Differential Potencies of Effector Genes in Adult *Drosophila*

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ABSTRACT

The GAL4/UAS gene expression system in *Drosophila* has been crucial in revealing the behavioral significance of neural circuits. Transgene products that block neurotransmitter release and induce cell death have been proved to inhibit neural function powerfully. Here we compare the action of the five effector genes *shibire*^{ts1}, *Tetanus toxin light chain (TNT)*, *reaper*, *Diphtheria toxin A-chain (DTA)*, and inwardly rectifying potassium channel (*Kir2.1*) and show differences in their efficiency depending on the target cells and the timing of induction. Specifically, effectors blocking neuronal transmission or excitability led to adult-induced paralysis more efficiently than those causing cell ablation. We contrasted these differential potencies in adult to their actions during development. Furthermore, we induced TNT expression in the adult mushroom bodies. In contrast to the successful impairment in short-term olfactory memory by *shibire*^{ts1}, adult TNT expression in the same set of cells did not lead to any obvious impairment. Altogether, the efficiency of effector genes depends on properties of the targeted neurons. Thus, we conclude that the selection of the appropriate effector gene is critical for evaluating the function of neural circuits.

Indexing terms: GAL4/UAS system; *shibire*^{ts1}; tetanus toxin; *reaper*; diphtheria toxin

Genetic modification in *Drosophila melanogaster* has been used to elucidate the functions of neural circuits in behavior (Sokolowski, 2001). The genes of choice ("effector genes") can conveniently and reproducibly be expressed in defined subsets of cells by using the GAL4/UAS system (Brand and Perrimon, 1993). The transcription factor GAL4, whose spatial and temporal expression is controlled by a flanking enhancer, determines the effector gene expression. Effectors that, for instance, block neurotransmitter release or induce cell death have been used to impair neural function (Brand and Dormand, 1995; Roman, 2004).

In addition to the spatial, temporal, and quantitative specificity of the GAL4 expression, conclusions from this approach depend on our understanding of processes induced by effector genes in the respective target cells. Thus, we quantitatively compared the influences of the five effector genes *shibire*^{ts1} (Kitamoto, 2001), *Tetanus toxin light chain (TNT)*; Sweeney et al., 1995), *reaper (rpr)*; White et al., 1996), *Diphtheria toxin A-chain* (Han et al., 2000), and human inward rectifying potassium channel (*Kir2.1*; Baines et al., 2001), in different cell types and at various developmental stages.

Misexpression of *shibire*^{ts1} (*shi*^{ts1}), a temperature-sensitive dominant negative Dynamin, blocks normal en-

docytosis for synaptic vesicle recycling, thereby causing an impairment of synaptic transmission (Kitamoto, 2001, 2002). Because of the temperature sensitivity of *shi*^{ts1}, synaptic output can be blocked by raising the temperature. This temperature-induced block can recover synaptic transmission within 1 minute by shifting back to the permissive temperature (Koenig and Ikeda, 1989; Kitamoto, 2002).

Another way of inhibiting neurotransmitter release is by expressing *TNT*, a protease specifically cleaving neuronal synaptobrevin (n-Syb; Sweeney et al., 1995; Martin et al., 2002). n-Syb is essential for neurotransmitter release, in that it regulates Ca²⁺-dependent fast synaptic

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vesicle fusion (Kidokoro, 2003). Despite the successful application of these two effector genes that mainly block chemical synapses (Kitamoto, 2002; Martin et al., 2002), electrical synapses, for example, are supposed to remain unaffected (White and Paul, 1999; Phelan and Starich, 2001).

Therefore, genetic ablation is an alternative intervention method; it can act regardless of synapse type (Sweeney et al., 2000). Ectopic *rpr* expression induces apoptosis by activating the caspase proteolytic cascade that finally leads to DNA fragmentation and chromatin condensation (White and Steller, 1995; Bergmann et al., 2003; Hay et al., 2004). Diphtheria toxin A (DTA), in turn, is an inhibitor of protein synthesis by ribosylating elongation factor-2 (Wilson and Collier, 1992). The toxicity of DTA is extreme; one molecule per cell is thought to be sufficient to cause cell death (Yamaizumi et al., 1978). Therefore, we used the attenuated mutant I of DTA (DTI) for cell ablation (Bellen et al., 1992; Han et al., 2000).

Another alternative method that can block neuronal activity regardless of the synapse type is electrical silencing (Baines et al., 2001; Nitabach et al., 2002). In *Drosophila*, the expression of the human inwardly rectifying potassium channel Kir2.1 hyperpolarizes neurons, thereby efficiently blocking action potential generation (Baines et al., 2001). In mammalian neurons, temporally controlled Kir2.1 expression can block neuronal excitability (Johns et al., 1999).

To examine adult behavior, it is often necessary to induce the appropriate effector gene specifically in the adult. A temperature-sensitive mutant of *GAL80* (*GAL80^{ts}*) has been introduced to control the *GAL4/UAS* gene expression system temporally (McGuire et al., 2003). *GAL80^{ts}* has been shown to suppress *GAL4* transactivation at 18°C but to become inactive at 30°C (McGuire et al., 2003). Thus, we prepared flies carrying both the effector gene and *GAL80^{ts}*, which allowed us to control temporally and spatially the expression of *TNT*, *rpr*, *DTI*, or *Kir2.1*.

By using this technique, we investigated the potency of the effectors in adult flies with different broadly expressing driver lines. Induction of paralysis by these effectors has been examined as a simple measure of the behavioral consequences. We contrasted this adult expression of the effectors with their actions during development. To examine further the interfering activities of *TNT* and *Shi^{ts1}* in a restricted subset of neurons, we targeted them to the mushroom bodies and tested these flies for olfactory learning. Formation of associative olfactory memory requires the mushroom bodies (for review see Heisenberg, 2003). These experiments indicated that the actions of effectors vary depending on the target cells. Thus, the selection of the appropriate effector genes is critical for evaluating the function of the neural circuit.

MATERIALS AND METHODS

Fly stocks and maintenance

All fly stocks were reared on standard *Drosophila* medium (cornmeal, agar, molasses, yeast, nipagin) under a constant light and dark cycle (14/10 hours) at 25°C and 60% relative humidity. Employed *GAL4* lines included *Act5C-GAL4* [II] (Ito et al., 1997), *elav-GAL4* [X] (Lin and Goodman, 1994), *D42* [III] (Yeh et al., 1995), and *MB247* [III] (Zars et al., 2000). Employed fly stocks carrying ef-

fector genes included *UAS-shi^{ts1}* [III] (Kitamoto, 2001), *UAS-TNT* [II; TNTE] (Sweeney et al., 1995), *UAS-rpr* [X] (Aplin and Kaufman, 1997), *UAS-DTI* [II] (Han et al., 2000), and *UAS-EGFP-Kir2.1* [II] (Baines et al. 2001). To control effector gene expression temporally, we generated fly strains carrying *Tub-GAL80^{ts}* [III] with the respective effector genes (except for *shi^{ts1}*), where *GAL80^{ts}* expression is ubiquitously driven by the *Tubulin* promoter (McGuire et al., 2003). All fly stocks have *w¹¹¹⁸* in their background; *w¹¹¹⁸* flies were used for all control crosses.

Behavioral assays

Adult paralysis and recovery. For all experiments, 50 virgin females were crossed with 20 males and kept to adulthood at 18°C. Groups of either 10 or 20 adult F1 flies were transferred 2 days after eclosion to fresh food vials by using an aspirator and were left there to recover overnight at 18°C. Flies were not treated with CO₂ to avoid potential negative effects of anesthesia. On the next day, these vials were placed in a 30°C incubator. To measure the paralytic effect of the effector genes, the numbers of immobilized flies were counted after gentle bumping of the vials at the given time points after the temperature shift. In experiments using *Act5C-GAL4/UAS-TNT*; *+Tub-GAL80^{ts}*, genders were separated before the experiment. Because both *elav-GAL4* and *UAS-rpr* are on the X chromosome, only the female progeny were examined. To assess the reversibility of the effector actions, fly vials in a 30°C incubator were shifted back to 18°C.

Temperature shift during development. Crosses were cultured at 18°C. Twenty-third-instar (wandering) larvae were collected with a brush and transferred to a new food vial. To induce effector gene activity, food vials containing collected animals were transferred to 30°C for 24 hours at three different developmental time points: third-instar larva (directly after stage selection), early pupa (pupal stages P1–P2), or late pupa (pupal stages P10–P12). After induction at 30°C, vials were placed again at 18°C and the numbers of eclosed adults were scored. Each measurement was repeated at least six times.

Olfactory associative learning. Standard Pavlovian training procedures reinforced by electric shock in a modified T-maze apparatus were applied (Tully and Quinn, 1985; Schwaerzel et al., 2002). A group of ~100 flies was trained by receiving a first odor for 1 minute in the presence of 12 pulses of electric shocks (90 V DC). After 1 minute of fresh air, the tube was scented for an additional minute with a second odor but without electric shock, followed by another period of 45 sec of air. The memory test started ~100 sec after the training trial by shuttling the flies into a choice point between the previously punished odor and the control odor. They were given 2 minutes to choose one of them. To measure associative learning, a reciprocal experimental design was employed: two groups of flies received either odor A with shock and B without or odor B with shock and A without. For both groups, the preference between odors A and B was measured after training. The learning index was then calculated by taking the mean preference of the two reciprocally trained groups (Tully and Quinn, 1985). Benzaldehyde or 3-octanol was used as odor presented in a cup of 5- or 14-mm diameter, respectively.

TABLE 1. Summary of Adult Paralysis: Time after the Onset of Expression to Immobilize 50% of the Adult Flies

	<i>Act5C</i>	<i>elav</i>	<i>D42</i>
<i>TNT; GAL80</i>	15 hours (♀) 6.5 hours (♂)	7.5 hours	9 hours
<i>shi^{ts1}</i>	— ¹	22 minutes	2.5 hours
<i>rpr; GAL80</i>	>5 days ²	>5 days ² (♀)	>5 days ²
<i>DTI; GAL80</i>	2.5 days	3.5 days	4.5 days
<i>Kir2.1; GAL80</i>	16 hours	15 hours	17.5 hours

¹No adult fly is available because of lethality in early development at 18°C.²No significant difference from control flies in the measured period (5 days).

Immunohistochemistry

Whole-mount preparation. Microdissection was performed in Ringer's solution to remove cuticle and connective tissues. The brains were fixed in phosphate-buffered saline containing 0.3% Triton X-100 (PBT; Sigma, St. Louis, MO) containing 4% formaldehyde for 2 hours at room temperature and subsequently rinsed with PBT three times. Blocking of samples was performed in 3% normal goat serum (Sigma) in PBT for 1 hour. The brains were incubated with the primary antibodies in the blocking solution at 4°C overnight. Mouse monoclonal anti-synapsin (3C11) for labelling presynapses (1:10; Klagges et al., 1996), rabbit polyclonal anti-GFP (1:1,000; Molecular Probes, Eugene, OR; A6455), or mouse monoclonal anti-TNT (1:400; courtesy of T. Binz, Hannover) was used as a primary antibody. The monoclonal antibody against the first open reading frame of the *Drosophila* Synapsin protein was previously shown to recognize multiple Synapsin isoforms on Western blots with wild-type heads, which were not detected in the deletion mutant (Klagges et al., 1996; Godenschwege et al., 2004). Consistently, the antibody did not give a signal on cryosections of the *synapsin* mutant brain, although it clearly stained the synaptic neuropils in the wild-type fly (Klagges et al., 1996; Godenschwege et al., 2004). The rabbit anti-GFP antiserum was raised against the wild-type GFP isolated directly from *Aequorea victoria* (according to the manufacturer's information). The antiserum specifically stains the transgenic GFP in the GAL4-expressing cells (see Fig. 3A). The specificity of the monoclonal antibody raised against the clostridial TNT light chain was verified in *Drosophila* by detecting transgenic TNT expression specifically in the targeted cells (Sweeney et al., 1995; see Fig. 3C). Samples were washed four times for 10 minutes with PBT. The brains were incubated with secondary antibodies in the blocking solution at 4°C overnight. Alexa Fluor 488- or Cy3-conjugated goat anti-rabbit or anti-mouse, respectively, was used to detect the primary antibody. After four 10-minute rinses with PBT, brains were mounted in Vectashield (Vector, Burlingame, CA). The experimental and control brains were stained in parallel under the same conditions. Confocal image stacks were taken with a Leica SP1. A stack of images was collected at 1-μm steps with a ×40 objective. Images of the confocal stacks were projected and analyzed with the software Image-J (NIH). The adjustments to contrast and brightness as well as rotations and organization of the images were performed in Photoshop (Adobe).

Agarose sections. Fly brains were fixed overnight at 4°C in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.0; Sigma, Steinheim, Germany). They were

TABLE 2. Adult Paralysis of Control Flies¹

Genotype	Percentage of motile flies 27 hours after temperature shift to 30°C
<i>Act5C/+</i>	100 ± 0.0
<i>elav/+</i>	100 ± 0.0
<i>D42/+</i>	100 ± 0.0
<i>TNT/+; GAL80</i>	99.0 ± 0.9
<i>shi^{ts1}/+</i>	96.0 ± 2.2
<i>rpr/+; GAL80</i>	98.3 ± 1.1
<i>DTI/+; GAL80</i>	95.0 ± 3.1
<i>Kir2.1/+; GAL80</i>	100 ± 0.0

¹The numbers represent the means ± SEM (%) based on 10 to 12 independent experiments.

embedded in 8% agarose and sectioned every 80–90 μm with a vibratome (Leica, Nussloch, Germany). The sections were washed for 5 × 20 minutes in PBT and then blocked with 7% normal goat serum in PBT for 1 hour. Sections were incubated overnight at 4°C with a polyclonal rat anti-n-Syb antibody (1:1,000; Wu et al., 1999). The rat polyclonal antiserum (R29) was raised against the full-length cytoplasmic domain of the *Drosophila* n-Syb protein (Wu et al., 1999). It stains a band of 32-kDa molecular weight on Western blots of the synaptic vesicle-enriched fraction but not of the cytosolic fraction (Fabian-Fine et al., 2003). Samples were washed for 5 × 20 minutes in PBT and incubated with a secondary antibody, Alexa 488-conjugated goat anti-rat (1:200; Molecular Probes), at 4°C overnight. Finally, sections were washed for 2 × 20 minutes in PBT and 4 × 20 minutes in PBS, and then embedded in Entellan (Merck, Darmstadt, Germany). Confocal image stacks were collected by using a Leica confocal microscope (see above under Whole-mount preparation).

RESULTS

Adult-induced paralysis

To evaluate the potency of effector genes in living adult *Drosophila*, we expressed *shi^{ts1}*, *TNT*, *rpr*, or *DTI* with different GAL4 drivers. We first determined the paralysis resulting from the gene products by counting the fraction of motile flies after mechanical agitation. We used three driver lines targeting *GAL4* expression: 1) ubiquitously (*Act5C-GAL4*), 2) panneuronally (*elav-GAL4*), or 3) predominantly to motor neurons (*D42*). The neuronal effects can be evaluated by comparing the mutant phenotypes caused by the first two drivers. For all the effector genes except for *shi^{ts1}*, *Tub-GAL80^{ts}* was used to prevent effector gene expression during development. Most GAL4 driver lines have expression during development (Hayashi et al., 2002), which may lead to defects persisting to adulthood.

We induced effector gene expression (or the dominant negative form of the *Shi^{ts1}* protein) by raising the temperature to 30°C after rearing the flies at 18°C. Table 1 summarizes the results of the paralytic effect in combination with each GAL4 line. With *TNT*, *shi^{ts1}*, or *Kir2.1*, experimental flies with all tested GAL4 lines caused paralysis. The paralytic effect was quickest in *shi^{ts1}* and slowest in *Kir2.1* if *elav-GAL4* or *D42* was used (Table 1). In contrast, flies expressing *rpr* or *DTI* caused no or slow paralysis with any of the GAL4 drivers. All the control flies without *GAL4* or effector genes were measured in

Fig. 1. Temperature-dependent paralysis with three driver lines targeting *GAL4* expression to all cells (*Act5C-GAL4*), all neurons (*elav-GAL4*), or predominantly motor neurons (*D42*) for three different effector genes. Fractions of motile flies as a function of time after temperature shift to 30°C are indicated with mean $\pm$ SEM ($n = 2-17$). Controls either with all driver lines alone or effector genes together with *Tub-GAL80^{ts}* (except *UAS-shi^{ts1}*) alone are tested in parallel (Table 2). **A:** *UAS-TNT* together with *Tub-GAL80^{ts}* in combination with *D42* (solid triangles), *elav-GAL4* (open circles), and *Act5C-GAL4* (female: open squares; male: solid squares). **B:** *UAS-shi^{ts1}* in combination with *D42* (solid triangles), *elav-GAL4* (open circles). **C:** *UAS-*

rpr together with *Tub-GAL80^{ts}* in combination with *D42* (solid triangles), *elav-GAL4* (open circles), and *Act5C-GAL4* (solid squares). **D:** *UAS-DTI* together with *Tub-GAL80^{ts}* in combination with *Act5C-GAL4* (solid squares), *elav-GAL4* (solid circles), and *D42* (solid triangles). As controls, *UAS-DTI* together with *Tub-GAL80^{ts}* (open diamonds) and the three driver lines, *Act5C-GAL4* (open squares), *elav-GAL4* (open circles), and *D42* (open triangles) are tested alone as a heterozygote. **E:** *UAS-EGFP-Kir2.1* together with *Tub-GAL80^{ts}* in combination with *D42* (solid triangles), *elav-GAL4* (open circles), and *Act5C-GAL4* (open squares).

parallel with the experimental flies. They were still motile 1 day after the temperature shift (Table 2).

Induced paralysis by blocking chemical synapses

In the case of TNT, all *GAL4* drivers were able to induce paralysis after shifting the temperature to 30°C, although the onset of the mutant phenotype took hours (Fig. 1A). The three *GAL4* lines had different kinetics of paralysis, with their half-life at 30°C varying between 6.5 and 15 hours (Table 1). *D42* caused the paralysis similar to the other *GAL4* lines, so induction of TNT in the motor neurons appears to be sufficient to deprive the fly of motility. We also found a particularly pronounced difference be-

tween genders with *Act5C-GAL4*. For unknown reasons, TNT-induced paralysis of females took twice as long as that of males (Table 1).

In contrast to the kinetics of TNT action, *Shi^{ts1}*, as reported elsewhere (Kitamoto, 2001), caused quick paralysis at the restrictive temperature. At the permissive temperature (18°C) with *elav-GAL4* or *D42*, flies were able to survive to adulthood. Within several minutes after shifting the temperature to 37°C, all experimental flies were paralyzed (data not shown). This quick paralysis was not complete if flies were transferred to 30°C (Fig. 1B). Especially with *D42*, shifting the temperature to 30°C failed to cause the total paralysis even after several hours, proba-

TABLE 3. Adult Eclosion Rate (%) after the Temporary Effector Gene Expression in Development¹

Genotype	Time point of temperature shift		
	Third-instar larvae	Early pupae	Late pupae
Experimental			
<i>TNT/Act5C/GAL80</i>	0.0 ± 0.0*	0.0 ± 0.0*	2.0 ± 1.3*
<i>TNT/elav/GAL80</i>	0.0 ± 0.0*	1.3 ± 1.3*	0.1 ± 0.1*
<i>shi^{ts1}/Act5C</i>	N.D. (lethal before third instar)		
<i>shi^{ts1}/elav</i>	11.7 ± 3.1*	1.7 ± 1.7*	0.0 ± 0.0*
<i>shi^{ts1}/D42</i>	85.0 ± 5.6	93.3 ± 2.1	90.0 ± 2.6
<i>rpr/Act5C/GAL80</i>	22.2 ± 3.6*	31.3 ± 5.2*	88.8 ± 3.0
<i>rpr/elav/GAL80</i>	2.5 ± 1.6*	23.8 ± 3.8*	92.5 ± 1.6
<i>DTI/Act5C/GAL80</i>	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*
<i>DTI/elav/GAL80</i>	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*
<i>DTI/D42/GAL80</i>	0.0 ± 0.0*	0.0 ± 0.0*	0.0 ± 0.0*
<i>Kir2.1/Act5C/GAL80</i>	0.0 ± 0.0*	2.5 ± 2.5*	0.0 ± 0.0*
<i>Kir2.1/elav/GAL80</i>	24.0 ± 3.7*	21.7 ± 3.1*	2.5 ± 1.6*
<i>Kir2.1/D42/GAL80</i>	2.9 ± 1.8*	2.5 ± 1.6*	22.5 ± 6.5*
Control			
<i>Act5C/+</i>	88.5 ± 2.0	91.7 ± 1.9	92.1 ± 1.8
<i>elav/+</i>	88.9 ± 2.0	92.4 ± 1.8	91.3 ± 2.0
<i>D42/+</i>	87.0 ± 2.6	97.8 ± 1.5	96.0 ± 1.6
<i>TNT/+GAL80</i>	88.8 ± 2.6	91.2 ± 3.0	92.0 ± 3.1
<i>shi^{ts1}/+</i>	91.4 ± 1.4	96.7 ± 2.1	98.3 ± 1.7
<i>rpr/+GAL80</i>	94.0 ± 2.2	90.0 ± 3.8	77.5 ± 5.3
<i>DTI/+GAL80</i>	96.7 ± 2.1	96.7 ± 2.1	96.7 ± 2.1
<i>Kir2.1/+GAL80</i>	84.0 ± 3.1	85.0 ± 4.3	97.5 ± 1.6

¹Numbers represent the mean ± SE (%) based on six to 10 individual experiments.
**P* < 0.01 (ANOVA, followed by Duncan's post hoc test); each experimental group is compared with its control with either GAL4 or UAS effector alone.

bly because of incomplete block of neurotransmission. Also in *elav-GAL4/+;UAS-shi^{ts1}/+* flies, the half-life of total paralysis was 22 minutes (Table 1, Fig. 1B). Given the similar TNT-induced paralysis by *D42* and *elav-GAL4*, the blocking effect of *Shi^{ts1}* appears to be sensitive to the combination of the amount of the expressed protein (strength of the GAL4 driver) and the restrictive temperature.

shi^{ts1}-expressing flies with *Act5C-GAL4*, however, failed to survive to adulthood even at 18°C, whereas *elav-GAL4* and *D42* did not cause detrimental effects during development. No third-instar larvae of this genotype were found, so the lethal period must lie early in development (Table 3). Moreover, because the *shi^{ts1}* mutant is viable at 18°C (Koenig et al., 1983) and animals with neuronal expression of *shi^{ts1}* with *elav-GAL4* survived until adulthood (Table 3), high levels of nonneuronal expression of the *Shi^{ts1}* protein even at the "permissive" temperature seem to be detrimental. Overall, the effect of *shi^{ts1}* on survival may vary with cell type, expression level, and temperature.

Genetic ablation and electrical silencing in adult flies

TNT and *Shi^{ts1}* are supposed to block chemical synapses but not electrical synapses (White and Paul, 1999; Phelan and Starich, 2001). Because it may, for some applications, be desirable to block neuronal signalling completely irrespective of the synapse type, we attempted genetic cell ablation as well as electrical silencing.

Surprisingly, none of the driver lines in combination with *rpr* or *DTI* quickly induced paralysis upon induction of effector gene transcription (Fig. 1C,D). Flies were motile even 1 day after inactivation of GAL80^{ts} (Fig. 1C). Experimental animals were indistinguishable from control flies either with the driver or with *UAS-rpr* alone (Fig.

1C, Table 2). Even after 5 days of permanent induction with *Act5C-GAL4* or *D42*, the *rpr*-expressing flies were still indistinguishable from controls in terms of their motility (data not shown). This may be due to the insufficient amount of Rpr protein, insofar as Rpr is active only at high dosage (White et al., 1996).

The effect of DTI on motility was also measured for 5 days after the induction. No fast induced paralysis was achieved with DTI; experimental flies were still motile 2 days after inactivation of GAL80^{ts}, as were control flies (Fig. 1D). In contrast to *rpr*, continuous induction of the *DTI* expression for 5 days deprived motility of the experimental flies with all GAL4 drivers (Fig. 1D). However, keeping the flies at 30°C for 5 days also reduced the survival rate of the control genotype (Fig. 1D). Overall, adult-induced *rpr* or *DTI* is less applicable for adult behavioral analysis because the onset of the behavioral phenotype is slow.

Adult-induced electrical silencing by *Kir2.1* could block motility of the flies (Fig. 1E). One day after the temperature shift, more than 80% of the flies became immotile with all three tested drivers (Fig. 1E). The kinetics of paralysis were similar with different drivers (Fig. 1E), although the effect generally took longer than with the effectors blocking chemical neurotransmission (Table 1).

Effector gene action during development

Because *rpr* and *DTI* expression in the inducible GAL4/UAS system did not cause quick adult-induced paralysis, we tested their efficiency during development. We shifted the temperature to 30°C for 24 hours at three different developmental periods (for details see Materials and Methods): third-instar larva, early pupa, or late pupa (Table 3). Survival of individuals to adulthood was used as a measure of killing efficiency. For additional comparison, we simultaneously examined *TNT*, *shi^{ts1}*, and *Kir2.1* under the same experimental conditions.

Induction of DTI for 24 hours effectively killed the animals in all three developmental stages (Table 3). These results suggest that more protein synthesis might be required during development for neuronal function or viability, in that the same 24-hour temperature shift in the adult caused no visible effect.

Induction of *rpr* either in early pupae or in larvae also had significant effects on viability (Table 3; *P* < 0.01). In contrast, the effect of the *rpr* was indistinguishable from control when induced in late pupae (Table 3; *P* > 0.05). The potency of *rpr* induction in the third-instar larva was the strongest of the three tested time points (Table 3; *P* < 0.01, one-way ANOVA followed by Duncan's post hoc test). Despite the more restricted expression pattern, *elav-GAL4* was more effective than *Act5C-GAL4* in combination with *UAS-rpr* when induced in the third-instar larvae (Table 3; *P* < 0.01, *t*-test). These results, together with those using DTI, suggest that developing cells might be more sensitive to genetic cell ablation.

TNT expression with the *Act5C-GAL4* and *elav-GAL4* drivers killed nearly all animals in any of the three induction periods (Table 3). Likewise, transient *Kir2.1* expression efficiently interfered with development in all three induction periods, although in some cases it could not kill all animals (Table 3). Keeping the flies with neuronal *shi^{ts1}* expression at the restrictive temperature for 1 day was efficient in all three periods, although the animals survived if it was expressed with *D42* (Table 3). *Act5C-*

Fig. 2. Blocking chemical synapses in the mushroom bodies. **A:** GAL4 expression pattern in *MB247* monitored by *UAS-mCD8-GFP* (green). Projection of the anterior half of the brain, where the mushroom body neurons have distinct terminals, is shown. *MB247* directs GAL4 expression predominantly in the alpha/beta and gamma lobes. Presynapses in the brain are visualized with the vesicle protein synapsin (magenta). Scale bar = 50 μ m. **B,C:** Olfactory learning of flies expressing *TNT* or *shi^{ts1}* in the mushroom bodies. The mean learning indices (bars) were plotted with SEM (error bars). **B:** Compared with

control flies without *GAL4* (*TNT/+Gal80*) or *UAS-TNT* (*247/+*), adult-induced *TNT* expression (*TNT/247/Gal80*) caused no significant impairment of olfactory memory (NS: $P > 0.05$, one-way ANOVA; $n = 8$ for all three groups). **C:** As reported, *shi^{ts1}* expression with *MB247* (*247/shi*) showed substantial memory decrease when trained and tested at 32°C [$*P < 0.01$, one-way ANOVA, followed by the multiple comparisons Fisher's PLSD; $n = 8-10$]. The control animals without either GAL4 driver (*shi/+*) or *UAS-shi^{ts1}* (*247/+*) were tested in parallel.

GAL4/UAS-shi^{ts1} could not be measured because of the lethality at 18°C prior to the third-instar larva.

Recovery from paralysis?

To investigate whether reactivation of *GAL80^{ts}* can retract the action of induced effector genes, we examined the recovery from paralysis by shifting flies back to the permissive temperature. At the time point when about 50% of the flies were still motile, we transferred the flies back to 18°C. All motile flies with *TNT* or *Kir2.1* induction got paralyzed from the temperature shift, and no individual recovered from paralysis within 1 day. The effects of *TNT* or *Kir2.1* were therefore not reversible with any of the employed GAL4 drivers. The progressive effect of *TNT*, possibly because of the residual *TNT* synthesis and its stability, is in line with the long half-life of paralysis after *GAL80^{ts}* inactivation (Fig. 1A, Table 1). A similar account may apply to the irreversible action of *Kir2.1*.

In contrast to the irreversible effects of induced *TNT* or *Kir2.1*, flies carrying *UAS-shi^{ts1}* with *elav-GAL4* or *D42* quickly recovered from paralysis with few exceptions. As reported (Kitamoto, 2001, 2002), 15 minutes at 18°C restored locomotion after complete paralysis at 37°C. The experimental flies with *elav-Gal4* or *D42*, however, exhibited aftereffects even after extended periods (~1 hour) at low temperature. About 20% of the recovered flies had a held-out wing phenotype, supposedly resulting from an irreversible defect in the motor neurons driving flight control muscles. Thus, we conclude that the dominant negative effect of *Shi^{ts1}* protein can quickly revert in many, but probably not all, neurons.

Effect of *TNT* or *shi^{ts1}* expression in the mushroom bodies

The adult paralysis observed with *TNT* and *Shi^{ts1}* using broadly expressing drivers prompted us to test these ef-

factor genes on a certain neuron type in the adult central nervous system. We targeted their expression to the Kenyon cells of the mushroom bodies (MBs), and evaluated their efficiency by measuring olfactory associative learning. In this paradigm, flies are conditioned to avoid a specific odor by pairing it with electric shock punishment (Tully and Quinn, 1985). The MBs have been shown to be necessary for olfactory short-term memory (for review see Gerber et al., 2004). With the GAL4 driver *MB247*, we directed effector gene expression to the adult MBs. *MB247* has a predominant expression in the alpha/beta and gamma lobes of the MBs (Fig. 2A).

Induced *TNT* expression had caused complete paralysis by shifting the temperature to 30°C for 24 hours (Fig. 1A), so we used this temperature regime again and applied it right before the olfactory learning experiments. Temporal blocking of Kenyon cells with *shi^{ts1}* had been shown before to interfere with olfactory learning and memory (for review see Heisenberg, 2003).

In contrast to the successful induction of paralysis by *TNT* in motor neurons, adult-onset *TNT* expression in the MBs unexpectedly failed to show a significant decrement in short-term memory (Fig. 2B; $P > 0.05$, one-way ANOVA). For comparison, we used *UAS-shi^{ts1}* in combination with the same GAL4 driver, *MB247*. At the restrictive temperature (32°C) for 15 minutes before and during the learning experiments, *UAS-shi^{ts1}* showed the expected defect in short-term memory (Fig. 2C; $P < 0.01$, one-way ANOVA; Schwaerzel et al., 2002). This result confirmed the integrity of the tested GAL4 line. We reasoned the ineffectiveness of adult-onset *TNT* expression in the MB as follows: 1) the concentration of *TNT* protein might be too low, 2) n-Syb might not be proteolyzed despite the presence of *TNT*, 3) neurotransmission might take place without n-Syb.

Figure 3.

Level of TNT expression in the MBs

To exclude the possibility of an insufficient amount of TNT in the MBs, we monitored it immunohistochemically in the flies used for behavioral experiments. *MB247* is likely to direct effector gene expression to restricted subsystems of MBs revealed by membrane-tethered GFP (CD8-GFP; Fig. 3A,B). In frontal sections of the MB peduncle, where the organization of the MB subsystems can be well discerned (Strausfeld et al., 2003), the gamma lobe (crescent staining) and the exterior part of the alpha/beta lobe of *MB247* are strongly labelled (Fig. 3B). The expression of reporter is not detectable in the alpha'/beta' subsystem, and the core region of the alpha/beta lobe is only weakly labelled (arrowheads in Fig. 3A,B).

As expected from the paralysis experiments, the TNT protein induced by *MB247* was abundantly expressed in the MBs, including the presynaptic regions: the alpha/beta and gamma lobe (Fig. 3C). Similar to the distribution of CD8-GFP, the immunoreactivity of TNT was enriched in the gamma and exterior part of alpha/beta subsystem at the peduncle section (arrows in Fig. 3B,D). Thus, low concentration of TNT was unlikely to be the cause for the inefficiency in blocking the MB output.

Next, we investigated the possibility that flies could still learn normally because n-Syb might be protected from proteolysis by adult-induced TNT in the Kenyon cells. To analyze the peduncle of the MBs, where the gamma subsystem is readily discernible by its crescent shape (arrow in Fig. 3F), we immunohistochemically examined the n-Syb protein in the agarose-sectioned brains. The n-Syb protein is widely distributed in the brain, including the peduncle regions (Fig. 3E,F). The n-Syb expression in the gamma subsystem, where TNT was expressed primarily by *MB247* (arrow in Fig. 3H), was reduced after TNT

induction compared with the control region (arrowhead in Fig. 3H). The core region of the alpha/beta subsystem can serve as an internal control, in that *MB247* has less GAL4 expression there (arrowheads in Fig. 3B,D). Despite the n-Syb reduction after TNT induction, the residual staining in the targeted region (gamma lobe neurons) might be due to incomplete degradation. Alternatively, the presynaptic terminals from extrinsic MB neurons and/or intrinsic neurons that are not labelled in *MB247* might contribute to the faint staining. Taken together, these results imply that induced TNT can cleave and consequently degrade n-Syb in the presynapses of the targeted cells (as also shown by Sweeney et al., 1995). Thus, the resistance of olfactory learning to adult-induced TNT might be explained by a mechanism of neurotransmission without n-Syb. This transmission should be independent of electrical synapses, insofar as it can be blocked by *Shi^{ts1}*.

DISCUSSION

The GAL4/UAS gene expression system has contributed to revealing the cellular basis underlying many different behaviors (for review see Roman, 2004). The parametric analyses in this study showed that the tested effector genes (*TNT*, *shi^{ts1}*, *rpr*, *DTI*, and *Kir2.1*) exerted differential interfering activities. Each one merits consideration as a tool for functionally dissecting the neural circuitry but also has its shortcomings.

Adult-onset cell ablation would be an attractive tool, insofar as it could intervene in all functions irrespective of cell type. Before eclosion, *DTI* seemed to be cell lethal (Table 3). However, it took several days from inactivation of *GAL80^{ts}* to impair motility when induced in adult flies; *DTI* might be of use for studies of larval behavior (Table 3).

In contrast, *rpr*-dependent ablation turned out to be of limited use in adult flies. In developing cells, it was more effective, similarly to the previously reported successful examples after chronic expression (Table 3; McNabb et al., 1997; Busto et al., 1999; Renn et al., 1999). Late pupal and adult neurons seemed to be resistant to *rpr*-dependent cell ablation (Fig. 1C, Table 3). Only larval expression resulted in lethality (Table 3). The restricted developmental time window of the sensitivity to *rpr* expression has been reported with ubiquitous expression by a heat shock promoter (White et al., 1996). Adult cells are more resistant to killing by X-rays, so age-dependent processes such as cell cycle progression may be required for triggering efficient apoptosis. Insofar as *rpr* acts with other proapoptotic genes, such as *head involution defective*, a combination could be more effective also in the adult-induced neuronal interference (Zhou et al., 1997; Wing et al., 1998; Keller et al., 2002). Cell ablation would still be attractive for functional analyses of neural circuits, so an effector gene causing neurodegeneration could also be a better candidate for adult-onset cell ablation (Driscoll and Gerstbrein, 2003).

For the time being, *Kir2.1*, *TNT*, and *shi^{ts1}* are the more practical effector genes with which to study the role of neural circuits. Although the onset of the adult-induced *Kir2.1* expression was relatively slow, the temporal expression caused the expected phenotype in adults and during development with all three GAL4 expression patterns. It silences action potential generation regardless of synapse types, so it may serve as a useful method for tracing the neural substrates of specific behaviors. TNT

Fig. 3. Adult-induced TNT expression in Kenyon cells of the mushroom bodies. The brains in C, E, and G are magnified in D, F, and H, respectively (F and H: boxed regions in E and G, respectively). All brains are frontal views oriented dorsal-top (about 45° tilted to left in A and B). Arrows and arrowheads, respectively, indicate the gamma and a part of the alpha/beta subsystem with little *GAL4* expression in *MB247*. Brains are prepared as either whole mounts (A–D) or agarose sections (E–H). Confocal sections of the mushroom body peduncle (B,D,F,H) are taken at the depth of the fan-shaped body (FB) of the central complex. **A:** Confocal section at the plane of the medial lobes (β , β' , γ) of *MB247/UAS-mCD8-GFP* flies (color code as in Fig. 2A). The gamma subsystem (arrow, γ) has GFP staining, but staining in the core of the beta lobe (arrowhead) is weak. **B:** Confocal section of the peduncle of the mushroom body in the same brain as in A at the plane of the FB. The crescent staining at the periphery of the peduncle section corresponds to the gamma subsystem (arrow). The staining in the core of the alpha/beta subsystem (arrowhead) is weak, as seen in A. **C:** Confocal projection for the anterior half of the brain where expression of TNT protein by *MB247* was induced at the presynaptic regions after the inactivation of *GAL80^{ts}*. **D:** Confocal section of the peduncle. TNT in the core of the alpha/beta lobe is stained little (arrowhead). **E–H:** n-Syb antibody staining in the sections of the FB of the control brain without *GAL4* driver (E,F) and the brain where TNT was induced by *MB247* after inactivation of *GAL80^{ts}* (G,H). **E:** Projected image of the brain section of a control fly. **F:** Confocal section of the peduncle. n-Syb is present in the gamma (arrow) and the alpha/beta subsystems (arrowhead). **G:** Projected image of the brain section after the induction of TNT in the mushroom body. **H:** Confocal section of the peduncle. The crescent staining of for the gamma cells is reduced (arrow; $n = 4$). Scale bars = 25 μm in A; 20 μm in B,D; 30 μm in C; 50 μm in E,G; 15 μm in F,H.

also caused an adult-specific paralytic phenotype similar to that of *Kir2.1*.

In contrast to these successful examples of blocking neurotransmission, the TNT action can be conditional. Previous studies already showed that the action of TNT can differ according to cell type (see, e.g., McNabb et al., 1997; Renn et al., 1999; Kaneko et al., 2000). In addition to the cell type specificity of TNT efficiency, adult-induced TNT can have significantly different effects from the continuous expression. Although chronic expression of TNT had impaired MB function (Martin et al., 1998; Liu et al., 1999), adult-induced TNT, unlike *Shi^{ts1}*, failed to impair olfactory learning (Fig. 2B), despite its induced expression (Fig. 3).

n-Syb is a member of the SNARE complex that is essential for synaptic vesicle fusion (Deitcher et al., 1998) and is widely distributed throughout the nervous system (Fig. 3). Therefore, TNT had been supposed to block synaptic vesicle release efficiently in neurons. Bhattacharya et al. (2002), however, reported that the absence of n-Syb can be compensated by ectopic expression of *c-synaptobrevin*, which is abundant in nonneuronal cells. Moreover, Rister and Heisenberg (2006) have found that the TNT-mediated inactivation of n-Syb in photoreceptor synapses is limited to certain developmental periods. The adult-onset TNT expression failed to block photoreceptor neurotransmission. These results are in line with the insufficient block of MB neurons by adult-induced TNT, suggesting that not all chemical neurotransmission is acutely sensitive to TNT. These results imply that continuous depletion of n-Syb may be required to block neurotransmission in certain cell types. Altogether, adult-induced TNT expression might not always be sufficient to block the neurons with chemical synapses.

With *UAS-shi^{ts1}*, we confirmed most of the expected phenotypes. In addition to its action on endocytotic processes (Kosaka and Ikeda, 1983), Dynamin has been shown to act on the processing of other membrane vesicles, including focal exocytosis (Di et al., 2003). Moreover, the involvement of Dynamin in hormone secretion at the Golgi apparatus suggests an effect of *Shi^{ts1}* also on the secretion of neuropeptides (Yang et al., 2001). These findings imply that *Shi^{ts1}* can impair neurotransmission more efficiently than the depletion of the synaptic vesicles by blocking endocytosis (Kitamoto, 2001).

So far, no case has been reported in which a chemical synapse has not been blocked by *shi^{ts1}*. However, its ectopic expression causes nonneuronal early developmental lethality at 18°C (Table 3), and its effect in certain types of neurons may be irreversible. Moreover, the blocking activities depend on the restrictive temperature and the expression level (Kitamoto, 2002). Selecting an effector gene for a behavioral analysis may always require special considerations, although our study adds to many previous examples demonstrating the outstanding usefulness of *shi^{ts1}* (Kitamoto, 2002).

Finally, this study confirmed the effectiveness of *GAL80^{ts}* (McGuire et al., 2003). In the present examples, the developmental expression of TNT, *rpr*, *DTI*, or *Kir2.1* was effectively suppressed in the presence of *Tub-GAL80^{ts}*. Without temporal control, many GAL4 driver lines have been found to be lethal with TNT (Sweeney et al., 1995; Martin et al., 2002). Because driver-dependent expression during development is, in general, a continuous source of concern for adult behavioral analysis using

the GAL4/UAS system, the additional temporal control by *GAL80^{ts}* is a substantial advance.

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