Potency of transgenic effectors for neurogenetic manipulation in 
*Drosophila* larvae

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ABSTRACT
Genetic manipulations of neuronal activity are a cornerstone of studies aimed to identify the functional impact of defined neurons for animal behavior. With its small nervous system, rapid life cycle and genetic amenability, the fruit fly *Drosophila melanogaster* provides an attractive model system to study neuronal circuit function.

In the past two decades, a large repertoire of elegant genetic tools has been developed to manipulate and study neural circuits in the fruit fly. Current techniques allow genetic ablation, constitutive silencing or hyperactivation of neuronal activity, and also include conditional thermogenetic or optogenetic activation or inhibition. As for all genetic techniques, the choice of the proper transgenic tool is essential for behavioral studies. Potency and impact of effectors may vary in distinct neuron types or distinct types of behavior. We here systematically test genetic effectors for their potency to alter the behavior of *Drosophila* larvae using two distinct behavioral paradigms: general locomotor activity and directed, visually guided navigation. Our results show largely similar but not equal effects with different effector lines in both assays. Interestingly, differences in the magnitude of induced behavioral alterations between different effector lines remain largely consistent between the two behavioral assays. The observed potencies of the effector lines in aminergic and cholinergic neurons assessed here may help to choose the best-suited genetic tools to dissect neuronal networks underlying the behavior of larval fruit flies.

INTRODUCTION
The binary GAL4/UAS system for targeted gene expression (Brand and Perrimon 1993) is widely used in *Drosophila* to manipulate or visualize neuronal networks and is an important tool that has largely contributed to the success of the fruit fly as a major model system in neuroscience. The availability of this expression system represents the starting point for the development of effector transgenes that allow to dissect the function of genetically identifiable neurons with high spatial and temporal precision. This has turned the fly GAL4/UAS system into one of the most powerful neurogenetic tools available. Notably, the impact of this tool in various experiments is highly dependent on the selection of an appropriate effector line. For example, UAS-tetanus toxin (TNT) was successfully used in various studies to inhibit neurotransmitter release (Thum et al. 2006, Tripodi et al. 2008, Kong et al. 2010b). TNT specifically cleaves neuronal Synaptobrevin (n-Syb), which is essential for synaptic vesicle release.
(Sweeney et al. 1995). In fly photoreceptors, however, TNT-resistant excitatory synapses exist along with TNT-sensitive ones (Rister et al. 2006). In addition, the potencies of the effector genes UAS-Kir2.1, UAS-TNT, UAS-Diphteria toxin A (UAS-DTA) and UAS-reaper (UAS-rpr) either expressed in motor neurons, in mushroom body neurons or pan-neuronally differed in the adult fly, depending on the properties of the defined target cells. For instance adult-induced paralysis was more efficiently induced by effector genes silencing neuronal transmission than effector genes causing cell ablation (Thum et al. 2006). Moreover, impairment of short-term memories was achieved by specific expression of UAS-shibire<sup>ts</sup> (UAS-sh<i>ts</i>), while fly learning performance was not affected by the expression of UAS-TNT (Thum et al. 2006). Thus, it is crucial to choose effector genes that work robustly and reliably in the neuron type and behavior of interest.

In this study we used the <i>Drosophila</i> larva to systematically assess and compare the potency of fifteen different effector lines in two distinct behaviors: four different effector genes causing cell ablation, four different effector genes that silence neuronal activity and seven different effector genes that increase neuronal excitability or intracellular signaling. In the last years, the <i>Drosophila</i> larva has emerged as a favorable model to investigate different neurobiological aspects based on its genetic accessibility, its reduced neuronal complexity in terms of cell numbers compared to adult flies, and its behavioral repertoire. Great advances were made in the understanding of neuronal networks required for larval learning and memory (Gerber et al. 2009; Selcho et al. 2009; Pauls et al. 2010; Von Essen et al. 2011, Selcho et al. 2014), olfaction (Vosshall and Stocker 2007; Stocker 2008; Gerber et al. 2009), vision (Keene et al. 2011; Kane et al. 2013), feeding (Cobb et al. 2009; Wang et al. 2013) and locomotor behavior (Saraswati et al. 2004; Selcho et al. 2012, Heckscher et al. 2012, Vogelstein et al. 2014) using the larva as a model system.

Here, we first manipulated larval locomotion by effector gene expression in octopaminergic/tyraminergic (OA/TA) neurons using a <i>Tdc2-Gal4</i> driver (Cole et al. 2005). Several studies have earlier on shown that OA and TA act antagonistically on muscle contraction, resulting in reduced locomotion in larvae lacking OA, whereas hypomorphic TA receptor mutants show longer track distances (Kutsukake et al. 2000; Nagaya et al. 2002; Saraswati et al. 2004; Selcho et al. 2012). A small set of about 40
OA/TA neurons within the ventral nerve cord is necessary to control normal locomotor activity in the larva (Selcho et al. 2012). Tdc2-positive cells within the central brain are dispensable for larval locomotion, but necessary for mediating non-nutritional sugar information during larval associative conditioning (Selcho et al. 2014).

In parallel, we compared the efficiency of the selected effector genes in light avoidance behavior by ectopic expression in photoreceptor neurons via the IGMR-Gal4 driver line (Moses and Rubin 1991; Keene and Sprecher 2012). In larvae, the visual system consists of two simple eyes (called Bolwigs Organ, BO) that are much simpler than the adult compound eyes. Each eye includes twelve photoreceptors, which are subdivided into two types: eight photoreceptors express green-sensitive rhodopsin6 (rh6) while four photoreceptors express blue-sensitive rhodopsin5 (rh5; Helfrich-Förster et al. 2002; Sprecher et al. 2007; Sprecher and Desplan 2008). Neuronal projections of photoreceptor cells innervate the larval optic neuropile (LON), where they connect to their target cells (Sprecher et al. 2011; Keene and Sprecher, 2012).

Feeding Drosophila larvae perform a stereotypic photophobic behavior when they are confronted to choose between light and darkness. Interestingly, for this avoidance behavior only rh5 but not rh6 is required. In addition, also neuronal silencing of the second-order interneurons 5th LN (lateral neuron) and DN2s (dorsal neurons) strongly impairs rapid light avoidance behavior (Keene et al. 2011).

By investigating larval locomotion and rapid light avoidance, we obtained similar, but not the same results for the different effector genes when genetically manipulating aminergic Tdc2-Gal4-positive and cholinergic IGMR-Gal4-positive neurons. The observed potency of the effector lines in aminergic and cholinergic neurons assessed here may help to choose the best-suited genetic tools to dissect neuronal networks underlying the larval behavior of Drosophila.

**MATERIALS AND METHODS**

**Fly strains**

Flies were cultured according to standard methods. For the behavioral experiments, all UAS-lines were crossed to either Tdc2-Gal4 or IGMR-Gal4 driver lines. Heterozygous controls were obtained by crossing Gal4-driver and UAS-effector to w1118. UAS-lines included in this study were UAS-rpr (UAS-reaper), UAS-grim, UAS-hid,rpr, UAS-Kir2.1, UAS-ΔOrk, UAS-shi6s (UAS-shibire6s), UAS-TNTE, UAS-DTI, UAS-
TRPA1, UAS-3xTRPM8, UAS-NaChBac, UAS-2xChR2 (UAS-ChR2-wt), UAS-ChR2-XXL, UAS-Paca, UAS-bPac and 10xUAS-myr::GFP (Table 1).

Behavioral assays
To analyze larval locomotor behavior we recorded single larva for one minute on 1.5% agarose in a 85mm diameter Petri dish under red light conditions. Recordings were made by a DMK22BUC03 video camera with a Pentax C2514-M objective in combination with IC capture software (www.theimagingsource.com). Offline tracking was done by the custom-made software package FlyTrace (J.P. Lindemann and E. Braun, www.uni-bielefeld.de/biologie/Neurobiology/) and a homemade MATLAB script to obtain crawling distances per minute for each larva. Experiments were performed at room temperature (~24°C) except for UAS-shi-ts, UAS-TRPA1 and UAS-TRPM8, indicated respectively. To induce temperature-dependent cell manipulation, larvae kept on 25°C were measured on restrictive temperature (33°C for UAS-shi-ts and UAS-TRPA1 and 16°C for UAS-TRPM8) after 5min incubation time. For optogenetic manipulation (UAS-ChR2-wt, UAS-ChR2-XXL, UAS-bPac and UAS-Paca) we used a 480nm LED with a light intensity of around 0,14 mW/cm². Although we used a cooling element, there was a slight increase in temperature (< 1°C) at the level of the arena due to the LED illumination. As published previously all-trans-retinal (~200µM) was added to the standard medium to counter limited cellular availability of all-trans-retinal and thus increase efficiency of ChR2 expression for the UAS-ChR2-wt lines, but not for UAS-ChR2-XXL (Schroll et al. 2006, Ullrich et al. 2013, Dawidow et al. 2014).

Light avoidance was performed under red light conditions. The behavioral arena is made of a Petri dish with a cover shading two out of four quarters of the arena thus consisting of a dark side and light-exposed side. Illumination intensity from a white light LED lamp was 780 lux. The dark side clouds everything. A group of 30 larvae was collected from a food vial that was kept in darkness for 30 minutes before the experiment. During the 5 min preference test larvae freely move on the plate. After 5 min, larvae were counted on lit and dark quarters to calculate a dark preference index:

\[ \text{PREF}_{\text{darkness}} = \frac{\text{larvae on dark quarters} - \text{larvae on lit quarters}}{\text{total number of larvae}} \]

Immunofluorescence
Central nervous system of third instar larvae were dissected in phosphate-buffered saline (PBS, pH 7.4; see Selcho et al. 2009). Afterwards, the specimen were fixated
in 4% paraformaldehyde in PBS for 40 min, washed four times in PBT (PBS with 0.3% Triton-X 100), and blocked with 5% normal goat serum in PBT. Specimens were incubated with either anti-Tyrosine decarboxylase 2 (anti-Tdc2; pab0822-p; covalab; dilution 1:200; Pech et al. 2013) or anti-GFP (A6455, Molecular Probes, dilution 1:1000) in blocking solution for one night at 4°C. Preparations were washed six times with PBT and incubated for one night at 4°C with the secondary antibody goat anti-rabbit IgG DyLight488 (111-485-144; Jackson ImmunoResearch; dilution 1:250). Finally, specimens were rinsed six times in PBT and mounted in 80% glycerol in PBS. Until scanning at a Leica TCS SPE confocal microscope, specimens were stored in darkness at 4°C.

Bolwigs organs (BO) of third instar larvae were dissected in PBS and immediately fixed in 4% paraformaldehyde in PBS for 20 min. Samples were washed 8-10 times with PBT and subsequently incubated with primary antibodies over night at 4°C. The following primary antibodies were used: rat anti-Elav 1:20, mouse anti-Chaoptin 1:20 (both from Developmental studies Hybridoma bank) and rabbit anti-GFP (Molecular probes; dilution 1:1000). The next day samples were washed every 30 minutes in PBT and subsequently incubated with secondary antibodies over night at 4°C. The following secondary antibodies were used: anti rat Alexa-647 (Jackson Immunoresearch), anti mouse Alexa-488 or anti rabbit Alexa-488 (both from Molecular Probes; 1:200). Next, samples were washed two times 15 min in PBT and two times 15 min in PBS before mounting in 50% Glycerol. Images were taken with Leica SP5 confocal microscope.

**Statistical methods**

For statistical comparison between genotypes, Wilcoxon rank sum test was used. To compare single genotypes against chance level, we used the Wilcoxon signed ranked test. All statistical analyses and visualizations were done with R version 3.0.2 (www.r-project.org). Data are presented as box plots, 50% of the values of a given genotype being located within the box, whiskers represent the entire set of data. Outliers are indicated as open circles. The median performance index is indicated as a bold line within the box plot. Significance levels between genotypes shown in the figures refer to the raw p values obtained in the statistical tests.
RESULTS
To evaluate the potencies of various effector genes to interfere with neurotransmission in the *Drosophila* larva, we expressed fifteen different effector genes in either OA/TA neurons in the central nervous system (CNS) or in photoreceptor neurons in the BO (Fig. 1). Based on the expression pattern of *Tdc2-Gal4* and *IGMR-Gal4*, we used larval locomotion and larval light avoidance as the behavioral read out. The results are summarized in Table 1. Information about the action of the tested effector genes in published behavioral studies on *Drosophila* larvae is compiled in Supplementary Table 1.
<table>
<thead>
<tr>
<th>name</th>
<th>genotype</th>
<th>insertion chromosome(s)</th>
<th>reference</th>
<th>mechanism</th>
<th>efficiency in OA/TA neurons</th>
<th>efficiency in photoreceptor neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>cell ablation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UAS-reaper (rpr)</td>
<td>w;1118 P[w+mC+UAS-rpr.CJ27]</td>
<td>1</td>
<td>Zhou et al. 1997</td>
<td>binding to IAPs and thus inducing cell death</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>UAS-grim</td>
<td>w*; P[UAS-grim.NI2]</td>
<td>2</td>
<td>Wing et al. 1998</td>
<td>binding to IAPs and thus inducing cell death</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>UAS-hid.rpr</td>
<td>w; P[UAS-hid.F], P[UAS-rpr]</td>
<td>1</td>
<td>Zhou et al. 1997</td>
<td>binding to IAPs and thus inducing cell death</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>neuronal silencing</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UAS-shibire (shib)</td>
<td>w; P[UAS-shib[1],K3]</td>
<td>3</td>
<td>Kitamoto 2001</td>
<td>temperature-sensitive dynamin impairs vesicle recycling</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>UAS-TENT-E</td>
<td>w; P[UAS-TeNT-C.D16]</td>
<td>2</td>
<td>Sweeney et al. 1995</td>
<td>cleaves n-syb and impairs vesicle docking</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>UAS-Kir2.1</td>
<td>w*; P[UAS-GhHpKCN1.2.EGFP]</td>
<td>2</td>
<td>Baines et al. 2001</td>
<td>inward rectifying K⁺ channel prevents membrane depolarization</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>UAS-dOrk</td>
<td>y¹ w*; P[UAS-dOrk1.1-AJ]</td>
<td>2</td>
<td>Nitabach et al. 2002</td>
<td>outward rectifying K⁺ channel prevents membrane depolarization</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>neuronal activation</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>UAS-TRP8</td>
<td>yw; P[UAS-TRP8.CA-A], P[UAS-TRP8.CA-A]</td>
<td>2;3</td>
<td>Peabody et al. 2009</td>
<td>temperature dependent cation channel opens in response to ~12-16°C</td>
<td>-</td>
<td>n.d.</td>
</tr>
<tr>
<td>UAS-TRP8I</td>
<td>w; P[UAS-TRP8I.B], Kapt16</td>
<td>2</td>
<td>Rosenzweig et al. 2005</td>
<td>temperature dependent cation channel opens in response to ~29°C</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>UAS-Paco</td>
<td>P[UAS-Zz2Z(PACa.SL)]</td>
<td>3</td>
<td>Schröder-Lang et al. 2007</td>
<td>eukaryotic photoactivatable adenyl cyclase increasing intracellular cAMP</td>
<td>-</td>
<td>n.d.</td>
</tr>
<tr>
<td>UAS-bPac</td>
<td>w*; P[UAS-bPac.S]</td>
<td>2</td>
<td>Stierl et al. 2010</td>
<td>bacterial photoactivatable adenyl cyclase increasing intracellular cAMP</td>
<td>-</td>
<td>n.d.</td>
</tr>
<tr>
<td>UAS-ChR2</td>
<td>a) P[UAS-ChR2.S2], P[UAS-ChR2.S2]</td>
<td>a) 2;3, b) 2</td>
<td>a) Nagel et al. 2003, b) Dawidow et al. 2014</td>
<td>photoactivatable cation channel (470nm) → cell depolarization</td>
<td>a) + b) +++</td>
<td>n.d.</td>
</tr>
<tr>
<td>UAS-NoChRac</td>
<td>y¹ w*; P[UAS-NaChRac-EGFP]</td>
<td>3</td>
<td>Nitabach et al. 2006</td>
<td>bacterial sodium channel increases sodium conductance → cell depolarization</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
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Gal4 lines

| Gal4 lines         |                                   |                          |                            |                                                                            |                              |                                    |
|--------------------|-----------------------------------|--------------------------|----------------------------|----------------------------------------------------------------------------|                              |                                    |
| iGMR-Gal4          | y¹ w*; w[+]/CyO; P[iGMR-GAL4.CJ2] | 3                        | Moses and Rubin 1991      | drives expression in photoreceptor cells                                  |                              |                                    |

**Table 1:** The genotypes, brief description of the mode-of-action and the observed efficiencies of the effector genes used in this study
**Effector genes inducing apoptotic cell death**

The crudest way to interfere with neuronal transmission is to ablate neurons by the expression of different pro-apoptotic genes. Several studies suggest that *Drosophila* cell death is highly dependent on DIAP1 protein, a member of the IAP (inhibitor of apoptosis) family (Yoo *et al.* 2002). IAPs bind to active caspases and inhibit their proteolytic function by triggering caspase degradation. IAPs also bind to pro-apoptotic genes like grim, rpr (reaper) and hid (head involution defective) which prevents caspase binding and inhibition and therefore causes cell death (Kornbluth and White 2005). Although the distinct proteins may act on different targets within the apoptotic cascade, they also seem to functionally interact (Wing *et al.* 1998). For example, embryonic midline studies revealed that Grim alone is sufficient to ablate CNS midline cells in contrast to Hid and Rpr indicating different apoptotic capabilities among the three gene products (Wing *et al.* 1998). In contrast, rpr expression alone is sufficient to ablate neurons (e.g. McNabb *et al.* 1997). Expression of the clostridian Diphteria toxin A (DTA) causes a general inhibition of protein synthesis and is therefore capable to induce cell death (Bellen *et al.* 1992, Han *et al.* 2000). Toxicity is achieved by enzymatic inactivation of eukaryotic elongation factor-2 (Pappenheimer 1977). As DTA is lethal, an attenuated version I is used in *Drosophila* (DTI; Bellen *et al.* 1992, Han *et al.* 2000).

Targeted expression of either UAS-rpr, UAS-grim or UAS-hid,rpr in OA/TA neurons significantly reduced the larval crawling distance per minute (Fig. 1A). Though not immobile, experimental larvae showed impaired forward locomotion reduced to about 50% of control level. Tdc2-Gal4/UAS-grim larvae moved significantly less compared to Gal4/+ and UAS/+ larvae (p<0.001). Similarly, the expression of UAS-rpr in Tdc2-Gal4-positive neurons led to reduced distances of the experimental larvae compared to controls (p<0.001). Since different studies suggested that a combined expression of pro-apoptotic genes might have synergistic effects (Wing 1998, Selcho *et al.* 2012), we expressed hid and rpr using Tdc2-Gal4 to ablate OA/TA neurons. Tdc2-Gal4/UAS-hid,rpr larvae crawled significantly less than Tdc2-Gal4/+ and UAS-hid,rpr/+ larvae (p<0.001), while the effect seemed to be at best just slightly stronger compared to the expression of grim and rpr alone. Inhibition of protein synthesis by expression of UAS-DTI significantly reduced larval crawling distances per minute compared to Tdc2-Gal4/+ and UAS-DTI/+ (p<0.001; Figure 1A).
Targeted expression of pro-apoptotic genes in IGMR-Gal4-positive photoreceptor neurons led to results similar to the locomotion assay. IGMR-Gal4/UAS-grim larvae showed reduced rapid light avoidance indicated by higher numbers of larvae remaining in the illuminated quarters during the preference test (Fig. 1B). Performance of experimental larvae was significantly reduced compared to IGMR-Gal4/+ and UAS-grim (p<0.001) controls. Experimental IGMR-Gal4/UAS-rpr larvae showed reduced preference scores compared to both control groups (p<0.001). Remarkably, expression of either grim or rpr alone did not lead to complete impaired light avoidance of experimental larvae (both p<0.05 against chance level). In contrast, performance was indistinguishable from chance level after expression of combined hid and rpr (p>0.05), whereas performance of control larvae was significantly over chance level (p<0.001 for IGMR-Gal4/+ and UAS-hid,rpr/+). Similarly, inhibition of protein synthesis in photoreceptor neurons by expression of UAS-DTI completely abolished light avoidance behavior (p<0.05 against chance level) while both control groups performed over chance level (p<0.0001 compared to IGMR-Gal4/+ and UAS-DTI/+).

**Fig.1:** A: Locomotor behavior after Tdc2-Gal4-driven ablation of OA/TA neurons with UAS-grim, UAS-rpr, UAS-hid,rpr or UAS-DTI. Experimental larvae showed significantly reduced performances compared to controls. B: Rapid light avoidance behavior after specific ablation of photoreceptor neurons using IGMR-Gal4. Expression of Grim, Rpr,
Hid together with Rpr or DTI significantly reduced larval dark preferences compared to controls. White box: experimental larvae, light grey box: Gal4/+ larvae, dark grey box: UAS/+ larvae. *<0.05, **<0.01, ***<0.001, n.s.>0.05. #: not significant different from chance level.

To confirm the efficiency of pro-apoptotic genes and DTI we visualized Tdc2-positive cells after expression of pro-apoptotic genes and DTI (Supplementary Figure 1A-C, E-G, I-K) by anti-Tdc2 immunostaining (Pech et al. 2013) that labels OA/TA neurons within the brain and ventral nerve cord. In addition, we expressed 10xUAS-myr::GFP (Pfeiffer et al. 2010) to analyze the efficiency of cell ablation and protein synthesis inhibition with the Gal4 expression pattern as anti-Tdc2 antibody labels neurons not included in the expression pattern of Tdc2-Gal4 (Supplementary Figure 1D, H, L). Expression of pro-apoptotic genes grim, rpr and combined hid and rpr in OA/TA neurons led to a similar strong reduction in cell number, showing the efficiency of the effectors. Nevertheless, in all cases a small, varying number of Tdc2-Gal4-positive neurons escaped apoptosis. The number of GFP-expressing cells seemed to be slightly lower in larvae expressing hid,rpr (6.6 ± 1.2 surviving cells) compared to larvae expressing either grim (9.4 ± 0.4 surviving cells) or rpr (10 ± 0.4 surviving cells). In detail, ventral paired median neurons (VPM) in thoracic neuromeres t1-t3 and abdominal neuromere a1 as well as one dorsal unpaired neuron in abdominal neuromere a9 seemed to consistently survive apoptosis, while ventral unpaired median neurons (VUM) in thoracic and abdominal neuromeres seemed to consistently die (Supplementary Figure 1). Furthermore, labeling of OA/TA neurons via anti-Tdc2 and anti-GFP, respectively, indicated weak but consistent expression of Tdc2 or GFP after expression of UAS-DTI (Supplementary Figure 1M, P; 34.6 ± 0.24 cells survived in comparison to 42 ± 0.57 cells in control CNS). In contrast, expression of DTI as well as grim, rpr, and hid,rpr in photoreceptor neurons using iGMR-Gal4 completely ablated all photoreceptor neurons based on anti-Elav labeling (Supplementary Figure 2C, F, I, L).

**Effector genes inducing neuronal silencing**

Impairment of neuronal transmission by genetical cell ablation is a crude manipulation and may cause various side effects. To avoid adaptations of the corresponding neuronal networks due to ablated cells, inhibition of synaptic transmission is probably a more cautious alternative to interfere with neuronal communication. Synaptic
transmission relies on Ca\textsuperscript{2+}-dependent neurotransmitter release from synaptic vesicles. At the presynapse, a multitude of proteins including SNARE proteins located in the vesicle (v-SNARE) and presynaptic membrane (t-SNARE) are required for proper fusion events. One of the key v-SNARE proteins for targeted vesicle fusion is neuronal synaptobrevin (n-Syb). n-Syb is targeted by clostridial Tetanus toxin that efficiently inhibits chemical transmission. Tetanus toxin consists of a heavy polypeptide chain required for proper binding to its neuronal target, plus a light chain. Expression of the light chain (UAS-\textit{TNT} or UAS-\textit{TeTxLc}) intracellularly cleaves n-Syb and thereby diminishes synaptic transmission (Sweeney \textit{et al.} 1995).

Another way to interfere with neurotransmission is the expression of UAS-\textit{shi}\textsuperscript{ts} (a temperature-sensitive dominant negative form of Dynamin) as this conditional effector can bypass developmental effects or synaptic compensation of constitutively silenced neurons (Kitamoto 2001). Dynamin encodes a GTPase required for normal endocytosis and is crucial for vesicle recycling and neuronal functionality. Ectopic expression of UAS-\textit{shi}\textsuperscript{ts} blocks neuronal transmission only at restrictive temperature (>29°C). Since neuronal inhibition by \textit{shi}\textsuperscript{ts} is only achieved at restrictive temperature, this tool allows a rapid and reversible inhibition in a spatially and temporally controlled manner (Kitamoto 2001). A limitation of this effector gene is the necessity to increase temperature during the experiment, which might cause side effects.

A third way to induce neuronal silencing is via over-expression of permanently open K\textsuperscript{+} channels (Baines \textit{et al.} 2001; Nitabach \textit{et al.} 2002). Kir2.1 is a human inward rectifying potassium channel. Neuronal over-expression of Kir2.1 hyperpolarizes neurons and reduces the probability of evoked action potential generation and neurotransmitter release at the presynapse, while spontaneous release of neurotransmitters seems to be unaffected (Baines \textit{et al.} 2001). Similar to Kir2.1 channels, the \textit{Drosophila} \textit{ΔOrk} outward rectifying potassium channels hyperpolarize neurons and inhibit normal synaptic transmission (Nitabach \textit{et al.} 2002). \textit{ΔOrk} channels act like a K\textsuperscript{+}-selective hole in the cell membrane without any voltage or time dependence of the open state inducing currents similar to natural leak conductance. The native function of this channel in \textit{Drosophila} is unknown (Goldstein \textit{et al.} 1996).

In our experiments, \textit{shi}\textsuperscript{ts} appeared to be the most potent transgenic tool to block neurotransmission (Kitamoto 2001). At 24°C, both larval locomotion and rapid light
avoidance was unaffected in Shi<sup>ts</sup>-expressing larvae (Fig. 2A, B; p>0.05). In contrast, at 33°C Tdc2-Gal4/UAS-shi<sup>ts</sup> larvae moved significantly less than Tdc2-Gal4/+ and UAS-shi<sup>ts</sup>/+ (p<0.001) controls. Although experimental larvae moved less than controls, mean distances for all groups were higher at 33°C than in any other experiment at 24°C, indicating a generally increased locomotor activity at higher temperatures. In some behavioral paradigms, this might strongly affect behavioral outcomes. In these cases, transgenic tools other than Shi<sup>ts</sup> might be better suited to silence synaptic transmission. We therefore tested Tdc2-Gal4/UAS-TNTE larvae that performed indistinguishable from both Tdc2-Gal4/+ and UAS-TNTE/+ (p>0.05) controls, indicating no efficient block of neuronal activity in OA/TA neurons. As both Shi<sup>ts</sup> and TNT affect chemical transmission and thus likely leave electrical synapses unaffected, we next tested the ion channels Kir2.1 or ΔOrk that alter the membrane potential. Tdc2-Gal4/UAS-Kir2.1 larvae showed significantly reduced distance scores (p<0.001). Along the same line, expression of UAS-ΔOrk significantly reduced the crawling distances of experimental larvae compared to controls (p<0.01). Notably, by trend the expression of UAS-Kir2.1 seemed to be more efficient than the expression of UAS-ΔOrk (see Fig. 2A).

Similar results for silencing synaptic transmission were obtained in the light avoidance assay. No significant dark preference was shown after conditional silencing of photoreceptor neurons using UAS-shi<sup>ts</sup> (p=0.91; Fig. 2B). In contrast, dark preferences of control larvae were significantly higher compared to lGMR-Gal4/UAS-shi<sup>ts</sup> larvae (p<0.01 for lGMR-Gal4/+ and p<0.05 for UAS-shi<sup>ts</sup>/+) and over chance level (both p>0.05). Similar to larval locomotion, expression of UAS-TNTE did not lead to any significant changes in light avoidance (p>0.05). However, lethality after the ubiquitous expression of UAS-TNTE driven by actin-Gal4 confirmed the general potency of this effector gene (data not shown). Furthermore we used anti-TNT labeling to underline the inefficiency of UAS-TNTE in OA/TA neurons or photoreceptor neurons, respectively. While immunolabeling after expression driven by Tdc2-Gal4 (Supplementary Figure 1Q) indicate presence of TNT in OA/TA neurons, no labeling was found in photoreceptors after expression driven by lGMR-Gal4. Thus, unaffected light avoidance in lGMR-Gal4/UAS-TNTE larvae might be due to a lack of TNT expression in photoreceptor neurons. Electrical silencing using either UAS-Kir2.1 or UAS-ΔOrk led to significantly reduced light avoidance of experimental larvae. The dark preference of lGMR-Gal4/UAS-Kir2.1 larvae was significantly reduced (p<0.001).
Although the dark preference of *IGMR-Gal4/UAS-ΔOrk* larvae was also reduced compared to controls (p<0.05 for *IGMR-Gal4/+* and p<0.01 for *UAS-ΔOrk/+*), ΔOrk seemed to be again -by trend- less potent than Kir2.1 as *IGMR-Gal4/UAS-Kir2.1* larvae performed not significantly different from chance level. Since both constructs are GFP-tagged, we used anti-GFP labeling to confirm effector gene expression and to investigate whether the expression of permanently open potassium channels throughout development changes neuronal morphology. Neither expression of ΔOrk or Kir2.1 obviously altered the morphology or arborisation pattern of OA/TA neurons within the VNC (data not shown).

<table>
<thead>
<tr>
<th>UAS-<em>shi</em>&lt;sup&gt;ts&lt;/sup&gt;</th>
<th>UAS-TNTE</th>
<th>UAS-<em>Kir2.1</em></th>
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**Fig.2: A:** Locomotor behavior after neuronal silencing of OA/TA neurons via expression of UAS-*shi*<sup>ts</sup>, UAS-TNTE, UAS-*Kir2.1* or UAS-ΔOrk. Expression of UAS-*shi*<sup>ts</sup> reduced performance of experimental larvae compared to controls specifically at restrictive temperature. In contrast, no obvious effect appeared due to TNTE expression. After electrical silencing of OA/TA neurons with Kir2.1 or ΔOrk channels, experimental larvae crawled significantly less compared to controls. **B:** *IGMR-Gal4/UAS-*shi*<sup>ts</sup> larvae performed indistinguishable from chance level in rapid light avoidance at restrictive temperature. In contrast, performance of control larvae was significantly over chance level. Expression of TNTE in photoreceptor neurons had no effect on light avoidance.
Expression of rectifier channels Kir2.1 or ΔOrk significantly reduced performances in experimental larvae compared to controls. White box: experimental larvae, light grey box: Gal4/+ larvae, dark grey box: UAS/+ larvae. *<0.05, **<0.01, ***<0.001, n.s.>0.05. #: not significant different from chance level.

Effector genes increasing neuronal excitability or intracellular signaling

While effector genes suppressing neuronal activity help to identify the necessity of defined neurons for a certain behavior, activators may also identify modulatory effects of these neurons onto the regarding circuit. To specifically activate neuronal activity or intracellular signaling in defined cells, we used effector genes encoding different cation channels, as neuronal activation can be achieved by influx of sodium or calcium or the decrease of potassium conductance. Two widely used activator genes are TRPA1 and TRPM8, members of the TRP (transient receptor potential) cation channel superfamily that are sensitive to different temperatures (Rosenzweig et al. 2005). TRPA1 channels activate in response to warm temperatures (Viswanath et al. 2003). In contrast, rat TRPM8 is responsible for sensing mild cold temperatures (Colburn et al. 2007; Peabody et al. 2009). UAS-Channelrhodopsin2 (UAS-ChR2-wt and UAS-ChR2-XXL), UAS-Paca and UAS-bPac expression was successfully used for optogenetic cell manipulation (see Supplementary Table 1). ChR2 is a light-activatable cation channel from the flagellate Chlamydomonas reinhardtii with seven transmembrane domains and an all-trans chromophore, which responds to blue light stimulation (~480nm) by opening the internal channel (Nagel et al. 2003; Zhang et al. 2007, Dawidow et al. 2014). The open state of these channels allows Na⁺ and to a lower extend Ca²⁺ to enter the cell which leads to membrane depolarization. Wild-type ChR2 (ChR2-wt) was shown to stimulate neuronal activity when additional all-trans-retinal is added to the food media to compensate the limited cellular retinal availability. Low light transmission through the cuticle is known to be the bottleneck for optogenetic approaches in adult flies (Dawidow et al. 2014). ChR2-variant ChR2-XXL was recently shown to bypass this limitation as photosensitivity is 10.000 times higher than in ChR2-wt. In addition, ChR2-XXL efficiently stimulates neuronal activity without retinal supplementation (Dawidow et al. 2014). PACα is a subunit of a light-activatable adenylyl cyclase from the flagellate Euglena gracilis. The photoactivated adenylyl cyclase (PAC) is composed of two subunits: PACα and PACβ (Schröder-Lang et al. 2007). Expression of PACα in
Drosophila allows cell manipulation by blue light stimulation due to increasing intracellular cAMP levels in a spatiotemporal manner. Additionally, a further Pac from sulfide-oxidizing Beggiatoa bacteria (bPac) was introduced to Drosophila for optogenetic approaches (Stierl et al. 2010). bPac carries a blue light-sensitive domain linked to a type III adenylyl cyclase allowing cell manipulation similar to UAS-Pacα. bPac cyclase activity seems to be 3-4 times higher than Pacα activity. bPac thus needs ~1000 times less light to induce similar cAMP changes in neurons (Stierl et al. 2010). Finally, expression of UAS-NaChBac, a bacterial voltage-gated sodium channel, can be used to permanently increase neuronal excitability (Ren et al. 2001; Nitabach et al. 2006). Expressed in oocytes, NaChBac was able to conduct Na⁺ inward currents with a lower activation threshold than endogenous Drosophila voltage-gated Na⁺ channels (Nitabach et al. 2006).

At 16°C, Tdc2-Gal4/UAS-TRPM8 larvae crawled similar distances per minute as Tdc2-Gal4/+ and UAS-TRPM8/+ (p>0.05) controls. Contrary, high temperature-induced opening of TRPA1 channels in OA/TA neurons led to significantly reduced crawling distances in experimental larvae compared to both control groups (p<0.001; Fig. 3A). Similar to the results achieved during the UAS-shb experiments, larval crawling was generally increased by high temperature (33°C; Fig. 2, 3). To test for the temperature specificity of TRPA1, we performed the experiment at room temperature (24°C). As expected, there was no effect between experimental larvae and control larvae. Tdc2-Gal4/UAS-TRPA1 larvae crawled significantly longer distances per minute than Tdc2-Gal4/+ larvae (p<0.05), but significantly shorter distances compared to UAS-TRPA1/+ larvae (p<0.05). While TRP channels allow conditional activation of neurons specifically during an experiment, expression of bacterial sodium channels via UAS-NaChBac constitutively activates neurons, which might cause unspecific side effects. Nevertheless, Tdc2-Gal4/UAS-NaChBac larvae showed significantly reduced crawling distances compared to both controls (p<0.001 for Tdc2-Gal4/+ and p=<0.01 for UAS-NaChBac/+).

Although successfully used by Peabody and colleagues (Peabody et al. 2009), there was no effect in rapid light avoidance at 16°C after expression of UAS-TRPM8 in photoreceptor neurons (p>0.05; Fig. 3B). To make sure that our temperature decrement is generally sufficient to activate TRPM8 channels, we expressed UAS-TRPM8 in motorneurons via OK6-Gal4. After short cold exposure at 16°C, larvae were...
immobile indicating this temperature decrement sufficient to activate TRPM8 channels and thus induce cell activity (data not shown). In contrast, at 33°C expression of UAS-
TRPA1 using *IGMR-Gal4* reduced dark preference scores of experimental larvae to zero (p>0.05). Both control groups performed over chance levels and preferred dark quarters significantly more compared to *IGMR-Gal4/UAS-TRPA1* (p<0.01 for *IGMR-Gal4/+* and p<0.001 for *UAS-TRPA1/+*). In accordance with the results obtained for larval locomotion, there was no effect at room temperature (24°C) indicating the temperature specificity of TRPA1 action (p>0.05; Fig. 3B).

Next, we expressed UAS-*NaChBac* in photoreceptor neurons. In contrast to the results obtained in the locomotion assay, *IGMR-Gal4/UAS-NaChBac* larvae performed not significantly different from controls (p>0.05 for *IGMR-Gal4/+* and UAS-*NaChBac/+*). The lack of significance, however, may rely on higher variance among this data set (Fig. 3B): while control larvae performed over chance level (both p<0.01), experimental larvae showed no significant preference for darkness (p>0.05) suggesting an increased excitability of photoreceptor neurons after expression of NaChBac channels.
Fig. 3: A: Locomotor behavior after artificial activation of OA/TA neurons using Tdc2-Gal4 directed expression of UAS-TRPM8, UAS-TRPA1 and UAS-NaChBac. Expression of UAS-TRPM8 did not affect larval locomotion at 16°C. In contrast, thermogenetic activation of OA/TA neurons via UAS-TRPA1 reduced performance in experimental larvae compared to controls specifically at restrictive temperature. Additionally, expression of bacterial sodium channels via UAS-NaChBac reduced crawling distances significantly in Tdc2-Gal4/UAS-NaChBac larvae compared to controls. B: Similar to the locomotion assay, rapid light avoidance was not affected in experimental larvae by TRPM8 expression. Contrary, dark preferences were abolished in experimental larvae after TRPA1 expression at restrictive temperature. Performance of control larvae was significantly different from chance level. In addition, lGMR-Gal4/UAS-NaChBac larvae showed no light avoidance behavior as performance scores were indistinguishable from chance level while controls performed over chance level. Here, experimental larvae performed not significantly different from controls. Finally, we tested the optogenetic effectors UAS-Paca, UAS-bPac and two different versions of UAS-ChR2 to activate OA/TA neurons in larval locomotion. The expression...
of these effector genes in photoreceptor neurons was omitted, as it seems counterproductive to activate light-sensitive neurons via blue light exposure. Expression of UAS-Paca in OA/TA neurons did not lead to any significant effect between experimental and control larvae (all p>0.05, Fig. 4A) indicating UAS-Paca to be ineffective, possibly since an increase of intracellular cAMP in Tdc2-positive neurons does not affect larval locomotion. Similarly, cellular manipulation using expression of UAS-bPac did not lead to significant changes at lit conditions since experimental larvae performed not significantly different from UAS control larvae (p>0.05; Fig. 4A).

Expression of UAS-ChR2-wt using Tdc2-Gal4 significantly reduced larval crawling distances compared to Tdc2-Gal4/+ (p<0.001) and UAS-ChR2/+ (p<0.05) larvae at blue light exposure (Fig. 4B). Also UAS-ChR2-XXL profoundly reduced larval locomotion after expression in OA/TA neurons, yet without retinal supplementation (p<0.001, Dawidow et al. 2014). To exclude unspecific effects induced by ChR2-activating blue light, we repeated the experiments under red light conditions. As expected, experimental larvae carrying either one of the two ChR2 variants performed indistinguishable from both control groups (all p>0.05).

Fig. 4: Locomotor behavior after optogenetic activation of OA/TA neurons. Expression of UAS-Paca and UAS-bPac did not affect larval locomotor behavior as experimental
larvae performed indistinguishable from control larvae during illumination. In contrast, blue light exposure specifically affected locomotor behavior of Tdc2-Gal4/UAS-ChR2-wt and Tdc2-Gal4/UAS-ChR2-XXL as they crawled significantly shorter distances compared to both controls. Remarkably, under red light conditions experimental larvae performed not significantly different from controls. White box: experimental larvae, light grey box: Gal4/+ larvae, dark grey box: UAS/+ larvae. *<0.05, **<0.01, ***<0.001, n.s.>0.05.

DISCUSSION
Comparison of various effector genes to manipulate neuronal activity
Relatively simple neuronal circuits in Drosophila flies and especially larvae facilitate neurogenetic manipulations (see e.g. Vogelstein et al. 2014) in order to investigate how the brain organizes behavior based on changing environmental information and innate needs. However, it is crucial to choose transgenic tools, which reliably and robustly manipulate the neuron type and behavior of interest. Here we compared the efficiency of fifteen different effector genes in larval aminergic and cholinergic neurons that were previously shown to affect neuronal activity and corresponding behavior in Drosophila (Venken et al. 2011).

First, expression of different pro-apoptotic genes appeared to be highly efficient to affect both OA/TA neurons and photoreceptor neurons and thus larval locomotor and light avoidance behavior. In light avoidance, the combined expression of hid and rpr showed the highest efficiency as larval dark preference dropped to zero. Though expression of grim and rpr alone also strongly affected light avoidance in experimental larvae, they still performed over chance level suggesting that the combined expression of two pro-apoptotic genes enhances the efficiency to induce cell death. This is in line with studies on adipokinetic hormone (AKH)-producing neurosecretory cells. Expression of UAS-hid,rpr was sufficient to consistently eliminate AKH cells whereas few cells survived after expression of UAS-hid or UAS-rpr alone (Isabel et al. 2005). GFP-labeling after expression of hid and rpr in OA/TA neurons revealed a slightly stronger reduction in cell number compared to larvae expressing rpr or grim, while in all cases a small varying number of Tdc2-Gal4 positive neurons escaped apoptosis, possibly due to the low Gal4 expression in these cells. However, it seems difficult to assume that the -at best- slightly stronger reduction in larval distances per minute in larvae expressing hid,rpr compared to larvae expressing rpr or grim might rely on the higher number of ablated cells. This is in line with previous findings showing that adult
paralysis is less efficiently induced by cell ablation than by neuronal silencing (Thum et al. 2006). Interestingly, in the larval locomotion assay not a single larvae turned out to be fully immobile after cell ablation, neuronal silencing or activation, respectively. This suggests that either OA/TA neurons modulate locomotion rather than command its initiation, or functional redundancy is in place to compensate for impaired OA/TA signaling.

Expression of Shi\textsuperscript{ts}, ΔOrk and Kir2.1 appeared to efficiently silence neuronal transmission in OA/TA neurons and photoreceptor neurons. UAS-\textit{shi\textsuperscript{ts}} strongly reduced crawling distances in the locomotion assay and similar to the combined expression of \textit{hid} and \textit{rpr} reduced larval dark preference to zero. Silencing by ectopic potassium channel expression was efficient to reduce larval locomotion and larval dark preferences, with Kir2.1 having a slightly stronger effect than ΔOrk. In contrast, \textit{TNTE} expression was insufficient to manipulate either larval locomotion or rapid light avoidance. Similarly, \textit{TNT} was shown to fail in silencing adult photoreceptor neurons (Rister et al. 2006) and mushroom body neurons (Thum et al. 2006) indicating the presence of TNT-resistant neurons.

Neuronal activation was achieved by UAS-\textit{TRPA1} and UAS-\textit{NaChBac}. Additionally, larval locomotion was affected in response to optogenetic activation using two variants of UAS-\textit{ChR2}. Here, the newly developed ChR2-XXL seemed to be more efficient compared to the wild-type ChR2-wt, which is in line with the reported extended open-state lifetime, elevated cellular expression and reduced dependence on retinal supplementation of ChR2-XXL (Dawydow et al. 2014). In this study we did not test light-inducible channels to activate photoreceptor neurons as photoactivation likely interferes with the light sensing pathways. In contrast to the effector genes discussed above, UAS-\textit{TRPM8}, UAS-\textit{bPac} and UAS-\textit{Pacα} failed to alter the behaviors used in this study. Pacα and bPac expression was not sufficient to affect larval locomotion after blue light stimulation. Basal activity of endogenous adenylyl cyclases in response to certain physiological states of the animal and thus higher levels of cAMP before induced Pacα or bPac stimulation by light exposure may underly this inefficiency. This is in particular likely for the OA/TA neurons, which are known to signal stress (Roeder et al. 2005). Thus, stress-stimulated elevated cAMP levels might have been induced by handling during or prior to the experiment. An alternative explanation is that elevated
intracellular cAMP levels in OA/TA neurons simply do not affect larval locomotor behavior. Furthermore it is noteworthy to mention that Pacα and bPac change intracellular signaling by altering cAMP levels rather that change neuronal excitability. Thus, the general potency of light inducible adenylyl cyclase (and probably for many more effector genes) seems to be highly dependent on the properties of the target neurons.

UAS-TRPM8 was expressed pan-neuronally using elav-Gal4 to test its functionality to different temperature decrements (Peabody et al. 2009). Here 100% of flies felt down after temperature shift from 24°C to ~15°C within 2.5 minutes using one or three copies of UAS-TRPM8, respectively. The mildest shift to 18°C was already sufficient to induce immobility in 60% of male flies, but not in female flies (Peabody et al. 2009). In our study we used a temperature shift from ~23°C to ~16°C which was not sufficient to affect either larval locomotion or larval light avoidance behavior. Moreover, UAS-TRPA1 and UAS-TRPM8 were used to screen and identify neurons controlling motor output in adult flies (Flood et al. 2013). Surprisingly, both transgenic lines induced contrary results driven by the same Gal4 lines, most probably based on differences in action potential frequency (Hamada et al. 2008; Peabody et al. 2009).

**Benefits and drawbacks of effector gene use in larval Drosophila**

In summary, UAS-hid,rpr, UAS-sh♯ and UAS-Kir2.1 seemed to be the most potent effector lines to impair neuronal transmission, since with these effector genes dark preferences of tested larvae were indistinguishable from chance level. UAS-TRPA1 turned out to be most efficient in activating photoreceptor neurons as also here dark preferences dropped to zero, while the efficiency of ChR2 variants to manipulate photoreceptor neurons was not tested in this study. In the locomotion assay, UAS-TRPA1 and UAS-ChR2-XXL seemed to be equally capable to induce neuronal activation.

While cell ablation is the crudest way to manipulate neuronal signaling, it comes with the plus that the efficiency of cell ablations can easily be assessed by antibody staining. Electrical synapses can be modulated by the expression of the rectifier potassium channels UAS-Kir2.1 and UAS-ΔOrk. Both lines are available as a GFP-tagged version (Baines et al. 2001; Nitabach et al. 2002; Supplementary Figure 1, 2) allowing fluorescent detection to verify the ectopic expression of rectifier potassium channels
and direct labeling of the manipulated neurons. In addition, UAS-ΔOrk is also available in a non-conducting version (Nitabach et al. 2002) to serve as a suitable genetic control. Both UAS-Kir2.1 and UAS-ΔOrk affected – albeit to a slightly different extent – larval locomotion and rapid light avoidance, indicating both to be a suitable choice. The largest benefit of UAS-shi is the possibility to induce fast and reversible conditional synaptic block by high temperatures. This eliminates possible developmental and adaptation effects. The same benefit applies for the usage of UAS-TRPA1 and UAS-TRPM8 (if functional in a given experiment). Also UAS-ChR2 variants and UAS-Paca or UAS-bPac (if functional in a given experiment) can be expressed to specifically activate neurons or neuron populations only during the experiment and thereby omit developmental side effects.

While all light and temperature inducible effector genes share the benefit of spatiotemporal conditional usage within the experiment, the larval locomotion experiment revealed disadvantages of these thermo- and optogenetic tools. Light and especially higher temperature during the experiment changed naïve behaviors in larvae. In addition, heat-inducible effector genes are less suitable to investigate temperature-dependent behaviors in Drosophila. The same is true for the combination of light-dependent behaviors and light-inducible effector genes such as UAS-ChR2 variants and UAS-Paca or UAS-bPac. It should also be kept in mind that all effector genes studied here share the caveat that the behavioral readout after their usage gives no indication about the identity of the transmitter underlying the observed effects. For example, biogenic amines and neuropeptides are often co-expressed with classical neurotransmitters like acetylcholine or GABA (Nässel and Homberg 2006; Nässel 2009). In these cases, neuronal manipulation will likely affect the release of both biogenic amines/neuropeptides and classical transmitters. On the other hand, there is also evidence for differences between the molecular release mechanisms of amine/peptide-containing dense core vesicles and small transmitter-containing synaptic vesicles (e.g. Renden et al. 2001, Park et al. 2014), and biogenic amine and neuropeptide release may not be restricted to active synaptic zones (e.g. Karsai et al. 2013). This may explain the inefficiency of TNTE in OA/TA neurons (Sweeney et al. 1995). Thus, to fully understand the function of defined neurons within a neuronal network, it is essential to identify the functional signaling substance for a certain behavior.
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COMPETING INTERESTS

The authors declare no competing interests

AUTHOR CONTRIBUTIONS


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**Supplementary Figure 1**: Labeling of Tdc2 by anti-Tdc2 after expression of UAS-\textit{grim} (A-C), UAS-\textit{rpr} (E-G), UAS-\textit{hid,rpr} (I-K) and UAS-\textit{DTI} (M-O) in \textit{Tdc2-Gal4} positive neurons. Despite expression of pro-apoptotic genes in all cases, a small number of OA/TA neurons escaped apoptosis (A, E, I). Weak expression of Tdc2 was remarkable after expression of UAS-\textit{DTI} (M). Expression of 10xUAS-\textit{myr::GFP} to confirm the efficiency of cell ablation and protein synthesis inhibition with the Gal4 expression pattern as anti-Tdc2 antibody might label neurons not included in the expression pattern of \textit{Tdc2-Gal4} (D, H, L, P). Scale bars: 50\mu m

**Supplementary Figure 2**: Bright field image of the cephalopharyngeal skeleton including the Bolwigs organ (BO) and close up of the BO (A’;B’;D’;E’;G’;H’;J’;K’;M and N) labeled with anti-Elav (red) and anti-Chp (green; A-L). Expression of UAS-\textit{grim} (C), UAS-\textit{rpr} (F), UAS-\textit{hid,rpr} (I) and UAS-\textit{DTI} (L) driven by \textit{IGMR-Gal4}. The expression of pro-apoptotic genes led to a complete loss of photoreceptor cells in all cases. Expression of UAS-\textit{Kir 2.1} and UAS-\textit{\Delta Ork}, labeled by GFP under the control of \textit{IGMR-Gal4} (M-N). Scale bars: black: 20 \mu m; white: 5\mu m.

**Supplementary Table 1**: List of publications using different effector gene combinations to manipulate different types of neurons and behaviors in the \textit{Drosophila} larva to the best of our knowledge.