}/let-7-C^{KO1}; P{W8, let-7-C$^{\Delta let\text{-}7}$}*, **F)** pupal brains as detected by locked nucleic acid (LNA) *in situ* hybridization.

Figure S2. *let-7-C* deficiency in γ neurons does not affect γ lobe formation

(**A**) A scheme depicting the possible types of GFP marked MB neurons obtained after heat-shock at different stages, which also depends whether MARCM recombination event was induced in MBNs or GMCs and differentiating neurons. (**B-C**) Upon induction of MARCM clones at the 1st instar larval stage, GFP positive control γ neurons (*hsFlp UAS CD8GFP; FRT 40A tubGal80/FRT 40A; tubGal4/+*) and GFP positive *Δlet-7* γ neurons (*hsFlp UAS CD8GFP; FRT 40A tubGal80/FRT 40A let-7 miR-125; tubGal4/P{W8, let-7-C$^{\Delta let\text{-}7}$}*) correctly remodel and project their axons to form γ lobes. Fas II (**B**) and Trio (**C**) mark γ lobes - red, GFP marks clonal neurons - green, DAPI marks nuclei – blue. MB lobes are outlined and marked.

Figure S3. Ecdysone signaling in the developing *Drosophila* brain

(**A-C**) Brains of wild type animals at the different stages of development in which active ecdysone signaling (*EcRE.lacZ*) was visualized via β-Gal staining. The highest signal for ecdysone signaling was observed at 48h APF (**B**).

Figure S4. The Apt expression pattern is not changed due to *let-7* deficiency

(**A**) *Drosophila* larval brain contains multiple neuroblasts, while in the pupal brain (**B**) only four MB neuroblasts per MB cell body cluster can be detected marked with a NB specific marker Miranda (Mira, arrows). (**C**). In the pharate brain only four MBNs (yellow arrow), and their direct progeny GMCs (white arrow) are mitotically active and positive for the mitotic marker phosphohistone-3, PH3. (**D**) At the pharate stage Ab staining is seen in differentiated neurons, but not in MBN (marked by Mira, **D'**). (**E**) In the pharate brain Apt staining is

restricted to MBNs (Mira positive cells) and few cells that are juxtaposed to MBNs (GMCs and immature neurons) (**E"**). (**F**) *let-7* deficiency does not affect the Apt expression pattern when compared to the control in Figure 3C. (**G**) Overexpression of Apt in *let-7-C* expressing neurons does not affect MB development.

Figure S5. Expression pattern of different MB specific drivers used in this study

(**A**) *201y-Gal4* - specific for γ lobe (larval brain), (**B**) *c305a-Gal4* - specific for α'/β' lobes (adult brain), (**C**) *309c-Gal4* - specific for all MB lobes, Fas II marks γ and α/β MB lobes.

Figure S6. Ab is required for α'/β' MB lobes formation.

(**A-B**) When induced at the L1 stage of development *ab* clonal GMCs give rise to normally developed γ lobe neurons (**A**, left side) and *ab* clonal MBNs generate γ and α/β, but not α'/β' lobe neurons (**A**, right side; **B**). (**C-E**) When induced at the L3 stage of development, *ab* clonal MBNs fail to form α'/β' lobe neurons, but produce underdeveloped α/β lobes that result from morphological defects such as midline crossing (**C**) or premature collapse (**D-E**) of mutant neurons. Blue arrows point to the places where α' lobe neurons should be seen if they would develop. Yellow outlines α/β lobes. (**F**) Ab overexpression in MB neurons *(hsFlp; act.CD2.Gal4;UAS-ab)* induced at L2 stage results in the axon guidance phenotype. (**G-H**) Forced expression of Ab in α/β neurons using α/β specific driver *c739Gal4* (**H**) decreases Fas II levels in α/β MB lobes in comparison to Control (**G**). MARCM MBN-derived clone induced at L1 contains early- and late-born neurons (GFP positive cells). After clonal induction at L1, larvae were fed EdU at L3 to mark the late born α'/β' neurons. Unlike wild type neurons born at L3 (EdU positive, GFP negative), *ab* mutant neurons (EdU positive, GFP positive) express Fas II. White dashed line outlines *ab* clone, yellow small circles mark EdU positive neurons that are born at L3.

Figure S7. The Cadherin expression pattern in *Drosophila* MBs

DE-Cadherin is expressed in the pupal MB cell body cluster and is enriched around MBNs and GMCs (**A**) and in the adult MB lobes (**B, B'**).

Supplementary Figures

Figure S1

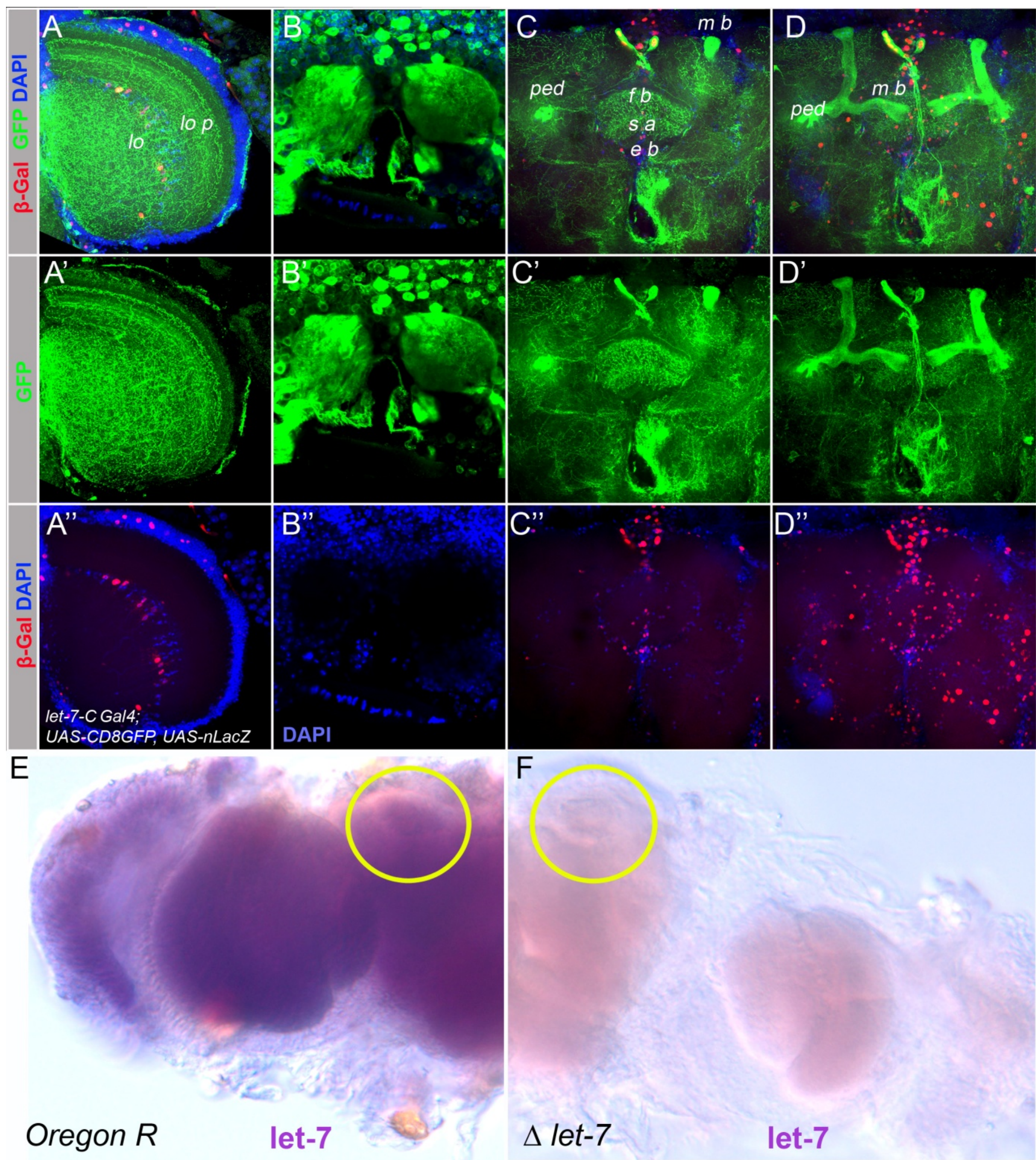


Figure S2

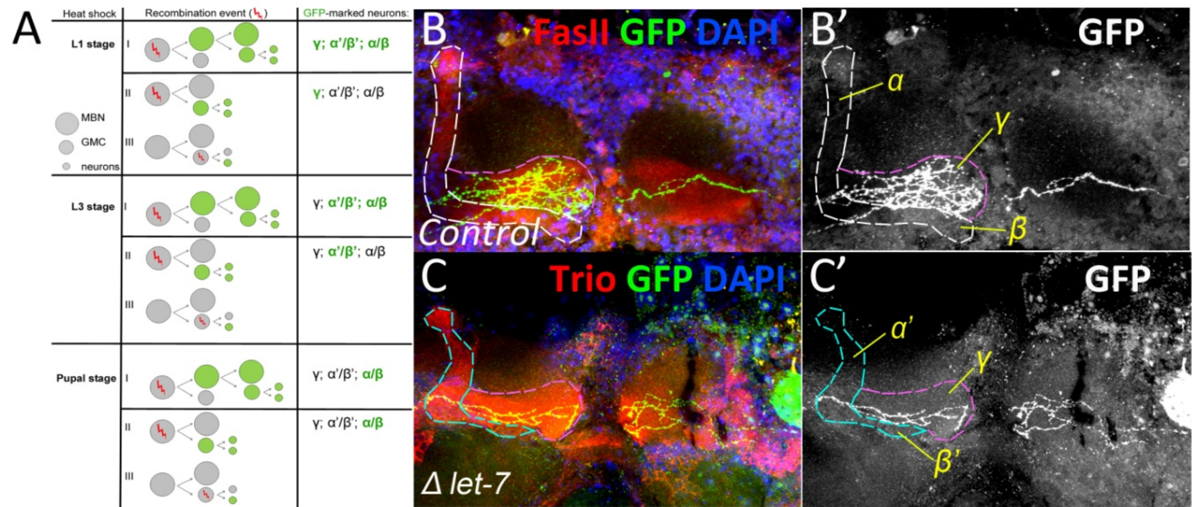


Figure S3

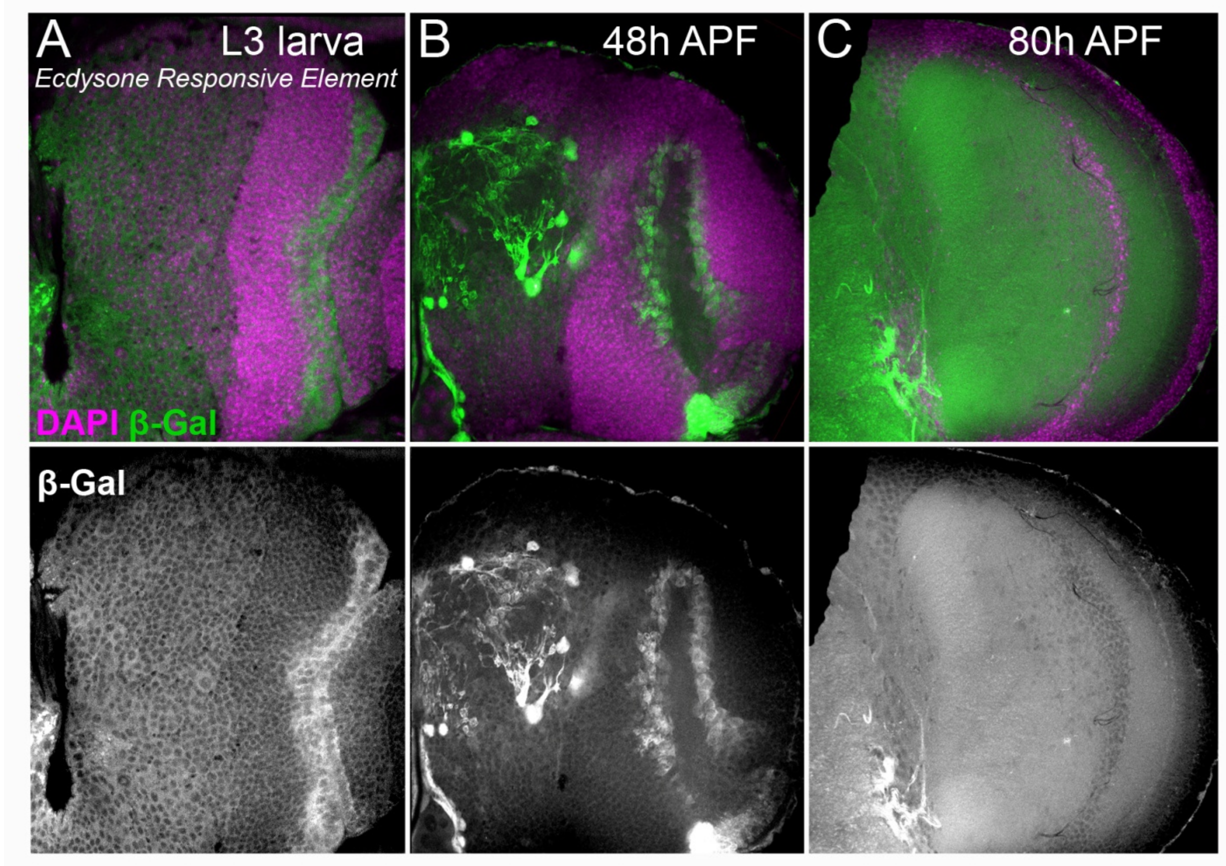


Figure S4

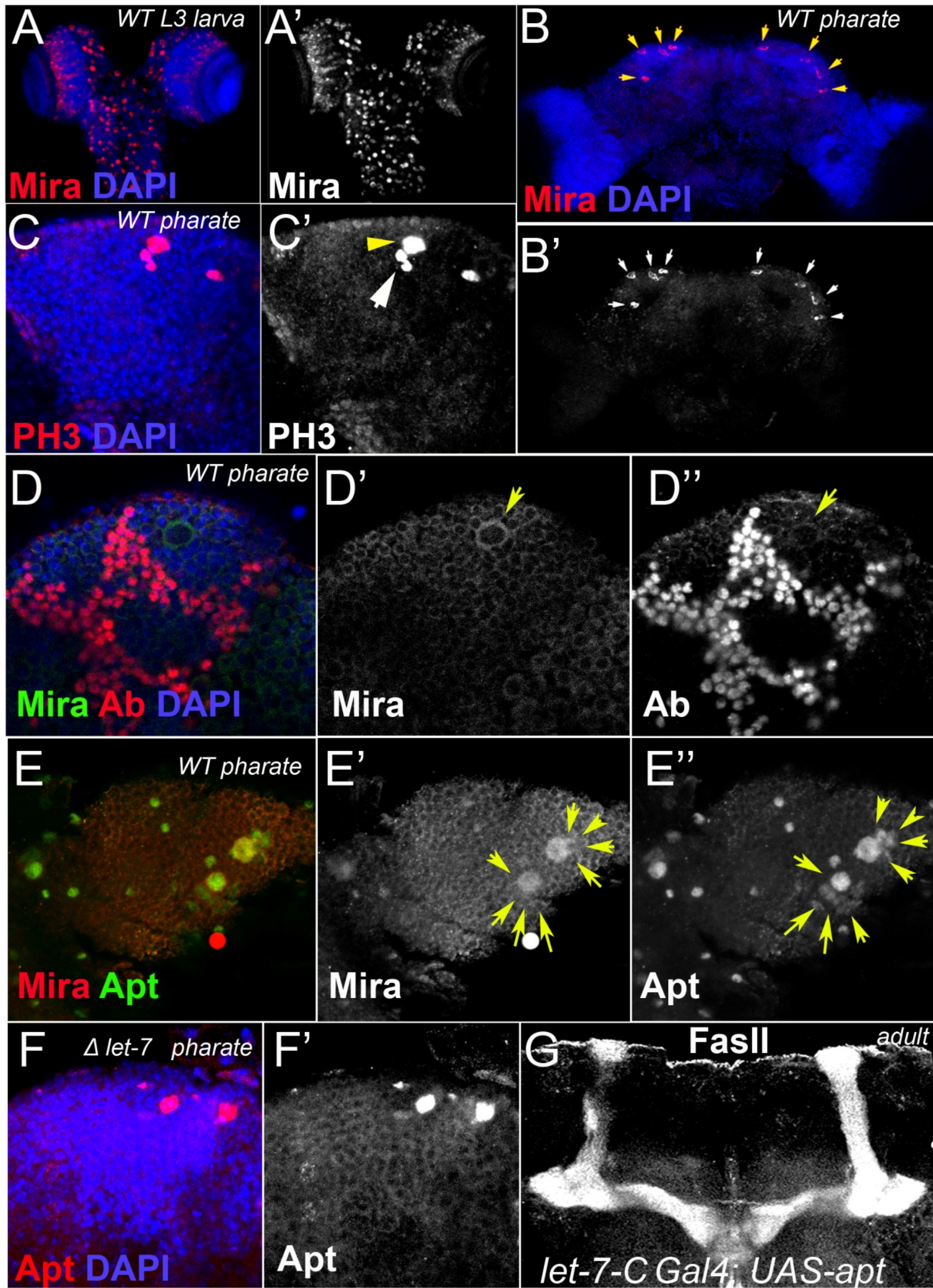


Figure S5

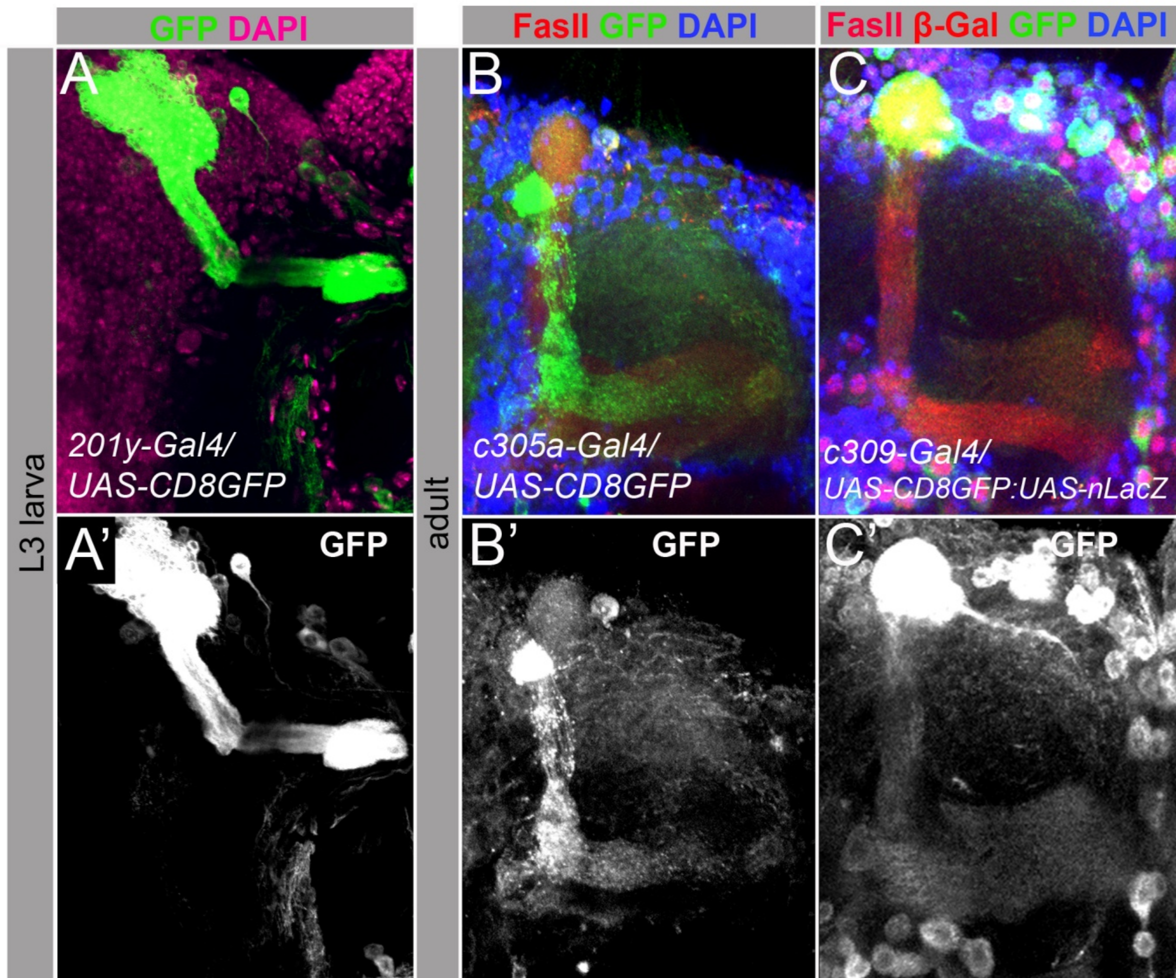


Figure S6

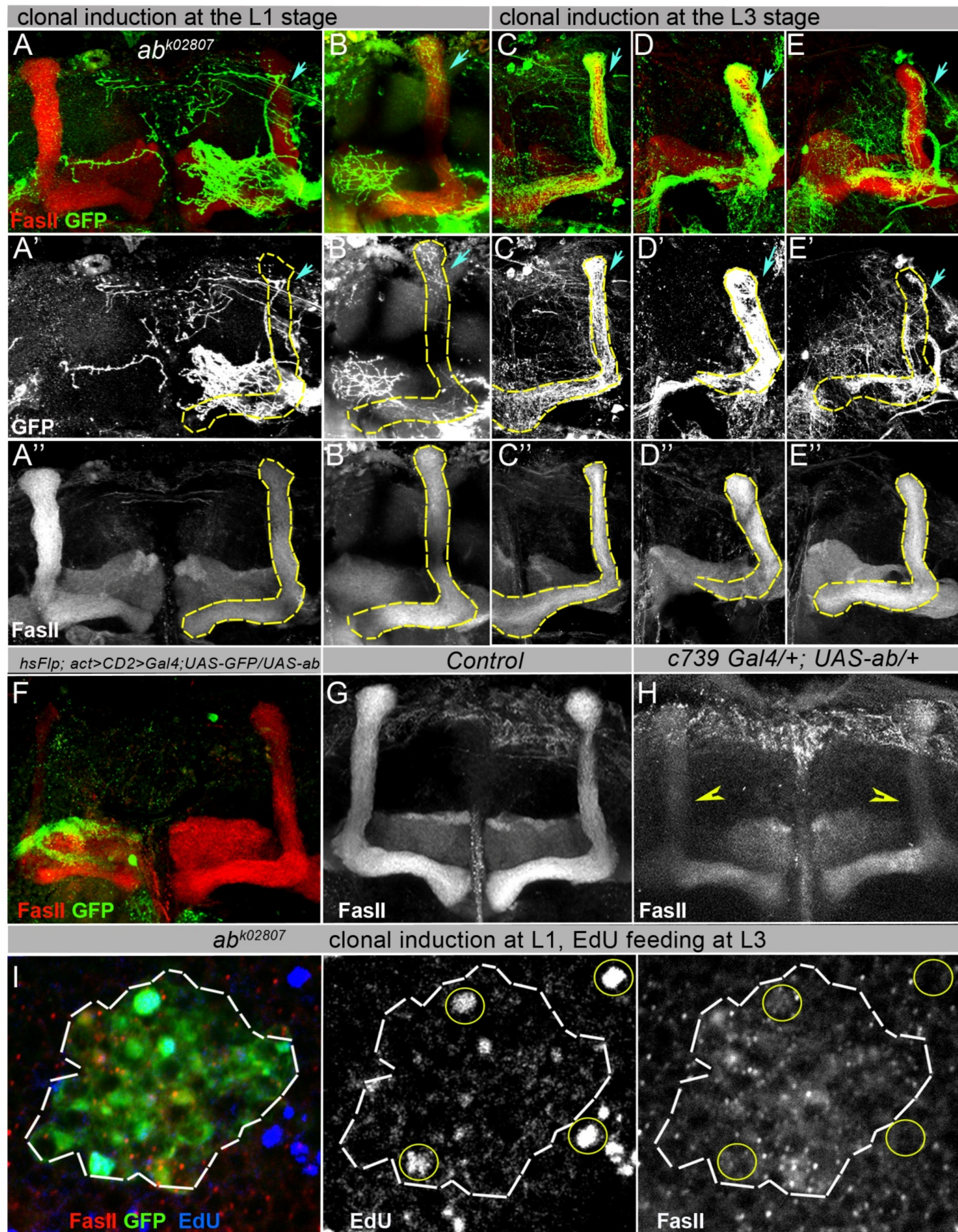
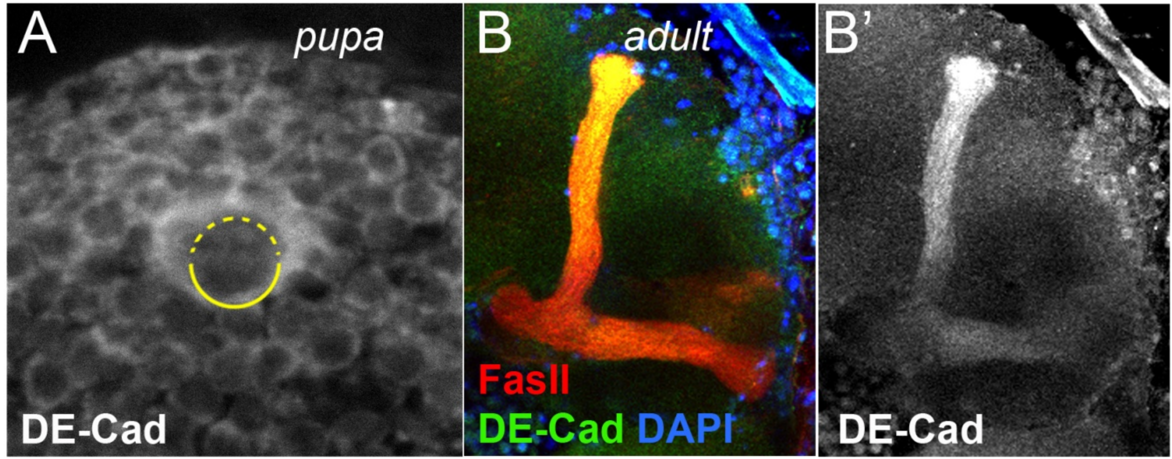


Figure S7



Supplementary References

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