Genetic Network Shaping Kenyon Cell Identity and Function in *Drosophila* Mushroom Bodies

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Abstract

Revealing the molecular mechanisms underlying neuronal specification and acquisition of specific functions is key to understanding how the nervous system is constructed. In the *Drosophila* brain, Kenyon cells (KCs) are sequentially generated to assemble the backbone of the mushroom body (MB). Broad-complex, tramtrack and bric-à-brac zinc finger transcription factors (BTBzf TFs) specify early-born KCs, whereas the essential TFs for specifying late-born KCs remain unidentified. Here, we report that Pipsqueak domain-containing TF *Eip93F* promotes the identity of late-born KCs by reciprocally regulating gene expression in KC subtypes. Moreover, *Eip93F* not only regulates the expression of calcium channel *Ca-α1T* in late-born KCs to functionally control animal behavior, but it also forms a genetic network with *BTBzf TFs* to specify the identities of KC subtypes. Our study provides crucial information linking KC subtype diversification to unique function acquisition in the adult MB.

Introduction

The nervous system contains a diverse ensemble of neuron types, which are generated during development in appropriate numbers under tightly controlled processes. A central question in developmental neurobiology concerns how diverse neuron types acquire unique cell identities with characteristic morphologies, distinct gene expression profiles and specific functionalities. Kenyon cells (KCs) are the intrinsic neurons in the *Drosophila* mushroom body (MB) [1] and can serve as an excellent model system for investigations into the molecular mechanisms of neuron subtype diversification. Around 2,000 KCs generated by four neuroblasts are grouped into three sequentially born subtypes, including γ, α'/β' and α/β neurons [1]. Functionally, these KC subtypes play different roles in short-term memory, acquisition, stabilization, and retrieval of memory [2, 3]. Morphologically, both α/β and α'/β' neurons have axons with two branches that project dorsally and medially into respective α and β, or α' and β' lobes, whereas γ neurons send out single axon branches that project medially into the γ lobe [1]. These three KC subtypes can be also distinguished by differentially expressed marker genes. For instance, broad-complex, tramtrack and bric-à-brac zinc finger transcription factor (BTBzf TF) Abrupt (Ab) is specifically expressed in γ neurons [4-6], whereas cell adhesion molecule Fasciclin II and Rho guanine nucleotide exchange factor Trio are expressed in different subsets of KC subtypes [4]. Therefore, specifying KC subtypes with proper cell numbers, unique cell identities and distinct functions is a key aspect of MB formation.

Previous studies have revealed that the other BTBzf TF, Chronologically inappropriate morphogenesis (Chinmo), exhibits a graded expression pattern in KCs and plays a crucial role in diversifying KC subtypes [7]. Chinmo is highly expressed in early-

born γ neurons, but its expression level is low in later-born α'/β' and absent in α/β neurons [7]. Notably, Chinmo controls the expression of the third BTBzf TF, Maternal gene required for meiosis (Mamo), functioning to specify the identities of KC subtypes at the larval stage via a fine-tuning process. In particular, a high expression level of Chinmo inhibits Mamo expression in γ neurons, whereas low Chinmo expression promotes Mamo production in α'/β' neurons [4]. As a gradual reduction of Chinmo expression occurs during development, Mamo, adding to its function in α'/β' neurons, takes over Chinmo's role of regulating the differentiation of γ neurons at the pupal stage [4, 5, 7]. In the absence of *chinmo* and *mamo*, molecular and morphological characteristics of γ and α'/β' neurons are shifted towards those of α/β neurons, constituting a cell identity transformation [4, 5, 7]. Therefore, the late-born α/β neural identity is hypothesized to be a default status upon the loss of the neural identity determinants for early-born KCs [4, 7]. However, it is also possible that key specification regulators of α/β neurons exist but have not yet been identified.

In this study, we leverage RNA-seq databases on KCs to identify subtype-specific markers as readouts for the cell identity of γ, α'/β' and α/β neurons [8, 9]. By doing so, we identified a phylogenetically conserved Pipsqueak domain-containing TF, Ecdysone-induced protein 93F (Eip93F/E93), with preferential expression in α/β neurons. Loss-of-function of *E93* not only downregulated α/β-specific gene expression, including a subunit of T-type like voltage gated calcium channel Ca-α1T, but it also upregulated γ-specific Ab in late-born KCs, implying that an identity shift towards early-born KCs had occurred. Intriguingly, RNAi knockdown of *E93* or *Ca-α1T* in α/β neurons further compromised animal behaviors, including foraging-related and night-time activities. In contrast, E93

overexpression precociously turned on Ca-α1T expression in early-born KCs at the expense of abolishing expression of early-born KC markers, such as Ab and Mamo. Notably, E93 was upregulated in early-born KCs in the absence of *chinmo* and *mamo* but diminished in late-born KCs upon Ab overexpression. Taken together, our results suggest that a hierarchical genetic network among *chinmo*, *mamo*, *E93* and *ab* with potential feedback loops controls the identity and function of KC subtypes during the construction of functional MBs.

Results

Identification of KC subtype-specific markers

By leveraging information from published RNA-seq studies showing preferentially expressed genes in adult KC subtypes [8, 9], we sought to identify a collection of KC subtype marker-expressing lines (available at stock centers) with GFP transgenes at genes of interest [10-12]. The results of this screen for KC subtype marker GFP lines are depicted in S1 Fig; highlights of certain KC subtype marker lines are described below. First, Abrupt (Ab)-GFP, a BAC clone-engineered GFP line, can be used to replace an excellent Ab antibody (generated by Dr. Crews laboratory but no longer available) for labeling γ neurons [4-6] (Fig 1A-B and S2 Fig). In addition, Lachesin (Lac; an Ig superfamily protein[13])-FSVS, a GFP-trapping line, expresses GFP-fused Lac specifically in α'/β' neurons starting from the early pupal stage (Fig 1C-D and S3A Fig). Notably, we also identified two other GFP-trapping lines, Ecdysone-induced protein 93F (E93)-GFSTF and Calcium channel protein α1 subunit T (Ca-α1T)-GFSTF, that express GFP-fused proteins enriched in α/β neurons. Enrichment in these neurons is evidenced by patterns of complementary expression to Trio, a marker of γ and α'/β' neurons [4] (Fig 1E-H). Consistent with the notion that generation of most of α/β neurons occurs at the pupal stage [1], E93-GFSTF and Ca-α1T-GFSTF were not detectable in KCs at the wandering larval (WL) stage (Fig 1E, 1G and S3B Fig). With this set of useful reagents, we set out to further explore the molecular mechanisms underlying cell identity specification of KC subtypes.

Loss-of-function of *E93* suppresses the α/β-neural identity and has behavioral consequences

Since E93, a Pipsqueak-domain containing TF, functions crucially in various biological processes [14-17] and E93-GFSTF was preferentially expressed in α/β neurons (Fig 1F), we sought to investigate whether *E93* acts as a critical regulator for specifying the α/β-neural identity. First, we found that the expression of a α/β-specific marker, Ca-α1T-GFSTF, was diminished in the context of *E93* mutation ($E93^{A11}$) and in a line with *E93* knockdown due to overexpression of *E93* RNAi by a pan-KC driver, GAL4-OK107[1] (Fig 2A-B and S4-S5 Fig). In contrast, the same RNAi knockdown elicited no discernible effects on the expression levels of Trio and Lac-FSVS in γ and α'/β' neurons (Fig 2A-B and S6 Fig). Since *Ca-α1T* encodes a subunit of a T-type like voltage gated calcium channel, which regulates sleep behavior [18], we wondered whether the loss of *E93* and *Ca-α1T* in α/β neurons could cause behavioral defects. To examine the behavior, we utilized a monitoring system to film and analyze the activities of individual flies (S7A-B Fig). The animal's speed of locomotion usually peaks around the day-to-night shift and gradually becomes reduced at night. In control samples (wild-type, RNAi-only, and GAL4-only lines), it took around eight hours for the moving speed to reach a minimum during the night period (Fig 2C and S7C-D Fig). Interestingly, night-time activity was significantly impaired (with a sharp reduction of moving speed to a minimal value at around two hours into the night period) when *Ca-α1T* and *E93* were knocked down using an α/β neural driver, c739-GAL4 [19] (Fig 2C and S7C-D Fig). Although the overall moving speed was lower and more variable in *E93* knockdown samples than in controls, it is possible that E93 also regulates factors that add on to causal effects from Ca-α1T. In addition to the effects on night-time activity, RNAi knockdown of *Ca-α1T* and *E93* appeared to compromise the foraging-related behavior. Compared to control flies, *Ca-α1T*- and *E93*-deficient animals

tended to explore regions without fly food during the day-time period (Fig 2C and S7E Fig). Taken together, these results suggested that Ca-α1T (expression is regulated by E93) acts in α/β neurons to potentially control animal behavior.

In addition to the observed reduction of Ca-α1T-GFSTF, *E93* knockdown in KCs substantially compromised the expression of another α/β-specific marker, myr::GFP driven by R44E04-LexA [5] (Fig 2D-E). Although these results seemed to indicate the loss of α/β neural identity due to loss of *E93* function, the findings also raised a possibility that the reduction of Ca-α1T-GFSTF and R44E04-LexA might simply be due to the absence of late-born KCs. However, this explanation was ruled out by the observation of relatively intact morphology of α and β lobes when we examined the expression of a third α/β-specific marker (myr::GFP driven by R70F05-LexA) upon *E93* knockdown in KCs [5] (Fig 2F-G). Moreover, compared to wild-type samples, the cell number of R70F05-LexA-positive neurons was not significantly altered in KCs when *E93* was knocked down (Fig 2H-I and S8 Fig). Taking advantage of R70F05-LexA as a putative α/β-neural marker, we further found that more than half of the R70F05-LexA-positive neurons ectopically expressed γ-specific Ab-GFP in *E93* knockdown samples (Fig 2H-I and S8 Fig), implying that α/β neurons had transformed into γ-like neurons. These results taken together suggested that E93 is required for specifying KCs towards the cell identity of α/β neurons, which are responsible for specific functions governing animal behaviors.

E93 overexpression promotes α/β neural traits in early-born KCs

We next asked if E93 indeed plays a crucial role in specifying α/β neural identity, i.e., does gain of E93 function transform the cell identity of other KC subtypes to α/β neurons? In

contrast to the undetectable Ca-α1T-GFSTF expression in wild-type with major KC subtypes of γ and α'/β' neurons at the WL stage, Ca-α1T-GFSTF expression was precociously upregulated in KCs when E93 was overexpressed by GAL4-OK107 (Fig 3A-B). Similarly, a portion of putative γ neurons exhibited α/β-neural like features (myr::GFP driven by R70F05-LexA) with axonal defects when E93 was overexpressed by the γ-neural driver, GAL4-201Y [1] (Fig 3C-D). Consistent with this observation, we further found that E93 overexpression driven by GAL4-OK107 (but not Worniu-GAL4, a pan-neuroblast driver [16]) compromised the expression of specific markers in γ neurons at various developmental stages, including Ab-GFP, the receptor for insect molting hormone ecdysone (EcR-B1) and Mamo isoforms (Fig 3E-J and S9-S10 Fig). Along with the effect of impairing γ-specific marker expression, we further found that E93 overexpression driven by GAL4-OK107 impaired the expression of markers in α'/β' neurons, including $Mamo^{D\sim G}$ isoforms and Lac-FSVS (Fig 3I-L). Its overexpression also caused morphological defects by perturbing the remodeling process that normally occurs in early-born KCs at 24-hour after puparium formation (APF; Fig 3K-L). These results taken together suggested that E93 when overexpressed is sufficient to cause specification of KCs toward the α/β neural identity.

Hierarchical genetic network of *chinmo*, *mamo*, *E93* and *ab* controls the cell identity of KC subtypes

Since Chinmo and E93 act as key TFs to regulate the cell identity of KC subtypes, we wondered whether *chinmo* and *E93* might form a genetic network to diversify KC subtypes. Since *chinmo* controls the expression of γ-specific Ab [4, 5] (S2 Fig), we decided to test

whether the loss of Ab-GFP by E93 overexpression (seen in Fig 3F) was caused by compromised Chinmo expression. However, we did not observe a reduction of Chinmo level upon E93 overexpression at the first instar larval stage (the most abundant Chinmo expression stage in the wild-type [7]), even though the same manipulation did block Ab-GFP expression (Fig 4A-B). In contrast, E93-GFSTF expression was precociously upregulated in early-born KCs in a *chinmo* mutation line (*chinmo*$^{[1]}$) and in the *chinmo* knockdown line (overexpression of *chinmo* RNAi driven by GAL4-OK107) at the WL stage (Fig 4C-D and S11 Fig). In line with the facts that microRNA *let-7* and RNA binding protein Syncrip (Syp) inhibit Chinmo expression [20, 21], we further found that E93-GFSTF expression was abolished by *syp* RNAi knockdown and partially promoted by *let-7* overexpression (S12 Fig). These results suggested that the adoption of γ neural identity by early-born KCs is in part due to suppression of the α/β-neural regulator E93 via Chinmo.

After the diminishment of Chinmo at the early pupal stage [7], we wonder whether E93 would be disinhibited in γ neurons and whether their neural identity would be transformed if this disinhibition indeed occurs? Since γ neurons exist in adult brains and their neural identity is crucially regulated by Mamo at the pupal stage [5], we then tested whether Mamo takes over Chinmo's role to suppress E93 expression in γ neurons. As such, *mamo* RNAi knockdown indeed caused the upregulation of E93-GFSTF in KCs (within enriched Trio expression) of adult brains but not brains at the WL stage (Fig 4E-G), suggesting that Mamo could inhibit E93 expression in γ neurons to ensure their neural identity. Since the E93-mediated α/β neural identity is accompanied by absence of certain γ-specific traits, such as Ab-GFP (Fig 2I and 3F), we next sought to explore whether Ab also plays a crucial role in controlling the KC identity. Intriguingly, we found that the

expression of α/β-specific Ca-α1T-GFSTF and E93-GFSTF was not detectable upon Ab overexpression (Fig 4H-J and S13 Fig). This was accompanied by the expansion of Trio in the cytosol in almost all KCs, indicating the transformation of KCs into γ neuron-like cells. We also noted that RNAi knockdown of *ab* neither abolished Trio expression nor upregulated the expression of Ca-α1T-GFSTF caused by *E93* knockdown in early-born KCs (S14-S15 Fig). Nonetheless, these results together suggested that *chinmo*, *mamo*, *E93* and *ab* form a hierarchical genetic network with potential feedback loops to control the identity of KCs (Fig 5).

Discussion

Drosophila MBs are primarily constructed by three sequentially generated KC subtypes, γ, α'/β' and α/β neurons [1]. Previous studies have revealed that BTBzf TFs, Chinmo and Mamo, function crucially in the specification of early-born KC subtypes into γ and α'/β' neurons [4, 7]. In this study, we revealed the mechanisms of KC subtype specification by identifying and characterizing Pipsqueak domain-containing TF E93 as a driver of KC identity towards α/β neurons. Our findings that E93 regulates the expression of calcium channel Ca-α1T to subsequently control animal behaviors also provide an illustration that neural identity specification is associated with acquisition of specific functions among KC subtypes. We further showed that the specification of KC subtypes is controlled via a genetic network formed by *chinmo*, *mamo*, *E93* and *ab*, which mainly controls the KC identity of γ and α/β neurons during construction of the adult MB (Fig 5).

Despite that reciprocal regulation between BTBzf TFs and E93 has been reported in multiple cell types during the *Drosophila* development [22, 23], here, we disclosed a novel strategy of using genetic networks of *BTBzf TFs* and *E93* to specify distinct cell identities among neurons derived from the same neuroblasts (Fig 5). To diversify KC subtypes, Chinmo and Mamo take turns to inhibit the E93 expression, thereby establishing the cell identity of γ neurons, as suggested by Ab expression [4] (Fig 2I and 4G). Without the inhibitory effects of Chinmo and Mamo on E93 expression, late-born KCs specify into α/β neurons by turning on the expression of α/β-specific genes and suppressing the expression of γ-specific genes, Ab included (Fig 2). Although how the genetic network of *BTBzf TFs* and *E93* specifies α'/β' neural identity has not yet been fully resolved, the Mamo expression promoted by the low level of Chinmo, which is regulated by the transforming

growth factor beta (TGF-β) signaling in MB neuroblasts, is crucial for KCs to adopt the α'/β' neural identity [4, 24]. Intriguingly, our results revealed that E93 overexpression can abolish the expression of Mamo to block KCs from adopting the α'/β' neural identity (Fig 3J and 3L). All these results taken together portray that the genetic network of *BTBzf TFs* and *E93* is very likely to control the KC identity during construction of the adult MB. In light of the phylogenetic conservation of the *E93* family and *tramtrack* (*TTK*)-type *BTBzf TFs*, including *chinmo*, *mamo* and *ab*, as Arthropoda-specific genes [25, 26], one cannot help but wonder whether *TTK*-type *BTBzf TF*s might have been evolutionally introduced to intervene with *E93* to regulate the development of multiple arthropodan tissues; perhaps future investigations may delineate their relationship on neuron-type diversification in the nervous system.

Once the identities of neuron subtypes are determined by key regulators, how the cells acquire specific functions in the nervous system to eventually participate in animal behaviors? Previous findings together with our current studies might shed some light on this topic. First, the expression of Chinmo and Ab is inhibited in late-born KCs, in part due to the preferential expression of *let-7* in α/β neurons [21, 27]. In addition, the neuronal (and KC) contribution of *let-7* regulates day- and night-time sleeping behaviors in developmental- and adult-restricted manners [28]. Furthermore, Chinmo and *let-7* potentially regulate the expression of E93, for which expression in α/β neurons governs the expression of a phylogenetically conserved sleep-modulatory calcium channel Ca-α1T to regulate animal behaviors [18] (Fig 2B-C, 4D and S11A Fig). These results together with the previous reported function of E93 in circadian rhythm provide an intriguing link between E93 and sleep-associated behaviors [29]. Since KC subtypes were reported to

function crucially in sleep/arousal and alcohol-induced sleep deficit behaviors [30-32], our finding of KC identity specification and Ca-α1T expression under the regulation of E93 suggests that this process may in some way endow α/β neurons with the capacity to control animal behaviors related to sleep. Since the intricate relationship between sleep and memory is well established [33-36], future deeper investigations on cell-type specification of memory-associated neurons are expected to provide insights into how to acquire for unique functions among neurons to regulate these two crucial traits of animals.

Figure legends

Fig 1. Expression patterns of KC subtype markers

(A-H) KC subtype-specific GFP markers (green) were counter-stained with Trio (magenta) to reveal expression patterns at the wandering larval (WL) (A, C, E and G) and adult (B, D, F and H) stages. (A-B) Ab-GFP was primarily expressed in cell bodies of γ neurons at both WL and adult stages. The Trio signal indicates locations of γ neurons for staining observed only in cytosol (arrows) and α'/β' neurons for staining in the entire cell (arrowheads). (C-D) Lac-FSVS expression was enriched in α' and β' lobes (arrowheads) of adult but not WL stage animals. The single section in the bottom panels of Fig. 1d reveal the lack of Lac-FSVS expression in the γ lobe. (E-H) E93-GFSTF and Ca-α1T-GFSTF were preferentially expressed in respective cell bodies and dendrites (the calyx) of α/β neurons (double-arrows) at adult but not WL stage animals. In addition to calyx expression, Ca-α1T-GFSTF was also seen in the protocerebral bridge (PB) of adult brains. Genotypes shown in all figures are summarized in Supplementary Table 1. Scale bar: 10 μm.

Fig 2. E93 specifies the α/β neural identity and affects animal behaviors

As compared to the wild-type controls (A and D), flies with overexpression of *E93* RNAi (B and E) by a pan-KC driver, GAL4-OK107, had significantly impaired expression of Ca-α1T-GFSTF and 44E04-LexA in α/β neurons (double arrows) in adult brains. As an internal control, the PB expression of Ca-α1T-GFSTF was intact under *E93* RNAi knockdown driven by GAL4-OK107. (C) In control samples, including yw, *Ca-α1T* RNAi-, *E93* RNAi- and GAL4-c739 (an α/β neural driver)-only flies, it took around eight hours for the minimal speed (purple spots) to be reached at night. However, flies took around two

hours to achieve a minimal speed when RNAi's for *Ca-α1T* and *E93* were overexpressed using GAL4-c739. Overall moving speed was lower and more variable in *E93* knockdowns. Moving speed (black line) was calculated as the overall traveling distance (mm) for 30 min. Standard deviation (in grey) for each time-point is shown. The bar graph depicts the duration of food region exploration (X to Z zones, from proximal to distal). All flies tended to explore more in the X zone in later days. Compared to control flies, *Ca-α1T* and *E93* knockdown flies explored less in the X zone, especially on day four. ZT: Zeitgeber time. The setting and analysis of the behavioral assay is detailed in S7 Fig. (F-G) *E93* knockdown did not block the expression of 70F05-LexA in α/β neurons to cause the detectable morphological defect in lobe regions (double-arrows). (H-I) However, compared to the wild-type, Ab-GFP expression was ectopically expressed in more than half of 70F05-LexA-positive neurons (double-arrows) when *E93* was knocked down in KCs. The expression levels of 44E04-LexA and 70F05-LexA were visualized by lexAop-myr-GFP in panels d-g and lexAop-mCD8::RFP in panels H and I. Cell numbers of 70F05-LexA- and Ab-GFP-positive neurons were counted in S8 Fig. Scale bar: 10 μm.

Fig 3. E93 is sufficient to shift the KC identity towards α/β neural-like fate

(A-B) Overexpression of E93 driven by GAL4-OK107 caused precocious expression of α/β-specific Ca-α1T-GFSTF in early-born KCs at the WL stage. (C-D) In addition, overexpression of E93 driven by a γ-neural driver, GAL4-201Y (magenta), ectopically turned on the expression of a α/β-specific 70F05-LexA driver in a portion of γ neurons (visualized by myr-GFP in green; arrow). (E-J) On the other hand, overexpression of E93 abolished γ-specific markers, including Ab-GFP (E-F), $Mamo^{H/I}$ (G-H), $Mamo^{D\sim G}$ (weak

green signal; I-J) and EcR-B1 (E-J), and α'/β'-specific Mamo$^{D\sim G}$ (strong green signal within yellow dashed-line; I-J) in the early-born KCs at the white pupal (WP) stage. (K-L) E93 overexpression also compromised the Lac-FSVS expression in α'/β' neurons and the morphology of MB lobes revealed by cell adhesion molecule Fasciclin II (Fas2, strong magenta for labeling α and β lobes) at 24 h after puparium formation (APF). An enhance-promoter (EP) line inserted at the proximal region of the *E93-A* 5'UTR was used to overexpress E93 in the gain-of-function experiments. The potency of the E93(EP) line was similar to two other in-house transgenic lines expressing E93-A and E93-B isoforms (see S9 Fig). Scale bar: 10 μm.

Fig 4. Genetic networks of *chinmo*, *mamo*, *E93* and *ab* control KC identity

(A-B) As compared to the wild-type, expression of Ab-GFP (green) was diminished in KCs at the first instar larval (L1) stage upon E93 overexpression driven by GAL4-OK107 (magenta). However, Chinmo expression (white) was not affected by E93 overexpression. (C-E) In contrast, RNAi knockdown of *chinmo*, but not *mamo*, driven by GAL4-OK107 (magenta) precociously turned on the expression of E93-GFSTF (green) in the early-born KCs at the WL stage. (F-G) However, RNAi knockdown of *mamo* driven by GAL4-OK107 ectopically turned on expression of E93-GFSTF (green) in KCs with weak cytosolically expressed Trio (magenta) in adult brains (magenta dash-lines). The weak Trio signal was possibly due to *mamo* RNAi knockdown in early-born KCs. E93-GFSTF was densely expressed in putative α/β neurons with negative Trio signal (region outside magenta dashed lines). (H-J) Ab overexpression driven by GAL4-OK107 diminished the expression of

E93-GFSTF and Ca-α1T-GFSTF in KCs of adult brains. Trio seemed to be expressed in the cytosol in almost all KCs upon Ab overexpression. Scale bar: 10 μm.

Fig 5. Hierarchical genetic networks govern the identity and function of Kenyon cell in the construction of MBs

(A-B) Scheme delineates hierarchical genetic networks among *chinmo*, *mamo*, *E93* and *ab* with feedback loops that control the cell identities of γ and α'/β' neurons. Syp and *let-7* are included in genetic networks to potentially link the regulation of E93 and sleeping modulatory calcium channel Ca-α1T [ref. #20, #21 #and 27] (S12 Fig). Based on the results of gain-of-function studies (Fig 3H-3J and 4H), possible feedback regulation in the genetic network is indicated with OE (as the abbreviation of overexpression). Since E93 regulates the Ca-α1T expression (Fig 2B) and since *let-7* is also crucial for the sleep behavior [ref. #28], E93 and Ca-α1T may be potentially associated with sleep and memory behaviors through KCs. Question marks (?) indicate possible regulation in the genetic network.

Materials and Methods

Experimental model and subject details

Flies were cultured in a room maintained at 25ºC (± 1.5ºC) and 50-65% humidity for all experiments. For most experiments, flies were used with no selection for sex; therefore, roughly equal numbers of males and females were used. However, females were selected for the analysis of Ca-α1T-GFSTF in Fig. 1G-H, 2A-B, 3A-B and 4I-J and S5A-B, S13 and S14D Figs due to the cytolocation of the *Ca-α1T* gene on the X chromosome. In addition, males were selected for the behavioral assay to mitigate complications of egg laying behaviors on locomotion tracking.

Fly strains

The fly strains used in this study were as follows. Most strains are available from either Bloomington *Drosophila* stock center (BDSC), Kyoto *Drosophila* stock center (DGGR) or Vienna *Drosophila* stock center (VDRC). (1) *Ab-GFP* (BDSC38626); (2) *Lac-FSVS* (DGGR115308); (3) *E93-GFSTF* (BDSC59412); (4) *Ca-α1T-GFSTF* (BDSC61800); (5) *hs-FLP*^{12}*,UAS-mCD8::GFP* (BDSC28832); (6) *tubP-GAL80,FRT*^{40A} (BDSC5192); (7) *chinmo*$^{[1]}$*,FRT*^{40A} (BDSC59969); (8) *GAL4-OK107* (BDSC854); (9) *UAS-mCD8::RFP [attp40]* (BDSC32219); (10) *UAS-mCD8::RFP [attp2]* (BDSC32218); (11) *UAS-E93 RNAi* (BDSC57868); (12) *UAS-E93 RNAi* (VDRC104390); (13) *UAS-Ca-α1T RNAi* (BDSC39029); (14) *GAL4-c739* (BDSC7362); (15) *44E04-LexA::P65* (BDSC52736); (16) *70F05-LexA::P65* (BDSC523629); (17) *lexAop2-myr::GFP*[37]; (18) *lexAop2-mCD8::RFP* [37]; (19) *FRT*^{82B}*,tubP-GAL80* (BDSC5135); (20) *FRT*^{82B} (BDSC86313); (21) *E93*$^{\Delta 11}$ (BDSC93128); (22) *E93(EP)* (BDSC30179); (23) *UAS-E93-A [VK37]* (this study); (24)

UAS-E93-B [VK37] (this study); (25) *GAL4-201Y* (BDSC4440); (26) *mamo$^{H/I}$-HA*[38]; (27) *mamo$^{D\sim G}$-HA* [38]; (28) *UAS-chinmo RNAi [VK37]* [5]; (29) *UAS-LUC-let7* (BDSC41171); (30) *UAS-Ssyp RNAi* (VDRC33011); (31) *UAS-mamo RNAi* (BDSC44103); (32) *UAS-ab* (BDSC23639); (33) *UAS-ab-HA* (FlyORF000705); (34) *UAS-Dcr2* (BDSC24651); (35) *UAS-ab RNAi* (VDRC104582). The *UAS-E93-A* and *UAS-E93-B* transgenes were generated using standard molecular biology methods to clone cDNA fragments derived from fully-sequenced EST clones, GH10557 and LP08695 (available from *Drosophila* Genomics Resource Center), carrying *E93-A* and *E93-B* isoforms into the attB-UAST vector. The generation of *UAS-E93-A* and *UAS-E93-B* transgenes and fly stains was performed by WellGenetics, Inc.

RNAi knockdown and overexpression experiments and MARCM clonal analyses

UAS-RNAi and *UAS-transgene* lines were crossed to GAL4-107 and GAL4-201Y for knockdown and overexpression of genes of interest in KCs. Mosaic clones for the MARCM studies were generated as previously described [39]. In short, mosaic clones of *chinmo$^{[1]}$* and *E93$^{\Delta 11}$* mutations were induced by 35 min of heat shock using *hs-FLP$^{[12]}$* in newly hatched larva. Dissection, immunostaining and mounting of adult brains were performed as described in a standard protocol [39]. Primary antibodies used in this study included guinea pig antibody against Chinmo (1:1000, Sokol laboratory [40]), rat monoclonal antibody against mCD8 (1:100, Thermo Fisher Scientific), rabbit antibody against GFP (1:750, Thermo Fisher Scientific), and mouse monoclonal antibodies against EcR-B1 (1:50, DSHB), Fas2 (1:100, DSHB) and Trio (1:50, DSHB). Secondary antibodies conjugated to different fluorophores (Alexa 488, Alexa 546 and Alexa 647; Thermo Fisher

Scientific and Jackson ImmunoResearch Lab, Inc.) were used at 1:750 dilutions. Immunofluorescence images were collected by confocal microscopy on a Zeiss LSM 700, projected using the LSM browser and processed in Adobe Photoshop CS6. All data are representative of more than 3 brains per genotype.

Distance measurement in the behavioral assay and statistical analysis

Individual fly activities were recorded and analyzed using the activity monitor system developed by DroBot, Inc. This system used an open-resourced software pySolo to track individual moving flies [41]. Average speed and standard deviation of individual flies were calculated according to total traveling distance within 30 min periods from the first day to the fifth day, as shown in Fig 2C. Box-plots and statistical analyses in S7C-D Fig were conducted using two online tools [(https://shiny.chemgrid.org/boxplotr/) and (https://astatsa.com/OneWay_Anova_with_TukeyHSD/)]. One-way ANOVA with post-hoc Tukey HSD test was used to compare datasets with six groups in S7D Fig. Student's t-test was used for statistical analysis to compare datasets with two groups in S8 Fig.

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Author contributions

Conceptualization, P.C.C. and H.H.Y.; Methodology, P.C.C., K.Y.K., S.Y.H. C.C., and H.H.Y.; Formal Analysis, P.C.C., C.C. and H.H.Y.; Investigation, P.C.C. and H.H.Y.; Writing: P.C.C. and H.H.Y.; Visualization, P.C.C., C.C. and H.H.Y.; Supervision: H.H.Y., Funding Acquisition, H.H.Y.

Competing interests

The authors declare no competing interests.

Materials and correspondence

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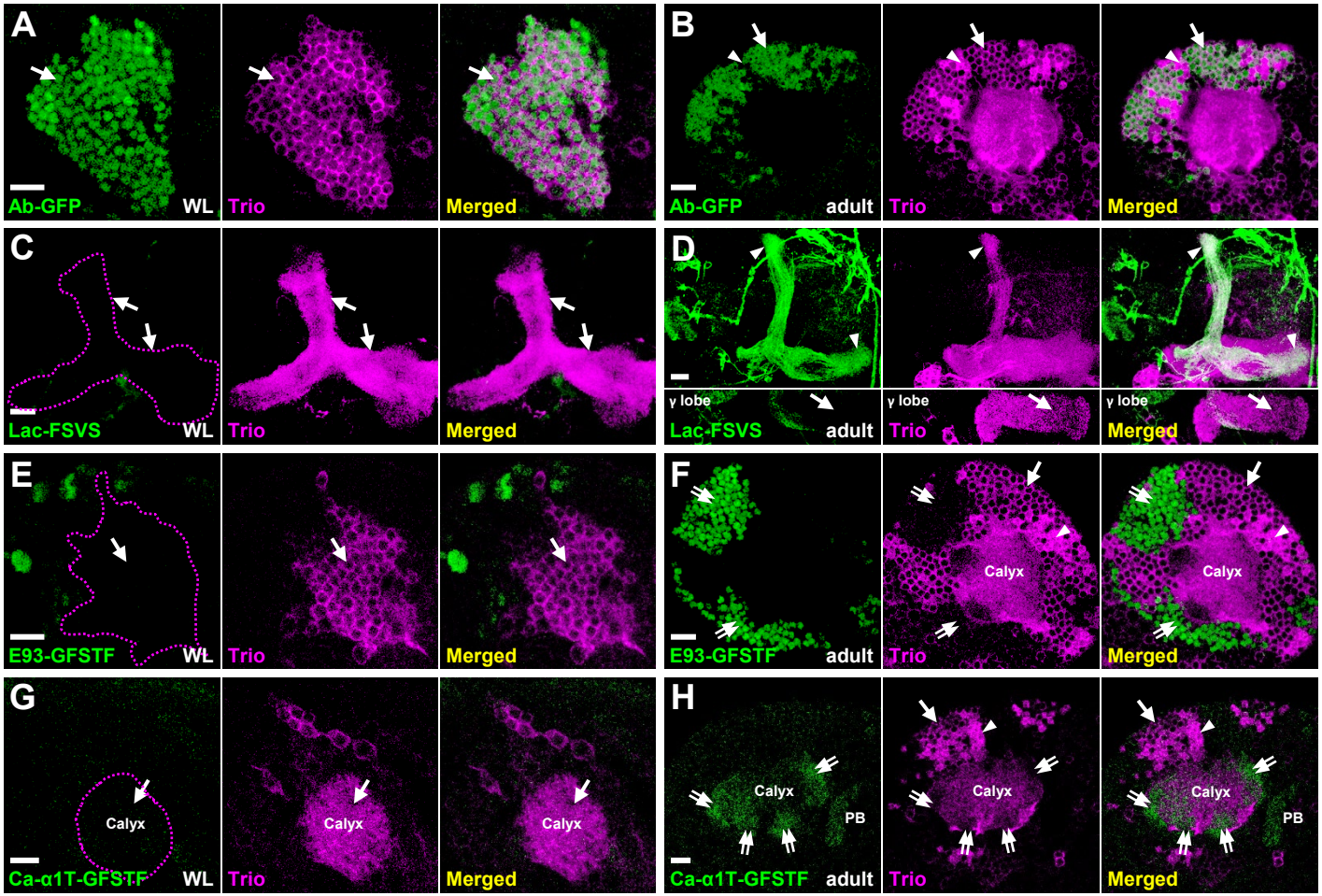
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Ab-GFP
WL
Trio
Merged
B
Ab-GFP
adult
Trio
Merged
C
Lac-FSVS
WL
Trio
Merged
D
γ lobe
Lac-FSVS
adult
γ lobe
Trio
γ lobe
Merged
E
E93-GFSTF
WL
Trio
Merged
F
E93-GFSTF
adult
Calyx
Trio
Calyx
Merged
G
Calyx
Ca-α1T-GFSTF
WL
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PB
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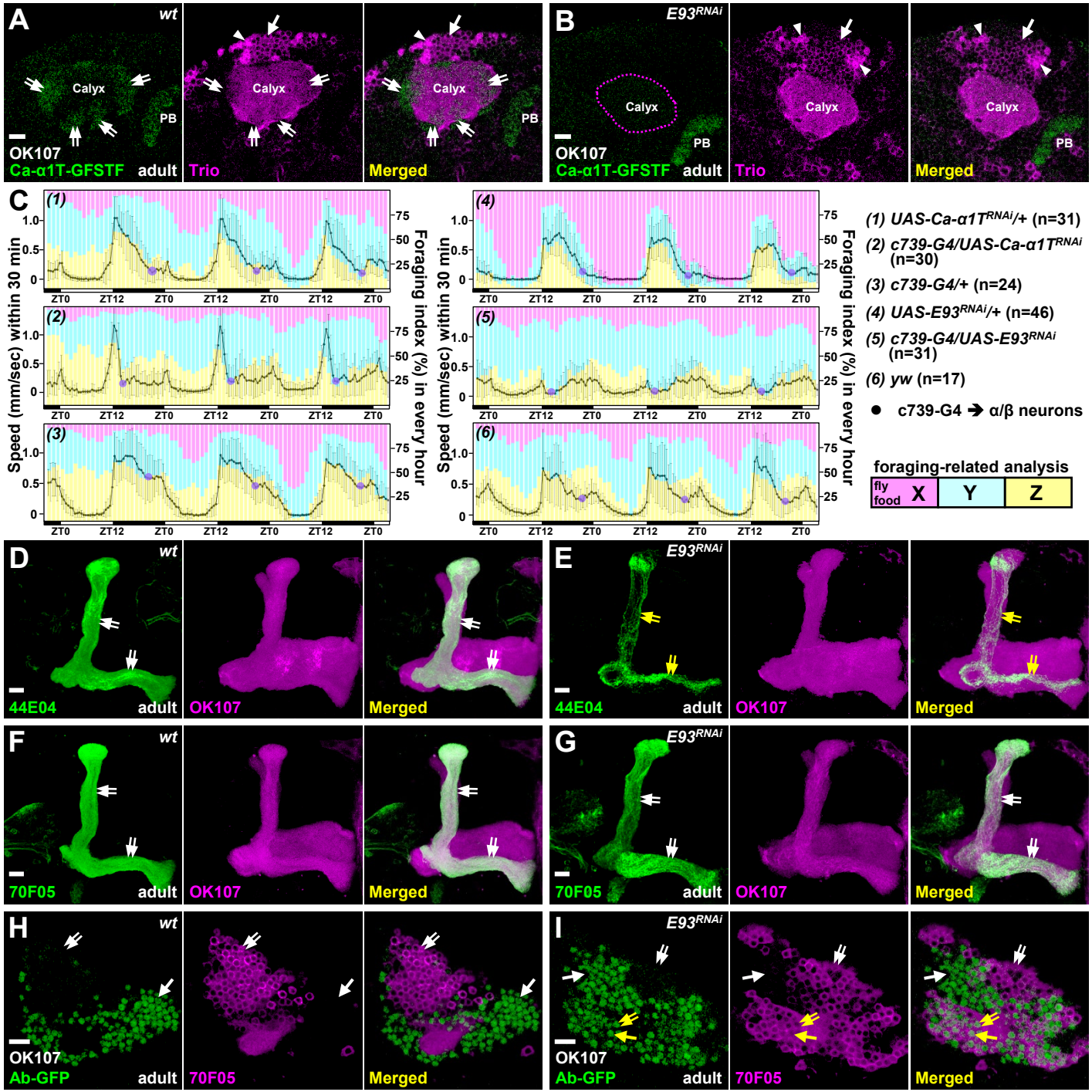
A
wt
Calyx
PB
OK107
Ca-α1T-GFSTF
adult
Trio
Merged
B
E93RNAi
Calyx
PB
OK107
Ca-α1T-GFSTF
adult
Trio
Merged
C
(1)
(2)
(3)
(4)
(5)
(6)
Speed (mm/sec) within 30 min
Foraging index (%) in every hour
ZT0
ZT12
(1) UAS-Ca-α1TRNAi/+ (n=31)
(2) c739-G4/UAS-Ca-α1TRNAi (n=30)
(3) c739-G4/+ (n=24)
(4) UAS-E93RNAi/+ (n=46)
(5) c739-G4/UAS-E93RNAi (n=31)
(6) yw (n=17)
● c739-G4 ➔ α/β neurons
foraging-related analysis
fly food
X
Y
Z
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F
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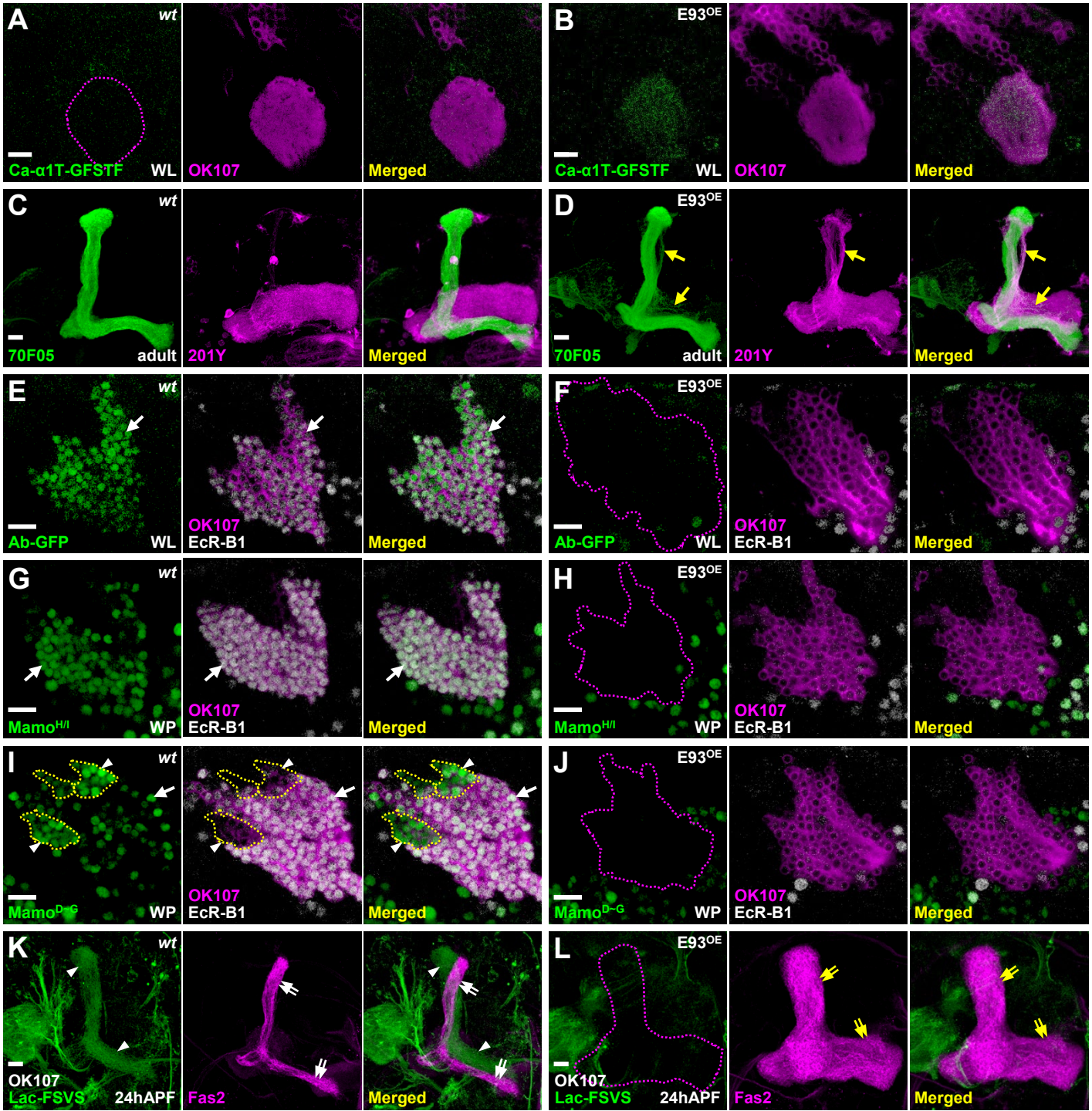
H
I
Ab-GFP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B
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C
wt
70F05
adult
201Y
Merged
D
E93OE
70F05
adult
201Y
Merged
E
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WL
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EcR-B1
Merged
F
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Ab-GFP
WL
OK107
EcR-B1
Merged
G
wt
MamoH/I
WP
OK107
EcR-B1
Merged
H
E93OE
MamoH/I
WP
OK107
EcR-B1
Merged
I
wt
MamoD-G
WP
OK107
EcR-B1
Merged
J
E93OE
MamoD-G
WP
OK107
EcR-B1
Merged
K
wt
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Fas2
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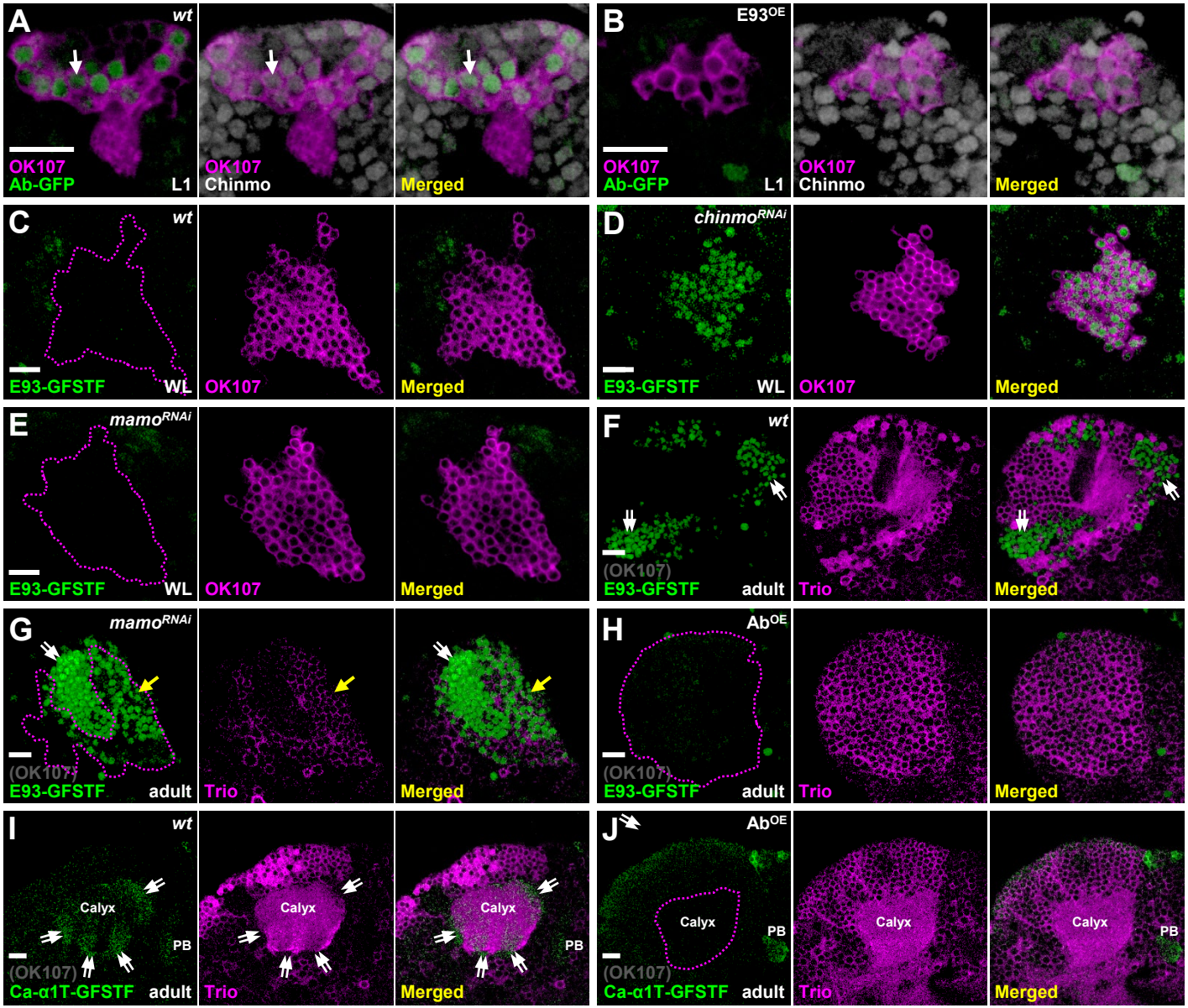
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L1
OK107
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B
E93OE
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L1
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Chinmo
Merged
C
wt
E93-GFSTF
WL
OK107
Merged
D
chinmoRNAi
E93-GFSTF
WL
OK107
Merged
E
mamoRNAi
E93-GFSTF
WL
OK107
Merged
F
wt
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Trio
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G
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Trio
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AbOE
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E93-GFSTF
adult
Trio
Merged
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Ca-α1T-GFSTF
adult
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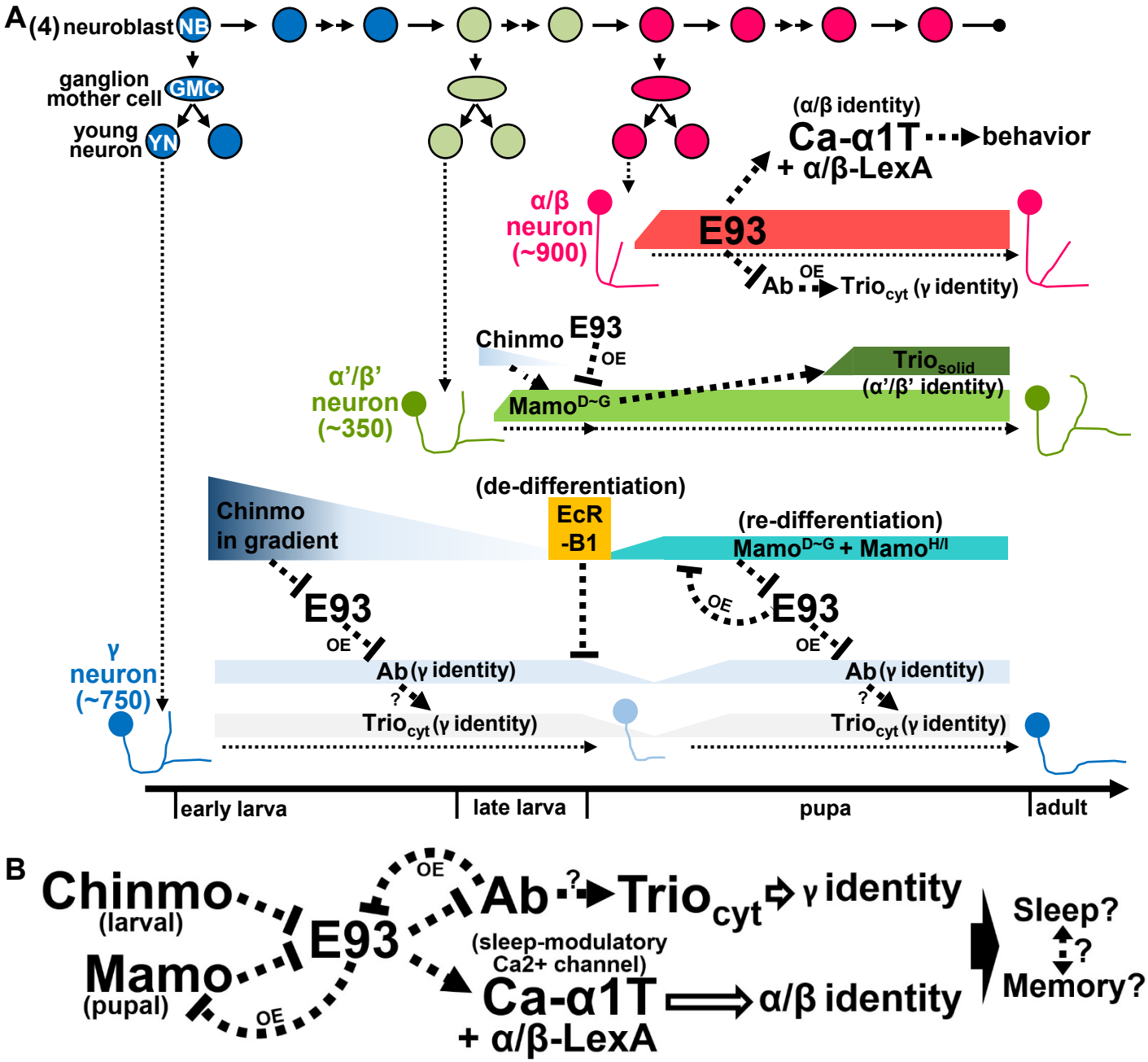
PB
(OK107)
Ca-α1T-GFSTF
adult
Trio
Merged


A
(4) neuroblast
NB
ganglion mother cell
GMC
young neuron
YN
(α/β identity)
Ca-α1T
behavior
+ α/β-LexA
α/β neuron (~900)
E93
OE
Ab
Trio$_{cyt}$ (γ identity)
Chinmo
E93
OE
α'/β' neuron (~350)
Mamo$^{D\sim G}$
Trio$_{solid}$
(α'/β' identity)
(de-differentiation)
Chinmo in gradient
EcR -B1
(re-differentiation)
Mamo$^{D\sim G}$ + Mamo$^{H/I}$
E93
OE
OE
E93
OE
γ neuron (~750)
Ab (γ identity)
?
Trio$_{cyt}$ (γ identity)
Ab (γ identity)
?
Trio$_{cyt}$ (γ identity)
early larva
late larva
pupa
adult
B
Chinmo
(larval)
Mamo
(pupal)
E93
OE
OE
Ab
?
Trio$_{cyt}$
γ identity
(sleep-modulatory Ca2+ channel)
Ca-α1T
+ α/β-LexA
α/β identity
Sleep?
?
Memory?