A versatile genetic tool for post-translational control of gene expression in *Drosophila melanogaster*

Sachin Sethi and Jing W. Wang*

Neurobiology Section, Division of Biological Sciences

University of California, San Diego, La Jolla, CA 92093, USA

*Correspondence: jw800@ucsd.edu (J.W.W)

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Abstract

Several techniques have been developed to manipulate gene expression temporally in intact neural circuits. However, the applicability of current tools developed for *in vivo* studies in *Drosophila* is limited by their incompatibility with existing GAL4 lines and side effects on physiology and behavior. To circumvent these limitations, we adopted a strategy to reversibly regulate protein degradation with a small molecule by using a destabilizing domain (DD). We show that this system is effective across different tissues and developmental stages. We further show that this system can be used to control *in vivo* gene expression levels with low background, large dynamic range, and in a reversible manner without detectable side effects on the lifespan or behavior of the animal. Additionally, we engineered tools for chemically controlling gene expression (GAL80-DD) and recombination (FLP-DD). We demonstrate the applicability of this technology in manipulating neuronal activity and for high-efficiency sparse labeling of neuronal populations.

Introduction

Tools for precise spatial and temporal control of gene expression are essential for understanding how neuronal circuits develop and function. For example, limiting genetic manipulation of a target gene to a specific time and a defined neuronal population permits the separation of the developmental role of the gene from its contribution to circuit function in the adult stage. In the vinegar fly, *Drosophila melanogaster*, bipartite expression systems (GAL4/UAS, LexA/LexAop, QF/QUAS) provide a powerful means to control gene expression in a spatially selective manner (Brand & Perrimon, 1993; Lai & Lee, 2006; Potter, Tasic, Russler, Liang, & Luo, 2010). Several modifications of these expression systems have been made to permit temporal control over the exogenous transcription factors (GAL4, LexA or QF) (Chan, Alekseyenko, & Kravitz, 2015; McGuire, Le, Osborn, Matsumoto, & Davis, 2003; Osterwalder, Yoon, White, & Keshishian, 2001; Potter et al., 2010). Much of the effort has been focused on using temperature or chemicals as means to control the gene expression systems. Temperature-dependent expression systems have been previously engineered by the direct fusion of a heat-inducible promoter to a gene of interest (Lis, Simon, & Sutton, 1983), or by using a temperature sensitive allele of GAL80, $GAL80^{ts}$. In the GAL4/UAS system, $GAL80^{ts}$ suppresses GAL4-induced gene expression at a low temperature (18 °C) but not at a high temperature (29 °C) (McGuire et al., 2003). Chemical-dependent tools include tetracycline-inducible systems (Bello, Resendez-Perez, & Gehring, 1998; Bieschke, Wheeler, & Tower, 1998), steroid hormone-inducible GAL4/LexA hormone receptor chimeras (Han, Stein, & Stevens, 2000; Osterwalder et al., 2001; Roman, Endo, Zong, & Davis, 2001) and the quinic acid-inducible QS/QF/QUAS system (Potter et al., 2010). For example, an RU486-inducible GAL4 was made by fusing the GAL4 DNA-binding domain to the human progesterone receptor and the p65 transcriptional activation domain (Roman et al., 2001). However, there are limitations associated with the existing tools for temporal control of gene expression. First,

temperature can have a significant impact on the physiology and behavior of a fly (Parisky, Agosto Rivera, Donelson, Kotecha, & Griffith, 2016; Sigrist, Reiff, Thiel, Steinert, & Schuster, 2003), which may prevent detection of the phenotype of interest. For example, temperature-dependent tools are unlikely to be suitable for the study of thermosensory circuits and related behaviors. Second, temporal control of gene expression using GAL80^{ts} can only be achieved with transcription factors containing the GAD-activation domain, making it incompatible with a majority of the driver lines (LexA, QF and split-GAL4) that do not have the GAD domain. Third, application of the current chemical-dependent tools requires the generation of new transgenic stocks, such as new promoter-GAL4 lines. Additionally, RU486, a chemical used to induce gene expression in the Geneswitch system, has been reported to cause developmental lethality in flies with pan-neuronal expression of the RU486-sensitive GAL4 (Li & Stavropoulos, 2016).

We propose an alternative chemically inducible system, in which gene expression is controlled at the post-translational stage, making it compatible with the existing library of GAL4 stocks. We adopted the destabilizing domain (DD) derived from dihydrofolate reductase (ecDHFR) of *E. coli* to control protein stability in a ligand-inducible manner (Cho et al., 2013; Iwamoto, Björklund, Lundberg, Kirik, & Wandless, 2010), a strategy that has been used to control gene expression in mice and worms (Cho et al., 2013; Iwamoto et al., 2010; Sando et al., 2013). On fusing the destabilizing domain to a protein of interest, the chimeric protein is degraded by the proteasome, but its degradation is blocked by trimethoprim (TMP), a cell-permeable ligand for DD (Fig. 1A) (Iwamoto et al., 2010). Thus, the protein of interest can be temporally controlled by TMP. A recent study demonstrated that TMP can regulate activity of the yeast I-SceI endonuclease in *Drosophila* larvae expressing the fusion protein I-SceI-DD (Janssen et al., 2016). Here, we characterized the efficiency and dynamics of this technology *in vivo* in the fly brain. We further use the DD technology to develop tools for mapping and manipulating neural circuits in *Drosophila*. As a proof of its utility, we fused DD to GAL80 and controlled GAL4-

dependent gene expression in a TMP-dependent manner. We show that TMP can activate GAL80-DD to manipulate neuronal activity in behaviorally relevant sensory neurons. Additionally, by fusing DD to the FLP recombinase, we devised a strategy to control the recombination frequency within a neuronal population by controlling the concentration of TMP in fly food. We further used the destabilized FLP recombinase to refine the expression pattern arising from the intersection of two transgenic lines by temporally limiting the availability of TMP. In summary, we present a chemically inducible system optimized for neurogenetics in *Drosophila* with broader utility than comparable conventional tools.

Results

Destabilized GFP. We first tested whether the ecDHFR-derived destabilizing domain (DD) can be used to control GFP expression levels. DD was genetically fused to the C-terminus of GFP and cloned into a 10XUAS construct (Fig. 1A, Figure 1 - supplement 1) to make *UAS-GFP-DD* transgenic flies. We reasoned that GFP expression should be conditioned on both the presence of the transcriptional activator GAL4 and the availability of the stabilizing ligand TMP. The expression of GAL4 in select neuronal populations affords spatial specificity. Feeding these flies with TMP at a specific time could provide a temporal control of GFP expression.

Using the pan-neuronal *nsyb-GAL4* to drive GFP-DD expression, we observed robust GFP expression throughout the brain of adult flies fed with TMP (Fig. 1B). In the absence of TMP, GFP expression was low throughout the brain; this is consistent with the previous studies in mice and nematodes showing that unbound DD is an effective tag to mark the fusion protein for degradation (Cho et al., 2013; Sando et al., 2013) (Fig. 1B). However, TMP levels may start to decline at the beginning of pupation, during which flies do not feed. To determine the efficiency of TMP-dependent GFP-DD stabilization during development, we measured GFP-DD expression throughout the brain from the larval to the adult stage (Figure 1 – supplement 2A).

We found substantial differences in GFP-DD expression between flies fed with solvent versus TMP during the larval (44 fold), 48 hr APF (19 fold) and three day old adult (27 fold) stages. Notably, even though flies do not feed for several days leading up to the late pupal stage (96 hr APF) and eclosion (<12 hr adult), we observed a five-fold difference in GFP-DD expression between flies raised on solvent versus TMP (Figure 1 – supplement 2A). In summary, the TMP-inducible DD system is maximally effective during the larval, early pupal and adult stages. During the late pupal stage, however, the utility of the system may be limited by unavailability of TMP.

Because TMP-dependent protein stabilization acts through post-translational modification of protein levels, it should be compatible with any GAL4, LexA, QF or split-GAL4 line. To illustrate this principle, we visualized TMP-dependent GFP-DD expression across several different cell types in the adult brain using previously characterized driver lines (Figure 1 – supplement 3A). We observed a significant difference in GFP-DD expression (ranging from 4 to 64 fold) between solvent and TMP feeding for every cell type that we tested. Maximum differences were observed in the axon terminals of sensory neurons (at least 45 fold). Interestingly, different cell types innervating the same anatomical region showed differential susceptibility to the DD system. For example, olfactory sensory neurons showed a much larger difference between TMP and solvent feeding (45 fold) compared to olfactory projection neurons (6 fold), even though both cell types innervate the antennal lobe. Similarly, PAM dopamine neurons showed a larger difference in GFP-DD expression (12 fold) compared to Kenyon cells (4 fold), even though both cell types innervate the mushroom body. Based on these results, we conclude that at least some of the differences between cell types arise from the variability in their proteasome activity levels. We also observed that the DD system was effective in non-neuronal tissues, such as ovaries (Figure 1 – supplement 3B). Finally, to demonstrate that the DD system can be used in combination with other binary systems apart from GAL4/UAS, we generated a *13XLexAop-*

GFP-DD transgenic fly line and observed similar TMP-dependent GFP expression in olfactory sensory neurons using the *Orco-LexA* driver line (Figure 1 - supplement 3D).

We next carried out experiments to determine the kinetics and dynamic range of this chemical induction system using *Orco-GAL4* to drive GFP-DD expression in olfactory sensory neurons. Feeding adult flies with food containing varying concentrations (0-5 mM) of TMP for 48 hours resulted in a dose-dependent change in GFP expression in the antennal lobe (Fig. 1C, D). The maximum GFP expression, induced by 1 mM TMP, was between 34 and 45 times higher than that of control flies fed with the solvent-containing food (Fig. 1D, Fig. 1 - supplement 3A). Results from an experiment in which flies were fed for varying durations (0-60 hrs) with food containing 1 mM TMP show that GFP levels increase initially but plateau within 36 hours (Fig. 1E, F). To test if TMP-dependent GFP expression is reversible, we fed flies with food containing 1 mM TMP for 48 hours and then switched them to regular food (Fig. 1G). We found that the GFP intensity was reduced by 73% within 24 hours (Fig. 1H). In sum, using GFP as a test molecule, we show that genetic fusion of the ecDHFR-derived destabilizing domain confers instability to a protein of interest in *Drosophila*. Feeding flies with TMP can control protein levels in a reversible and dose-dependent manner with a large dynamic range.

We then investigated whether TMP has adverse effects on survival and behavior. Feeding adult flies with a defined medium containing TMP ranging from 0 to 10 mM did not have any detectable effect on their survival (Fig. 1 - supplement 4A). Moreover, feeding adult flies with 1 mM TMP for 48 hours did not alter their locomotion speed (Fig. 1 - supplement 4C) or their ability to locate an odor source (Fig. 1 - supplement 4D) during foraging. We then tested if TMP affects the development of the fly. Flies raised on TMP ranging from 0 to 10 mM throughout development were equally likely to survive to adulthood (Figure 1 - supplement 4B). However, we observed a delay in the developmental time for flies raised on 1 mM TMP (11.2 +/- 0.1 days to eclosion) and 10 mM TMP (15.3 +/- 0.3 days) as compared to solvent (10.2 +/- 0.1 days)

(Figure 1 - supplement 4B). In summary, feeding adult flies TMP at 1 mM can induce GFP-DD expression at saturation level (Figure 1D) without any observable side effects on their survival or behavior. Notably, however, the same dosage of TMP delays development by about one day. Thus, we settled on the concentration of 1 mM TMP for further experiments except for cases when a lower level of induction was desirable.

Destabilized GAL80. To evaluate the utility of the DD system for manipulating circuit function, we next investigated whether expression of GAL80 could be controlled by TMP. Binding of GAL80 to GAL4 prevents GAL4-mediated transcriptional activation in the GAL4/UAS expression system (Lee & Luo, 1999). We engineered a chemically inducible GAL80 by fusing DD to the C-terminus of GAL80. GAL80-DD was cloned downstream of a pan-neuronal promoter, *n-synaptobrevin* (*nsyb*). Addition of GAL80-DD to the GAL4/UAS expression system could allow TMP to control gene expression. Indeed, we found that *nsyb-GAL80-DD* was able to suppress GAL4-dependent GFP expression in olfactory sensory neurons (Fig. 2A). This suppression of GFP expression in flies carrying the *Orco-GAL4*, *UAS-GFP* and *nsyb-GAL80-DD* transgenes was TMP-dependent (Fig. 2A). This feature can be used to control gene expression to perturb neuronal function in a stage-dependent manner. For example, RNAi expression could be targeted to specific neurons during the adult stage by removing TMP from the food, which causes the degradation of GAL80. As a proof-of-concept experiment, we fed flies with TMP throughout development and up to 3 days post-eclosion (Fig. 2B, C). When flies were moved from TMP-containing food to regular food, GFP expression started to increase after 24 hours, and peaked at 72 hours post-TMP removal (Fig. $2B_1$, C_1). In contrast, flies fed with TMP continuously, from embryo to adult, showed low GFP expression throughout the course of the experiment (Fig. $2B_2$, C_2). Furthermore, flies that were raised on regular fly food throughout showed high GFP expression (Fig. $2B_3$, C_3). To determine how soon after eclosion it is possible to activate gene expression, we raised flies on TMP during development up to eclosion and

measured GFP expression in the brains of young adult flies (Figure 2- supplement 1A). We observed a difference between flies raised on TMP and those switched to solvent-containing food starting at one day post-eclosion (Figure 2- supplement 1B, C). However, we did not detect any GFP expression in flies younger than eight hours for either condition (Figure 2- supplement 1B,C). This lack of GFP expression even in the absence of TMP is most likely due to the time that is required to inactivate GAL80-DD upon TMP withdrawal (Figure 1H), although it is formally possible that the expression level of *Orco-Gal4* is low at eclosion. Either way, these results suggest that fusion of the ecDHFR-derived destabilizing domain to GAL80 permits TMP to control GAL80 activity, providing a chemically inducible system to control gene transcription in a temporal manner.

We further tested if GAL80-DD can be used to manipulate neuronal activity underlying behavior. We focused on the innate olfactory aversion to CO_2 in a T-maze assay. Olfactory aversion to CO_2 can be abolished by silencing Gr21a-expressing sensory neurons (Suh et al., 2004). We controlled the expression of tetanus toxin, a potent inhibitor of synaptic transmission (Sweeney, Broadie, Keane, Niemann, & O'Kane, 1995), in Gr21a neurons using *nsyb-GAL80-DD* and TMP feeding (Fig. 2D). *Gr21a-GAL4*-derived tetanus toxin expression was blocked in the presence of GAL80-DD when flies were fed TMP, but not when they were fed the solvent (Fig. 2D). Accordingly, aversion to CO_2 was observed only when flies expressing *Gr21a-GAL4, UAS-TNT, nsyb-GAL80-DD* were fed with TMP, and not when they were fed with the solvent (Fig. 2E). In comparison, control flies with GAL4 alone had high avoidance for both solvent and TMP feeding conditions. We observed a difference between solvent- and TMP-fed flies expressing *UAS-TNT, nsyb-GAL80-DD* (Fig. 2E). However, the avoidance indices of both solvent- and TMP-fed *UAS-TNT, nsyb-GAL80-DD* flies were significantly higher than the avoidance index of *Gr21a-GAL4, UAS-TNT, nsyb-GAL80-DD* flies fed with the solvent (Fig. 2E). In summary, we show that

GAL80-DD can be used to manipulate GAL4-dependent expression of neuronal effectors and thereby alter the function of neuronal circuits underlying behavior.

Destabilized flippase. Flippase-mediated removal of a stop cassette has been widely used for lineage analysis and sparse neuronal labeling (Lee & Luo, 1999; Marin, Jefferis, Komiyama, Zhu, & Luo, 2002; Wong, Wang, & Axel, 2002). Lineage analysis requires transient high-level expression of flippase (FLP) at specific developmental stages. On the other hand, sparse neuronal labeling requires low-level FLP expression in post-mitotic neurons for the stochastic removal of a stop cassette. Owing to the large dynamic range of the DD system, we reasoned it could be used to control FLP expression at different levels by varying TMP concentrations in fly food, thereby accommodating both sparse labeling and lineage mapping. The heat-shock promoter has been used previously to drive different levels of FLP expression by varying the duration of the heat-shock pulses. However, heat-shock driven FLP activity cannot be limited to a subset of cells due to the ubiquitous expression of the heat shock promoter. This limitation restricts the utility of *hs-FLP* for lineage analysis in an intersectional manner.

We fused DD to the C-terminus of FLP and incorporated the coding sequence into a 10XUAS construct (*10XUAS-FLP-DD*). We tested the destabilized flippase in olfactory projection neurons using *GH146-GAL4* to drive *UAS-FLP-DD* and a GFP stop-cassette reporter, *UAS(FRT.STOP)CD8GFP*. In these flies, stabilization of FLP-DD by TMP should permit FLP-mediated excision of the stop cassette, resulting in GFP expression in certain projection neurons. We observed that the number of GFP-positive olfactory projection neurons was correlated to the TMP dosage (Fig 3A, B). By varying the concentration of TMP (0.01 – 1 mM) in fly food, we could control the number of labeled projection neurons (Fig 3A). Furthermore, there were similar numbers of labeled neurons in both brain hemispheres for a given sample (Fig. 3B). For flies fed with standard fly food without TMP, 42% of the brain hemispheres had only one GFP-positive cell (Fig. 3C). This feature of FLP-DD can be used to generate single-cell

clones at a reasonable probability for connectomics applications. As a proof-of-concept, we analyzed GFP-labeled neurons in the brains of 36 flies fed with solvent only. Out of 72 brain hemispheres, 30 had only a single GFP-positive projection neuron (see Fig. 3D for examples). In summary, dose-dependent expression of FLP-DD can be used to control the number of genetically manipulated cells within a population.

Restricting the activity of FLP-DD in a spatial and temporal manner should further refine expression patterns which arise from the intersection of two expression systems (eg. GAL4/UAS and QF/QUAS). To illustrate this principle, we focused on the intersection of *GH146-QF* and *NP21-GAL4*. It has been reported that the expression patterns for *NP21-GAL4* and *GH146-GAL4* overlap only in the DA1 lateral projection neurons (lPNs) in the adult brain (Potter et al., 2010), which we validated (Fig. $4A_1$,A_2). However, when UAS-FLP expression is driven by *NP21-GAL4*, the adult intersection pattern includes additional olfactory projection neurons, ellipsoid body neurons and neurons with cell bodies close to the lateral horn (Fig. $4B_1$,B_2). Similarly, when *QUAS-FLP* is driven by *GH146-QF*, the adult intersection pattern includes additional neurons (visual projection neurons in this case) (Fig $4C_1$,C_2). This discrepancy between the overlap and the intersection patterns arises because of the broader expression patterns for *GH146-QF* and *NP21-GAL4* before the adult stage. Thus, the stop cassette is prematurely excised during development in neurons outside of the overlapping adult pattern. We reasoned that the adult expression pattern can be recapitulated by limiting TMP feeding to the adult stage thereby inactivating FLP during development. Indeed, when *UAS-FLP-DD* was expressed by the *NP21-GAL4* line and 1 mM TMP was fed to flies only during the adult stage, GFP expression was limited only to DA1 lPNs in the whole brain (Fig $4D_1$,D_2). In comparison, flies fed with solvent alone did not have GFP expression in any neurons in the brain (Fig $4E_1$,E_2). Furthermore, flies fed with 1 mM TMP throughout development have GFP expression in additional olfactory projection neurons (Fig $4F_1$,F_2). We noted that the expression pattern in

UAS-FLP-DD flies fed with 1 mM TMP throughout development was a subset of that observed with *UAS-FLP* flies (Fig. 4B, F). It is possible that TMP levels decline in the fly brain during metamorphosis after the larvae stop feeding. In fact, similar results have been observed in the context of RU486-induced FLP activity (Harris, Pfeiffer, Rubin, & Truman, 2015). To mitigate this potential decline of TMP, we fed flies with 10 mM TMP throughout the larval stage and obtained a larger portion of the *UAS-FLP* expression pattern (Fig. 4B, figure 4 – supplement 1). In sum, we show that TMP can be used to limit FLP-DD activity temporally in a way such that the intersection pattern is identical to the overlap in the adult expression patterns.

Discussion

Here we report a chemically inducible system to control gene expression in a dose-dependent and reversible manner. The DD system broadens the functionality of the *Drosophila* genetic toolkit as it provides an independent axis of control which can be used in combination with existing reagents. The DD system provides several advantages over existing chemical-dependent tools. First, TMP-induced DD stabilization is dose-dependent over several orders of magnitude of TMP concentration. This dose-dependency can be exploited for titration of *in vivo* gene expression levels. Here, we use this dose-dependent relationship to predictably alter the number of labeled projection neurons in the fly brain. Second, in contrast to existing chemical reagents, tools such as GAL80-DD can be combined with existing GAL4 lines to knockdown targeted genes by RNAi or to perform neuronal silencing screens in a temporally refined manner. Third, when TMP is withdrawn, the degradation kinetics of the DD fusion protein are most likely faster than those of the native protein. Thus, it is possible that the DD system offers fast temporal control in experiments which require reversible gene expression. Finally, it is worth noting that the cost of TMP is almost 150 times less than RU486 or quinic acid at their

respective working concentrations, making the DD system conducive to large scale behavioral screens.

The applicability of the DD system for a given cell type is limited by two factors: 1) the cell type should have an active proteasome; 2) orally-fed TMP should be able to reach the cell. In the absence of TMP, the level of proteasome activity in a given cell type may influence the residual level of a DD chimeric protein. For instance, low proteasome activity may result in high residual levels of the chimeric protein. It may be possible to reduce the residual expression by co-expressing components of the protein degradation machinery, similar to how co-expression of Dicer enhances RNAi efficiency (Dietzl et al., 2007). As it is, the background accumulation of the DD-fusion protein and the sensitivity of the downstream targets should be taken into consideration while designing experiments using the DD-system. As TMP is a cell-permeable ligand which can cross the blood-brain barrier, it should be accessible to all tissues during the adult and larval stages. However, the effectiveness of TMP in stabilizing the protein of interest during late pupal stages is reduced due to lack of feeding for several days leading up to this stage (Figure 1- supplement 2). This may lead to undesirable leaky expression when GAL80-DD is used with a GAL4 line that is highly expressed during the late pupal stage. Therefore, the level of gene expression controlled by GAL80-DD should always be determined using reporters before manipulating neuronal activity with effector transgenes. Due to the nature of chemical delivery, the utility of the DD-system is also limited to applications which can tolerate gene expression at a relatively slower rate. We anticipate that it will take roughly 24 hours to activate or inactivate the DD-system to an appreciable degree. However, the optimal delay from the start or stop of drug feeding is dependent on the level of gene expression required for a specific application. It may also be possible to achieve faster induction by using photocaged forms of trimethoprim (Ballister, Ayloo, Chenoweth, Lampson, & Holzbaur, 2015). During the course of our experiments, we observed a detrimental effect of the solvent DMSO (greater than 0.1%) on

the survival of larvae (see Methods). This toxicity can be circumvented by using a water-soluble form of TMP- trimethoprim lactate or by mixing pure TMP directly into the food. Finally, as trimethoprim is an antibiotic, experiments using the DD system should incorporate appropriate controls to rule out the effect of gut microbes on the phenotype of interest.

DD-based tools are conducive for mapping and manipulating neural circuits underlying behavior. We illustrate this concept by using destabilized GAL80-DD to chemically manipulate neural activity in olfactory sensory neurons. To our knowledge, GAL80-DD is the first construct that allows control of GAL80 activity *in vivo* by a small molecule. Conventional experimental designs utilize the heat-inducible $GAL80^{ts}$ to suppress GAL4-depdendent transcription at low temperatures. While $GAL80^{ts}$ may still be preferable in experiments that require low background expression, GAL80-DD is a useful alternative for experiments that are disrupted by temperature manipulations. To further illustrate the utility of the DD-system for circuit mapping, we engineered FLP-DD for sparse neuronal labeling at high-efficiency and temporally controlled genetic intersection. In a previous study, an RU486-inducible FLP recombinase was constructed by fusing it with the human progesterone receptor (Flp-Switch) (Harris et al., 2015). Although this construct can be chemically induced similar to FLP-DD, further experiments will be required to compare the efficacy and dose-dependency of the two recombinases. All DD-fusion proteins presented in this study are soluble molecules which function in the cytoplasm or the nucleus. However, previous studies have used ecDHFR-derived destabilized domains to conditionally alter membrane protein expression (Iwamoto et al., 2010). Therefore, we predict that this tool can be used to temporally control expression of membrane proteins such as ion channels and G-protein-coupled receptors.

In addition to chemically inducible forms of GAL80 and FLP, the DD technology can be used in flies for several other applications. DD can be knocked-in and fused to endogenous proteins to control their expression by limiting TMP feeding. This can be done using custom-designed

genome editing strategies or by integration into the large number of available MiMIC sites within coding introns (Venken et al., 2011). DD can also be fused to a variety of effector genes for the purpose of inducible neuronal silencing or genome editing (Maji et al., 2016). Due to its inducible nature, GFP-DD can be coupled with knock-in GAL4 lines to compare gene expression in individual cells across time points spanning only a few hours, such as circadian fluctuation of gene expression. GFP-DD may also be useful as a sensor for proteasome activity.

In conclusion, we have developed a new set of tools for chemical control of gene expression in *Drosophila* which has broad-ranging applications and several advantages over existing tools of a similar nature. We characterized its efficiency and temporal limitations, and demonstrated its utility by engineering tools for chemical control of gene expression, recombination and neuronal activity.

Materials and Methods

Fly husbandry

Flies were raised on standard fly food (unless otherwise noted) at 25°C in a 12:12 light-dark cycle. The following transgenes were used in this study - nsyb-GAL4 (Riabinina et al., 2015) (BDSC_51941), Orco-GAL4 (Kreher, Kwon, & Carlson, 2005) (BDSC_23292), UAS-GFP, GH146-GAL4 (Stocker, Heimbeck, Gendre, & de Belle, 1997)(BDSC_30026), Gr5a-GAL4 (Thorne, Chromey, Bray, & Amrein, 2004), R58E02-GAL4 (Liu et al., 2012), P1^{a}-split GAL4 (Hoopfer, Jung, Inagaki, Rubin, & Anderson, 2015), MB434B-split GAL4 (Aso et al., 2014), Tdc2-GAL4 (Cole et al., 2005), MB247-GAL4 (Zars, Fischer, Schulz, & Heisenberg, 2000), UAS-(FRT.STOP)mCD8-GFP (Potter et al., 2010) (BDSC_30032) and UAS-(FRT.STOP)GFP.myr (BDSC_55810), UAS-6XmCherry-HA(Shearin, MacDonald, Spector, & Steven Stowers, 2014) (BDSC_52267), QUAS-6xGFP(BDSC_52264)(Shearin et al., 2014),

20XUAS-FLPD5 (Nern, Pfeiffer, Svoboda, & Rubin, 2011)(BDSC_55805), GH146-QF(Potter et al., 2010) (BDSC_30014), QUAS(FRT.STOP)GFP (Potter et al., 2010) (BDSC_30134), NP21-GAL4 (Hayashi et al., 2002) (BDSC_30027), Actin5C-GAL4 (BDSC_4414), Orco-LexAVP16 (Lai, Awasaki, Ito, & Lee, 2008), Gr21-GAL4 (Scott et al., 2001), UAS-TNT (Sweeney et al., 1995), 10XUAS-GFP-DD (this study), 10XUAS-FLP-DD (this study), nsyb-GAL80-DD (this study), 13XLexAop-GFP-DD (this study). See supplementary information for list of fly genotypes for every experiment, cloning details for generation of new stocks and key resources table.

TMP feeding

Trimethoprim (Teknova Inc., CA) was maintained as a 100 mM stock solution in DMSO. To prepare food containing TMP for adult flies, standard fly food was heated to a liquid state. After cooling, TMP (or pure DMSO) was added to the food and vortexed to achieve a homogenous mixture of the required concentration. Food was poured into standard fly vials and allowed to solidify. Adult flies were transferred to new vials with TMP-containing food every 3 days. 1% DMSO was found to severely affect survival of larvae. Therefore, to feed flies with TMP from the embryo stage, pure TMP in powder form was mixed in fly food to attain the required concentrations. Detailed information on the feeding regimen for every experiment can be found in the supplementary information.

Histology

Tissue samples were prepared for imaging using protocols that have been previously described (Lin, Chu, Fu, Dickson, & Chiang, 2013). Tissues were dissected in cold PBS and fixed in 4% (w/v) paraformaldehyde for 3 minutes on ice in a microwave. Next, tissues were fixed in 4% (w/v) paraformaldehyde containing 0.25% Triton-X-100 for 3 minutes on ice in a microwave. Fixed tissues were placed in blocking solution (2% Triton X-100, 0.02% sodium azide and 10% normal goat serum in PBS) and degassed in a vacuum chamber for 6 x 15 mins to expel

tracheal air. For the purpose of quantification in Figures 1 and 2, samples were not immunostained. All samples for a given experiment were prepared and imaged in parallel to allow for comparison among them. Rabbit anti-GFP (Invitrogen A-11122, 1:200), mouse anti-bruchpilot nc82 (DSHB AB_2314866, 1:50), mouse anti-HA (Biolegend 901501, 1:500) and rabbit anti-TeTx antibody (POL 016, Statens Serum Institut, 1:1000) were used as primary antibodies in this study. Alexa Fluor 488 anti-rabbit immunoglobulin G (Invitrogen A-31628; 1:100) and Alexa Fluor 647 anti-mouse immunoglobulin G (Invitrogen A-21235, 1:100) were used as secondary antibodies. Brains were incubated in primary antibodies in dilution buffer (1% normal goat serum, 0.02% sodium azide and 0.25% Triton X-100 in PBS) for 48 hours at 4°C, rinsed for 3 x 15 mins in washing buffer (1% Triton X-100, 3% NaCl in PBS), incubated in secondary antibodies in dilution buffer for 24 hours at 4°C, and rinsed again for 3 x 15 mins in washing buffer. Samples were mounted in Focusclear (Cedarlane Labs) between glass coverslips separated by spacer rings.

Samples were imaged with a 10X/0.3 or 20X/0.75 objective using a Zeiss LSM 510 confocal microscope to collect Z-stacks at 2-µm intervals. During the course of an experiment, the laser power and gain were held constant to allow for comparison among images from different experimental conditions. To quantify GFP expression, maximum intensity Z-projections were prepared using ImageJ (NIH). Average fluorescent intensity in the background was subtracted from the sample fluorescent intensity and the result was used as a proxy for GFP expression.

T-maze assay

Flies were raised in standard fly food or food containing 1 mM TMP from embryo to adult stages up to the time of the experiment. Behavioral tests were performed as described previously (Su, Menuz, Reisert, & Carlson, 2012). About 30 flies were transferred from food vials into a 15 mL centrifuge tube (Fisher scientific, 14959B) using a funnel. The tube containing the flies was connected to the T-maze apparatus and the flies were transferred into a horizontal elevator in

the dark. Flies were held in the elevator for one minute before being pushed forward to choose between the test and the control arm. A fluorescent lamp was switched on at this point to phototactically draw flies out of the elevator. Flies were given one minute to choose between either arm, following which the elevator was retracted to separate the flies in the test arm from those in the control arm. The tubes serving as the test and the control arms were detached and flies in them were counted.

Flies were forced to choose between the control arm containing air and the test arm containing 0.28% CO_2. 400 μL of 10% CO_2 was injected into the test arm using a syringe (Becton Dickinson, 10 mL). The positions of the test arm and the control arm were alternated for each trial. The avoidance index was calculated as (no. of flies in the control arm - no. of flies in the test arm) /(no. of flies in the test arm + no. of flies in the control arm).

Survival assay

Adult flies were raised on a defined medium (1 M sucrose, 1% agar) with 0-10 mM TMP from eclosion to death. Each experimental vial contained 5 male and 10 female flies. Flies were transferred to new vials every two days. Number of living flies was recorded every day. To quantify survival during development, 20 eggs were manually placed using forceps in a vial of fly food containing 0-10 mM TMP. Vials were observed daily to quantify the developmental timing for puparium formation and time to eclosion.

Odor localization and locomotion assay

Odor localization ability and walking speed were measured using a setup described previously (Root, Ko, Jafari, & Wang, 2011; Zaninovich et al., 2013). Single flies were introduced in custom built chambers (60 mm diameter, 6 mm height) and tracked at 2 Hz under 660 nm LED illumination using custom software written in Labview (V.8.5, National Instruments). Wild type flies were fed with regular fly food containing 1 mM TMP or 1% DMSO for 48 hours before the

experiment. The average walking speed of each fly during the first 50 seconds of each trial was determined using a custom macro with Igor Pro (V.6, Wavemetrics, Inc.). To perform the odor localization experiment, flies were transferred to starvation vials containing water with 1 mM TMP or 1% DMSO in Kimwipes (Kimberly-Clark) 24 hours prior to the experiment. 1% apple cider vinegar in low melting agarose was used as the odor source. Latency to localization is defined as the elapsed time before a fly spends more than 5 seconds within 5 mm of the odor source.

Statistical Analysis

Statistical results (*p* value, effect size, n) are indicated in figure legends corresponding to each experiment. All statistical analyses were performed in Igor Pro (V.6, Wavemetrics, Inc.). Sample size for each experiment was pre-determined based on variation in experimental groups in pilot experiments. Most experiments were performed at least twice to confirm results. Data from one representative experiment is shown.

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Author contributions

S.S. and J.W.W. designed the research and wrote the paper. S.S. generated the reagents and performed the experiments detailed in this paper.

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Figure legends

Figure 1

GFP-DD expression and degradation kinetics. (A) Schematic showing the destabilizing domain (DD) system. ecDHFR = *E. coli.* Dihydrofolate reductase, POI = protein of interest, TMP = Trimethoprim. (B) TMP-dependent GFP expression in the adult brain. Flies were fed 1 mM TMP-containing food from embryo stage up to dissection. (C, D) Dose-dependent change in GFP-DD expression in the axonal terminals of olfactory sensory neurons. *Orco-Gal4, UAS-GFP-DD* flies were fed with TMP (0 – 5 mM) for 48 hours before dissection (n=5-6, $p<0.001$, $F=41.37$, one-way ANOVA with Tukey's post-hoc test). (E, F) GFP-DD expression is dependent on duration of TMP feeding. All flies were fed with fly food containing 1 mM TMP (n=5-6, $p<0.001$, $F= 87.34$, one-way ANOVA with Tukey's post-hoc test). (G, H) GFP degradation kinetics. Flies were fed with 1 mM TMP for 48 hours and then switched to standard fly food. GFP-DD expression in the antennal lobe was observed at 12-hour intervals following the switch. (n=8-10, $p<0.001$, $F=71.43$, one-way ANOVA with Tukey's post-hoc test). Error bars indicate SEM. Significant differences between conditions ($p<0.05$) are denoted by different letters. Scale bar = 100 μm (B), 50 μm (C, E, G).

Figure 1 – supplement 1

(A) Schematic of the *10XUAS-GFP-DD* construct. Restriction sites are shown in red to facilitate cloning. All shown sites are unique apart from *XhoI* which has two cut sites (both shown). (B) DNA and protein sequence of the destabilizing domain.

Figure 1 – supplement 2

TMP-dependent GFP expression in the whole brain across developmental stages – (from left to right) – Larva, Early pupa (48 hr APF), Late pupa (96 hr APF), Adult (<12 hr post eclosion),

Adult (3 day old). Fluorescence intensity has been normalized to the mean fluorescence intensity from the brains of flies fed with the solvent. Scale bar = 100 μm. Differences in GFP expression between solvent and TMP fed flies are significant (unpaired t-test, two-tailed) across all developmental stages - Larva (p<0.001, t=18.6, n=6), Early pupa (p<0.001, t=10.89, n=5-6), Late pupa (p=0.025, t=3.46, n=5-6), Adult at eclosion (p=0.022, t=3.25, n=6), 3 day old adult (p<0.001, t=7.55, n=6).

Figure 1 – supplement 3

(A) TMP-dependent GFP expression in different cell types in the adult brain. Fluorescence intensity has been normalized to the mean fluorescence intensity from the brains of flies fed with the solvent. Flies were fed with solvent or TMP-containing food from embryo stage to five days post-eclosion. Differences in GFP expression between solvent and TMP-fed flies are significant for all cell-types shown (n=4-6, p<0.003, t>5.3, unpaired t-test, two-tailed, for all cell types). (B, C) TMP-dependent GFP expression in ovaries (B) and the foreleg (C). (D) TMP-dependent GFP expression in olfactory sensory neurons using the LexA/LexAop system. Scale bar = 50 μm (A, D), 100 μm (B), 150 μm (C).

Figure 1 – supplement 4

Effect of TMP on survival and behavior (A) Survival of wild-type flies fed with 0-10 mM TMP from eclosion to death. 15 flies were placed in each vial, four vials per condition. Error bars indicate SEM. (B) Survival (top) and developmental timing (bottom) of flies raised on 0-10 mM TMP. 20 eggs were placed in each vial, four vials per condition, one-way ANOVA with Tukey's post-hoc test. Significant differences between conditions (p<0.05) are denoted by different letters. Survival to pupa (n=4, p=0.186, F=1.88), survival to adult (n=4, p=0.245, F=1.58), time to pupation (n=4, p<0.0001, F=389.04), and time to adulthood (n=4, p<0.0001, F=251.73) (C) Walking speed of wild-type (CS) flies fed with 1 mM TMP or solvent for 48 hours prior to assay

(n=148-161, unpaired t-test, two-tailed). Bar indicates median. Whiskers indicate 90% percentile. (D) Percentage of flies reaching the food odor is plotted against time. Food odor: 1% apple cider vinegar. Error bars indicate SEM.

Figure 2

Chemically inducible control of GAL4-dependent expression using destabilized GAL80 (nsynaptobrevin-GAL80-DD). (A) GAL4-driven GFP expression in olfactory sensory neurons can be suppressed by destabilized GAL80 (*nsyb-GAL80-DD*) in a TMP-dependent manner (n=5, unpaired t-test, two-tailed, t=13.25). (B, C) GAL80-DD can be used to temporally control GFP expression. (B_1, C_1) *Orco-Gal4, UAS-GFP, nsyb-GAL80-DD* flies were fed with food containing 1 mM TMP up to 3 days post-eclosion, following which flies were switched to standard fly food and dissected for quantification. GFP expression was compared to flies fed with 1 mM TMP throughout (B_2, C_2) or solvent throughout (B_3, C_3) (n=4-5). 0 hour time point in C_1 and C_2 represent the same sample. (D) Tetanus toxin expression in the V antennal lobe glomerulus of flies fed with 1 mM TMP or solvent. (E) CO_2 avoidance index of flies fed with 1 mM TMP or solvent. One arm of the T-maze contained 0.28% (v/v) CO_2 and the other arm had air. GAL80-DD can restore CO_2 aversion by suppressing TNT expression in the presence of TMP. n=11 per condition, two-way ANOVA indicated a significant interaction between feeding condition and genotype, F=23.66, p<0.001. Significant differences between conditions (p<0.05) are denoted by different letters (Tukey's post-hoc test). All flies were between 4-7 days old. Error bars indicate SEM. Scale bar = 50 μm (A), 25 μm (B,D).

Figure 2 – supplement 1

(A) *Orco-Gal4, UAS-GFP, nsyb-GAL80-DD* flies were fed with food containing 1 mM TMP up to eclosion, following which flies were switched to standard fly food or maintained on 1 mM TMP. (B,C) After switching to standard fly food, GFP expression is visible in the antennal lobe starting

at 1 day post-eclosion. No GFP is observed in either TMP or solvent-fed flies right after eclosion (<8 hr adults). n=5-6 per condition, two-way ANOVA indicated a significant interaction between feeding condition and age, F=16.22, p<0.001. Significant differences between conditions (p<0.05) are denoted by different letters (Tukey's post-hoc test). Error bars indicate SEM. Scale bar = 25 μm.

Figure 3

Chemical control of recombination frequency using destabilized flippase (*10XUAS-FLP-DD*). (A) GFP expression in a sub-population of olfactory projection neurons following excision of the STOP cassette by FLP-DD. Scale bar = 50 μm. (B) Number of GFP-positive projection neurons can be controlled by varying TMP dosage. The number of GFP-labeled cells within a sample is similar across both brain hemispheres. Each point represents number of cells in one brain. (C) Pie chart indicating the number of labeled projection neurons for flies fed with solvent. 42% of all hemispheres had a single GFP-labeled cell. (D) Examples of labeled single projection neurons (D - dorsal, V - ventral, M - medial, L - lateral). Red = anti-Bruchpilot nc82, Green = GFP. Scale bar = 25 μm.

Figure 4

Refining intersection patterns by temporally limiting FLP-DD expression. (A) Z-stack projections showing expression patterns of *GH146-QF* (green) and *NP21-GAL4* (red). Both transgenic lines overlap in a single population of DA1 lPNs (arrow in A_2). Between one to three overlapping neurons can be observed across all samples. Antenna was ablated from the brain sample shown in A_2 to visualize projection neurons in the absence of sensory neuron axon terminals in the antennal lobe. (B-C) Intersection using constitutively expressed flippase generates expanded patterns with additional expression in other olfactory (B) or visual (C) projection neurons. (D) Temporally limiting FLP-DD expression by feeding 1 mM TMP

exclusively in adult stage results in GFP expression only in DA1-lPNs. (E) No GFP expression is observed in the absence of TMP. (F) GFP expression in additional olfactory projection neurons can be observed using FLP-DD if TMP is fed continuously. Scale bar = 25 μm.

Figure 4 – supplement 1

Intersection pattern between *GH146-QF* and *NP21-GAL4* in flies fed with 10 mM TMP continuously. Scale bar = 25 μm.

Figure 1

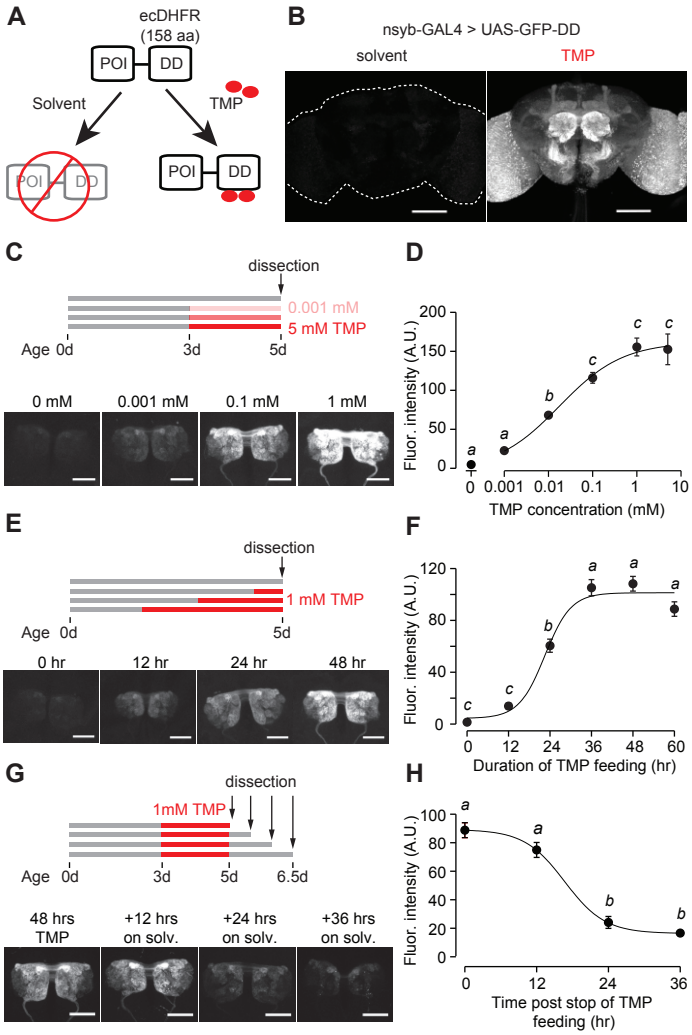
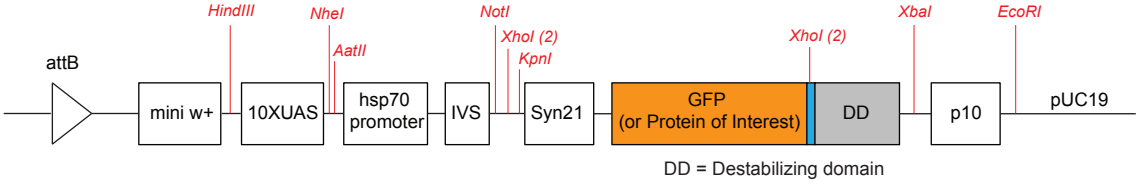

A
ecDHFR
(158 aa)
POI
DD
Solvent
TMP
B
nsyb-GAL4 > UAS-GFP-DD
solvent
TMP
C
dissection
0.001 mM
5 mM TMP
Age 0d
3d
5d
0 mM
0.001 mM
0.1 mM
1 mM
D
Fluor. intensity (A.U.)
TMP concentration (mM)
E
dissection
1 mM TMP
Age 0d
5d
0 hr
12 hr
24 hr
48 hr
F
Fluor. intensity (A.U.)
Duration of TMP feeding (hr)
G
dissection
1mM TMP
Age 0d
3d
5d
6.5d
48 hrs TMP
+12 hrs on solv.
+24 hrs on solv.
+36 hrs on solv.
H
Fluor. intensity (A.U.)
Time post stop of TMP feeding (hr)

Figure 1 - Supplement 1

A



B

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            10        20        30        40        50        60        70        80        90       100       110       120
             *         *         *         *         *         *         *         *         *         *         *         *
  1 NNNNNNNNNNNNNNNNNNCTCGAGATCTCCCTGATTGCCGCCCTGGCCGTGGACCACGTGATTGGCATGGAAAATGCCATGCCCTGGAACCTGCCCGCCGATCTGGCCTGGTTCAAGCGC 120
   X  X  X  X  X  X  L  E  I  S  L  I  A  A  L  A  V  D  H  V  I  G  M  E  N  A  M  P  W  N  L  P  A  D  L  A  W  F  K  R
                        >>>>
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Figure 1 - Supplement 2

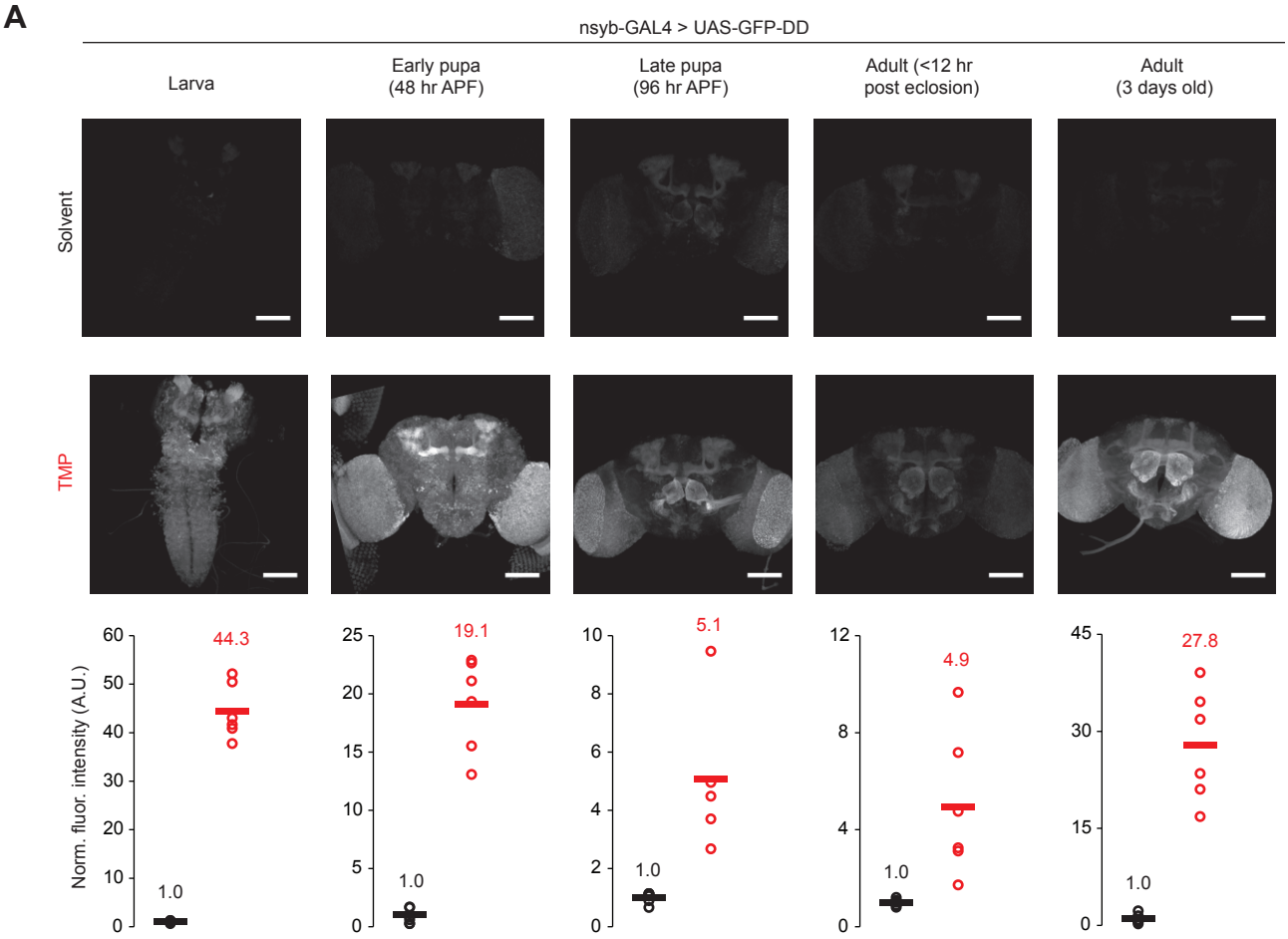


Figure 1 - Supplement 3

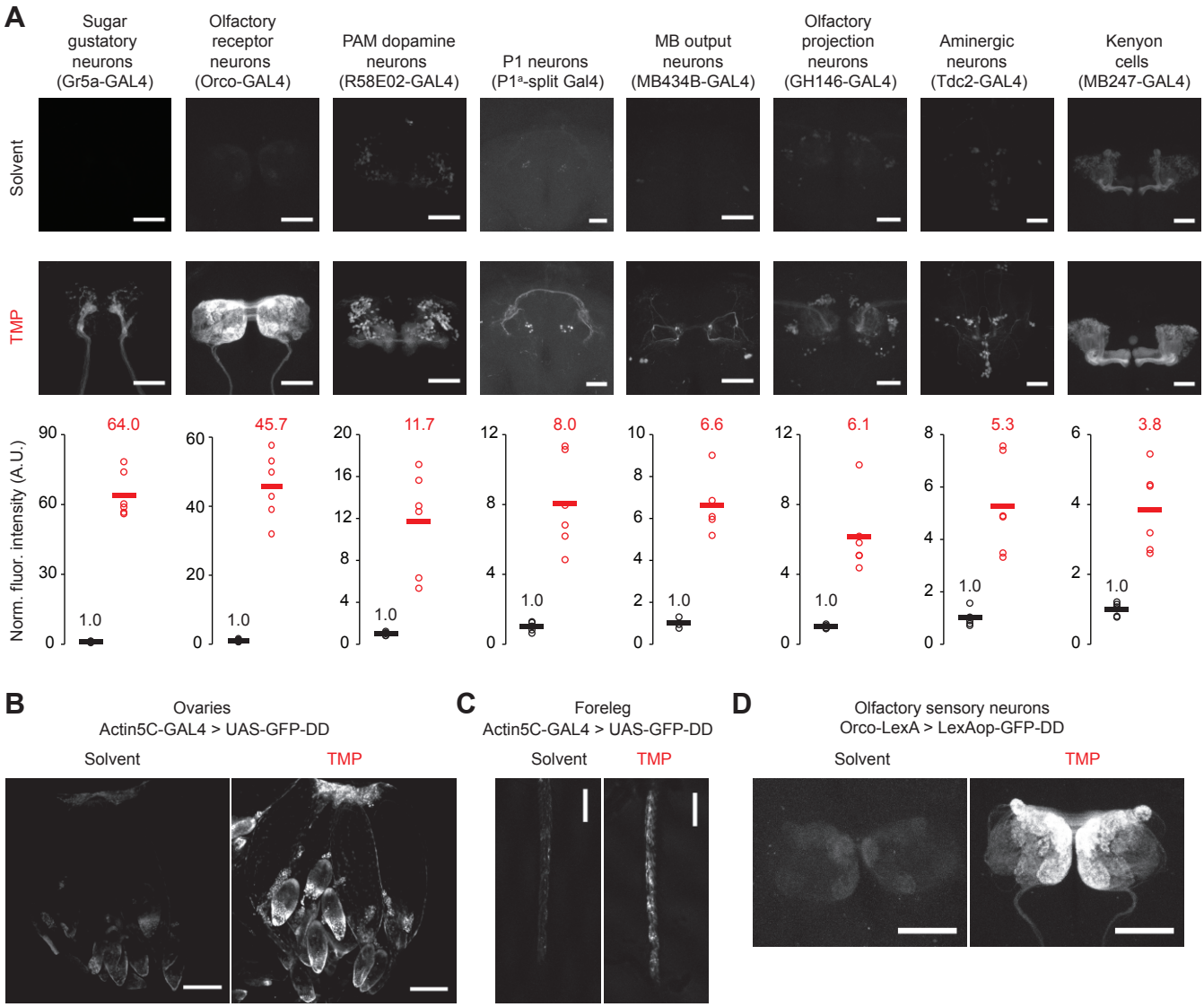


Figure 1 - Supplement 4

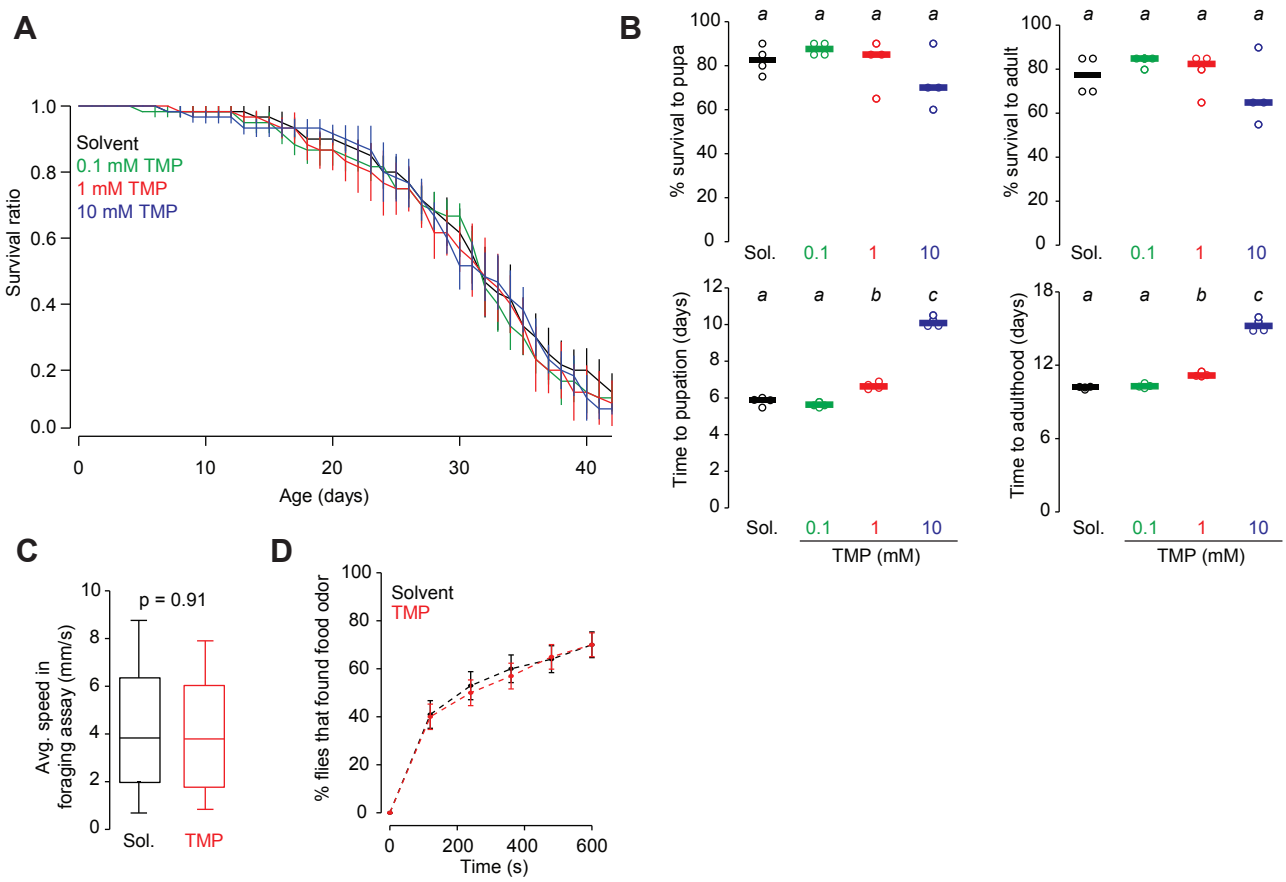


Figure 2

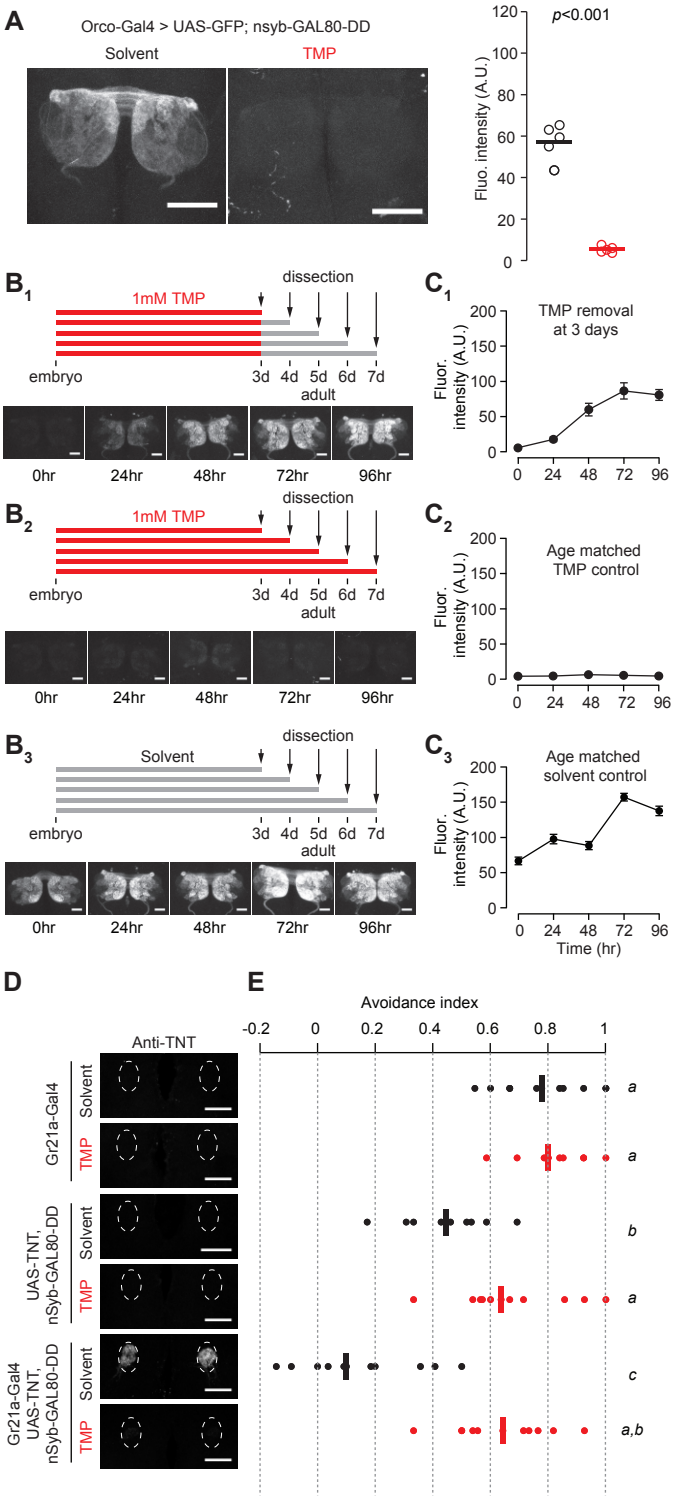

A
Orco-Gal4 > UAS-GFP; nsyb-GAL80-DD
Solvent
TMP
p<0.001
Fluo. intensity (A.U.)
B1
1mM TMP
dissection
embryo
3d 4d 5d 6d 7d
adult
0hr 24hr 48hr 72hr 96hr
C1
TMP removal at 3 days
Fluor. intensity (A.U.)
B2
1mM TMP
C2
Age matched TMP control
B3
Solvent
C3
Age matched solvent control
Time (hr)
D
Anti-TNT
Gr21a-Gal4
UAS-TNT, nSyb-GAL80-DD
Gr21a-Gal4 UAS-TNT, nSyb-GAL80-DD
E
Avoidance index
a
a
b
a
c
a,b

Figure 2 - Supplement 1

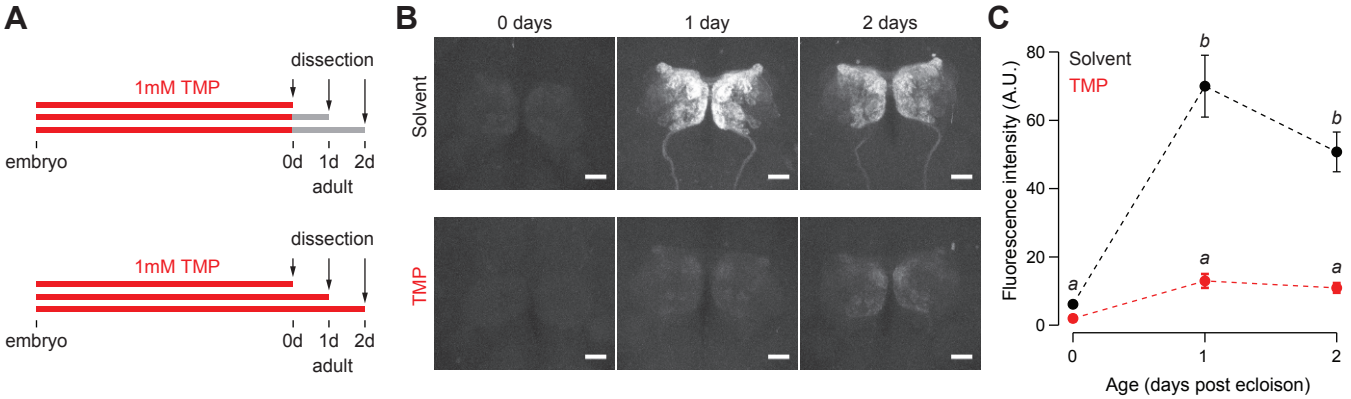


Figure 3

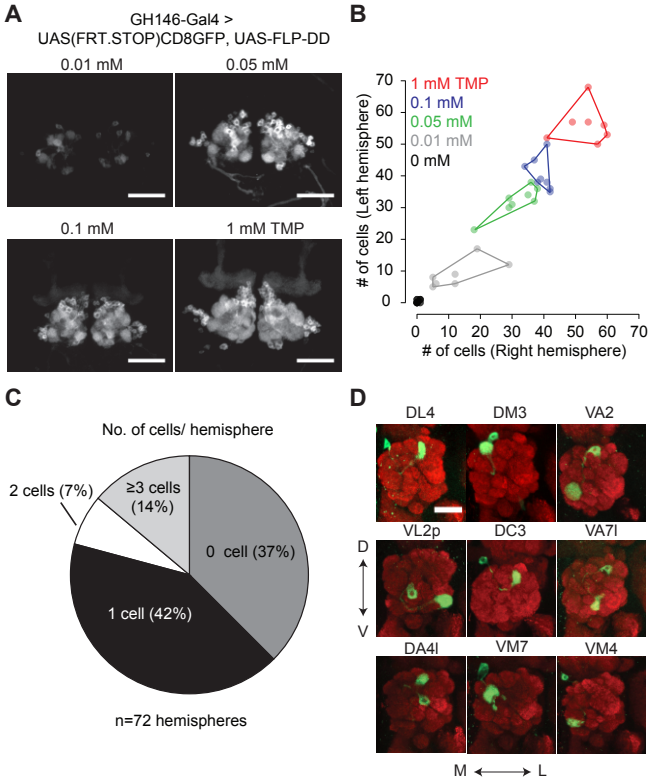

A
GH146-Gal4 >
UAS(FRT.STOP)CD8GFP, UAS-FLP-DD
0.01 mM
0.05 mM
0.1 mM
1 mM TMP
B
1 mM TMP
0.1 mM
0.05 mM
0.01 mM
0 mM
# of cells (Left hemisphere)
# of cells (Right hemisphere)
0
10
20
30
40
50
60
70
C
No. of cells/ hemisphere
2 cells (7%)
≥3 cells (14%)
0 cell (37%)
1 cell (42%)
n=72 hemispheres
D
DL4
DM3
VA2
VL2p
DC3
VA7l
DA4l
VM7
VM4
D
V
M
L

Figure 4

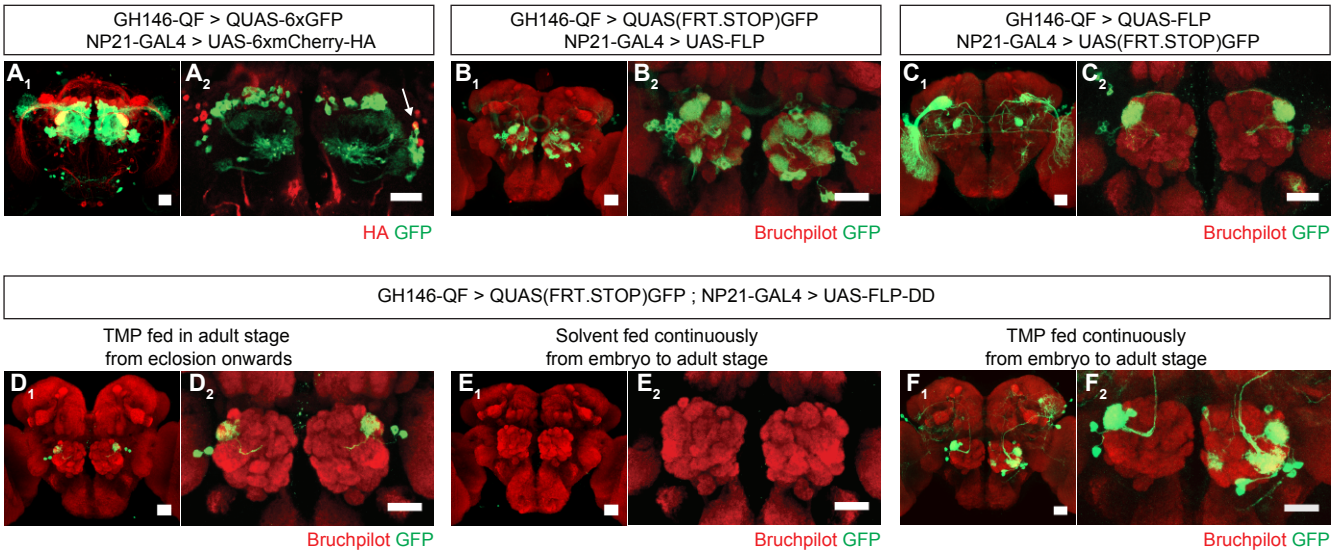
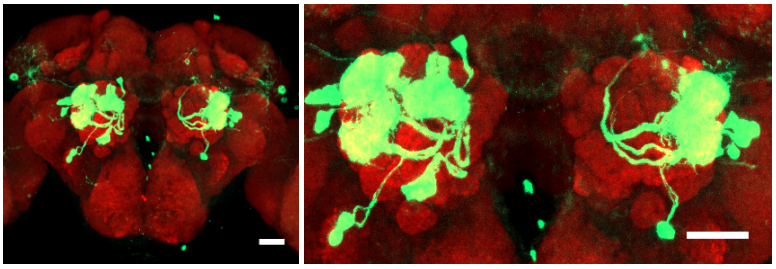

GH146-QF > QUAS-6xGFP
NP21-GAL4 > UAS-6xmCherry-HA
GH146-QF > QUAS(FRT.STOP)GFP
NP21-GAL4 > UAS-FLP
GH146-QF > QUAS-FLP
NP21-GAL4 > UAS(FRT.STOP)GFP
A1
A2
B1
B2
C1
C2
HA GFP
Bruchpilot GFP
Bruchpilot GFP
GH146-QF > QUAS(FRT.STOP)GFP ; NP21-GAL4 > UAS-FLP-DD
TMP fed in adult stage
from eclosion onwards
Solvent fed continuously
from embryo to adult stage
TMP fed continuously
from embryo to adult stage
D1
D2
E1
E2
F1
F2
Bruchpilot GFP
Bruchpilot GFP
Bruchpilot GFP

Figure 4 - figure supplement 1

GH146-QF > QUAS(FRT.STOP)GFP, NP21-GAL4 > UAS-FLP-DD

10 mM TMP fed continuously from embryo to adult stage



Bruchpilot GFP

Supplementary methods

Transgenic fly generation

Drosophila codon optimized destabilized domain (DD) was synthesized with 5' *XhoI* and 3' *XbaI* overhangs by Genewiz, Inc. (La Jolla, CA). Plasmids were generated using standard protocols for PCR, restriction digestion and ligation.

Destabilized GFP

To generate the 10XUAS-GFP-DD fly, DD was ligated to the c-terminus of GFP in the pJFRC81 vector (Pfeiffer, Truman, & Rubin, 2012). GFP was subcloned from the pJFRC81 plasmid using primer P1 and P2. DD was ligated to the c-terminus of GFP using the *XhoI* cut site. GFP-DD was ligated into the pJFC81 vector between the *PshAI* and *XbaI* cut sites. To generate the 13XLexAop2-GFP-DD fly, GFP-DD was cut from the 10XUAS-GFP-DD and ligated to the pJFRC95 plasmid(Pfeiffer et al., 2012) between the *NotI* and *XbaI* sites. Both GFP-DD constructs were transformed using phiC31 integrase mediated recombination into the attP2 landing site(Groth, Fish, Nusse, & Calos, 2004) by Genetic Services Inc. (Cambridge, MA).

Destabilized GAL80

To generate the nsyb-GAL80-DD fly, DD was ligated to the c-terminus of GAL80. GAL80 was subcloned with 5' *EcoRI* and 3' *XhoI* overhangs from pAC-GAL80 plasmid (Addgene #24346) using primers P3 and P4. DD was subcloned from the 10XUAS-GFP-DD plasmid with 5' *XhoI* and 3' *AatII* overhangs using primers P5 and P6. GAL80-DD was triple ligated between *EcoRI* and *AatII* sites in the cut nsyb-GAL4-hsp70 plasmid (Addgene #46107)(Riabinina et al., 2015). The resulting construct was transformed using phiC31 integrase mediated recombination into the VK00005 landing site(Groth et al., 2004) by Genetic Services Inc. (Cambridge, MA).

Destabilized FLP

The 10XUAS-FLP-DD plasmid was generated by ligating DD to the c-terminus of FLPD5. FLPD5 was subcloned with 5' *NotI* and 3' *XhoI* overhangs from pCaSpeR-DEST5 (DGRC #1031) using primers P7 and P8. FLP was ligated between the *NotI* and *XhoI* sites in the cut 10XUAS-GFP-DD plasmid. The construct was transformed using phiC31 integrase-mediated recombination into the attP2 landing site by Bestgene Inc. (Chino Hills, CA).

Primers

Primer No.	Sequence
P1	GGAGTAGTCCCGATATTGGTTG
P2	TTCATCTCGAGCTTGTAGAGCTCATCCATGCCGT
P3	ATCATCGACAGCCGAATTCCAACATGGACTACAACAAGAGATCTTCG
P4	GCGGCAATCAGGGAGATCTCGAGTAAACTATAATGCGAGATATT
P5	CTGGTTTCCAAACTGATCGGTC
P6	CGACGGTATCGATAGACGTCTATTAACGGCGCTCCAGAATCTCGAA
P7	TACTTCAGGCGGCCGCGGCTGGAGGGTACCAACTTAAAAAAAAAAATCAAAATG CCACAATTTGATATATTATGT
P8	ATCAGGGAGATCTCGAGTATGCGTCTATTTATGTAGGATG

Recommended steps for generation of new POI-DD constructs

To generate new protein of interest (POI)-DD fusion constructs driven by UAS, the POI can be cloned in a non-directional manner (between *XhoI* restriction sites) or in a directional manner (between *NotI* and *XhoI*) (See Figure 1- supplement 1) using the UAS-GFP-DD plasmid as template. Note that for directional cloning between *NotI* and *XhoI*, the *XhoI* site upstream of Syn21 must be mutated during primer design (see P7 for example).The Syn21 sequence (21 bp) can be included within the primer if desired (see P7 for example).

Table of genotypes and feeding condition

Figure	Genotype	Feeding condition
1B	*w⁻ ; + ; nsyb-GAL4/10XUAS-GFP-DD*	Flies fed with standard fly food or food containing 1 mM TMP from embryo stage to adult up to dissection
1C, D	*w⁻ ; + ; Orco-GAL4/10XUAS-GFP-DD*	Flies fed with standard fly food containing 0 – 5 mM TMP for 48 hours
1E, F	*w⁻ ; + ; Orco-GAL4/10XUAS-GFP-DD*	Flies fed with standard fly food containing 1 mM TMP for 0 - 60 hours
1G, H	*w⁻ ; + ; Orco-GAL4/10XUAS-GFP-DD*	Flies fed with standard fly food containing 1 mM TMP for 48 hours, and then moved to standard fly food without TMP for 0-36 hours
1-S2A	*w⁻ ; + ; nsyb-GAL4/10XUAS-GFP-DD*	Flies fed with standard fly food or food containing 1 mM TMP from embryo up to dissection.
1-S3A	Sugar gustatory neurons: *w⁻ ; + ; Gr5a-GAL4/10XUAS-GFP-DD* Olfactory receptor neurons: *w⁻ ; + ; Orco-GAL4/10XUAS-GFP-DD* PAM dopamine neurons: *w⁻ ; + ; R58E02-GAL4/10XUAS-GFP-DD* P1[a] neurons: *w⁻ ; R15A01-AD/+ ; R71G01-DBD /10XUAS-GFP-DD* MB output neurons: *w⁻ ; R30E08-p65AD/+; R53C10-DBD /10XUAS-GFP-DD* Olfactory projection neurons: *w⁻ ; GH146-GAL4/+; 10XUAS-GFP-DD/+* Aminergic neurons: *w⁻ ; Tdc2-GAL4/+; 10XUAS-GFP-DD/+* Kenyon cells: *w⁻ ; + ; MB247-GAL4/10XUAS-GFP-DD*	Flies fed with standard fly food or food containing 1 mM TMP from embryo up to dissection.
1-S3B,C	*w⁻; Actin5C-GAL4/+ ; 10XUAS-GFP-DD/+*	Flies fed with 1 mM TMP or 1% DMSO in standard fly food for 48 hours before dissection.
1-S3D	*w⁻; + ; Orco-LexA/13xLexAop-GFP-DD/+*	Flies fed with 1 mM TMP or 1% DMSO in standard fly food for 48 hours before dissection.

1-S4A	Wild-type (w1118)	Flies fed with 0 -10 mM TMP in 1 M sucrose, 1% agar from eclosion to death
1-S4B	Wild-type (w1118)	Flies raised in 0-10 mM TMP in standard fly food from embryo onwards.
1-S4C	Wild-type (Canton-S)	Flies fed with 1 mM TMP or 1% DMSO in standard fly food for 48 hours before behavioral assay.
1-S4D	Wild-type (Canton-S)	Flies fed with 1 mM TMP or 1% DMSO in standard fly food for 24 hours, following which flies were transferred to starvation vials containing water with 1 mM TMP or 1% DMSO for 24 hours before behavioral assay.
2A	*w^- ; UAS-GFP/+ ; Orco-GAL4/nsyb-GAL80-DD*	Flies fed with standard fly food or food containing 1 mM TMP from embryo stage to adult up to dissection
2B, C	*w^- ; UAS-GFP/+ ; Orco-GAL4/nsyb-GAL80-DD*	B1, C1: Flies fed with food containing 1mM TMP from embryo stage to 3 days post eclosion. Flies were then transferred to standard food without TMP up to dissection. B2, C2: Flies fed with standard food from embryo stage to adult up to dissection. B3, C3: Flies fed with food containing 1mM TMP from embryo to adult up to dissection.
2D, E	*w^-/+ ; Gr21a-GAL4/+ ; +* *w^-/+ ; UAS-TNT/+ ; nsyb-GAL80-DD/+* *w^-/+; Gr21a-GAL4, UAS-TNT/+; nsyb-GAL80-DD/+*	Flies fed with standard fly food or food containing 1 mM TMP from embryo stage to adult up to behavioral assay
2-S1B,C	*w^- ; UAS-GFP/+ ; Orco-GAL4/nsyb-GAL80-DD*	Flies raised on food containing 1 mM TMP from embryo stage up to eclosion. Flies were then transferred to standard food without TMP (B, top) or maintained on food containing 1 mM TMP (B, bottom).
3A, B	*w^- ; GH146-GAL4/+ ; UAS(FRT.STOP)mCD8GFP/ 10XUAS-FLP-DD*	Flies fed with standard fly food containing 0 – 1 mM TMP from embryo stage up to dissection.
3C,D	*w^- ; GH146-GAL4/+ ; UAS(FRT.STOP)mCD8GFP/ 10XUAS-FLP-DD*	Flies fed with standard fly food without TMP from embryo stage up to dissection.

4A	*w⁻; GH146-QF/UAS-6xmcherry-HA; NP21-GAL4/QUAS-6xGFP*	Flies fed with standard fly food from embryo stage up to dissection
4B	*w⁻; GH146-QF/ QUAS(FRT.STOP)mCD8GFP ; NP21-GAL4/ UAS-FLP*	Flies fed with standard fly food from embryo stage up to dissection
4C	*w⁻; GH146-QF/ UAS(FRT.STOP)GFP.myr; NP21-GAL4/ QUAS-FLP*	Flies fed with standard fly food from embryo stage up to dissection
4D-F	*w⁻; GH146-QF/ QUAS(FRT.STOP)mCD8GFP ; NP21-GAL4/ UAS-FLP-DD*	D: Flies fed with standard fly food without TMP from embryo stage up to eclosion. After eclosion, flies were switched to food containing 1 mM TMP for 4-5 days before dissection. E: Flies fed with standard fly food from embryo stage up to dissection F: Flies fed with fly food containing 1mM TMP from embryo stage up to dissection.
4-S1	*w⁻; GH146-QF/ QUAS(FRT.STOP)mCD8GFP ; NP21-GAL4/ UAS-FLP-DD*	Flies fed with fly food containing 10 mM TMP from embryo stage up to dissection.

Key Resources Table

Reagent type	Designation	Source or reference	Identifiers	Additional information
genetic reagent (fly line)	nsyb-GAL4	Riabinina et al., 2015	BDSC_51941	
	Orco-GAL4	Kreher, Kwon, & Carlson, 2005	BDSC_23292	
	UAS-GFP			
	GH146-GAL4	Stocker, Heimbeck, Gendre, & de Belle, 1997	BDSC_30026	
	Gr5a-GAL4	Thorne, Chromey, Bray, & Amrein, 2004		
	R58E02-GAL4	Liu et al., 2012	BDSC_41347	
	P1a-split GAL4	Hoopfer, Jung, Inagaki, Rubin, & Anderson, 2015		
	MB434B-split GAL4	Aso et al., 2014		
	Tdc2-GAL4	Cole et al., 2005	BDSC_9313	
	MB247-GAL4	Zars, Fischer, Schulz, & Heisenberg, 2000		
	UAS-(FRT.STOP)mCD8-GFP	Potter et al., 2010	BDSC_30032	
	UAS-(FRT.STOP)GFP.myr		BDSC_55810	
	UAS-6XmCherry-HA	Shearin, MacDonald, Spector, & Steven Stowers, 2014	BDSC_52267	
	QUAS-6xGFP	Shearin, MacDonald, Spector, & Steven Stowers, 2014	BDSC_52264	
	20XUAS-FLPD5	Nern, Pfeiffer, Svoboda, & Rubin, 2011	BDSC_55805	
	GH146-QF	Potter et al., 2010	BDSC_30014	
	QUAS(FRT.STOP)GFP	Potter et al., 2010	BDSC_30134	
	NP21-GAL4	Hayashi et al., 2002	BDSC_30027	
	Actin5C-GAL4		BDSC_4414	
	Orco-LexAVP16	Lai, Awasaki, Ito, & Lee, 2008		
	Gr21-GAL4	Scott et al., 2001		

	UAS-TNT	Sweeney et al., 1995		
	10XUAS-GFP-DD	this study		see supplementary methods
	10XUAS-FLP-DD	this study		see supplementary methods
	nsyb-GAL80-DD	this study		see supplementary methods
	13XLexAop-GFP-DD	this study		see supplementary methods
antibody	Rabbit anti-GFP	Invitrogen	A-11122	1:200
	mouse anti-bruchpilot nc82	DSHB	AB_2314866	1:50
	mouse anti-HA	Biolegend	901501	1:500
	rabbit anti-TeTx	Statens Serum Institut	POL 016	1:1000
	Alexa Fluor 488 anti-rabbit immunoglobulin G	Invitrogen	A-31628	1:100
	Alexa Fluor 647 anti-mouse immunoglobulin G	Invitrogen	A-21235	1:100
recombinant DNA reagent (plasmids)	pJFRC81	Pfeiffer, Truman, & Rubin, 2012	Addgene_36432	
	pJFRC95	Pfeiffer, Truman, & Rubin, 2012		
	nsyb-GAL4-hsp70	Riabinina et al., 2015	Addgene_46107	
	pCaSpeR-DEST5	DGRC	1031	
	pAC-GAL80	Addgene	24346	
	10XUAS-GFP-DD	this study		see supplementary methods
	10XUAS-FLP-DD	this study		see supplementary methods
	nsyb-GAL80-DD	this study		see supplementary methods

	13XLexAop-GFP-DD	this study		see supplementary methods
chemical compound, drug	Trimethoprim	Teknova Inc., CA	T1205	
software, algorithm	Igor Pro V6.0	Wavemetrics, Inc.		
	Foraging assay behavior quantification	Zaninovich et al., 2013		
other	Focusclear mounting reagent	Cedarlane Labs	FC-101	

References

Groth, A. C., Fish, M., Nusse, R., & Calos, M. P. (2004). Construction of transgenic Drosophila by using the site-specific integrase from phage phiC31. *Genetics*, *166*(4), 1775–82. http://doi.org/10.1534/genetics.166.4.1775

Pfeiffer, B. D., Truman, J. W., & Rubin, G. M. (2012). Using translational enhancers to increase transgene expression in Drosophila. *Proceedings of the National Academy of Sciences*, *109*(17), 6626–6631. http://doi.org/10.1073/pnas.1204520109

Riabinina, O., Luginbuhl, D., Marr, E., Liu, S., Wu, M. N., Luo, L., & Potter, C. J. (2015). Improved and expanded Q-system reagents for genetic manipulations. *Nature Methods*, *12*(3), 219–222. http://doi.org/10.1038/nmeth.3250