Development/Plasticity/Repair

# Constitutive Activation of $Ca^{2+}$/Calmodulin-Dependent Protein Kinase II during Development Impairs Central Cholinergic Transmission in a Circuit Underlying Escape Behavior in *Drosophila*

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**Development of neural circuitry relies on precise matching between correct synaptic partners and appropriate synaptic strength tuning. Adaptive developmental adjustments may emerge from activity and calcium-dependent mechanisms. Calcium/calmodulin-dependent protein kinase II (CaMKII) has been associated with developmental synaptic plasticity, but its varied roles in different synapses and developmental stages make mechanistic generalizations difficult. In contrast, we focused on synaptic development roles of CaMKII in a defined sensory-motor circuit. Thus, different forms of CaMKII were expressed with UAS-Gal4 in distinct components of the giant fiber system, the escape circuit of *Drosophila*, consisting of photoreceptors, interneurons, motoneurons, and muscles.**

**The results demonstrate that the constitutively active CaMKII-T287D impairs development of cholinergic synapses in giant fiber dendrites and thoracic motoneurons, preventing light-induced escape behavior. The locus of the defects is postsynaptic as demonstrated by selective expression of transgenes in distinct components of the circuit. Furthermore, defects among these cholinergic synapses varied in severity, while the glutamatergic neuromuscular junctions appeared unaffected, demonstrating differential effects of CaMKII misregulation on distinct synapses of the same circuit. Limiting transgene expression to adult circuits had no effects, supporting the role of misregulated kinase activity in the development of the system rather than in acutely mediating escape responses. Overexpression of wild-type transgenes did not affect circuit development and function, suggesting but not proving that the CaMKII-T287D effects are not due to ectopic expression. Therefore, regulated CaMKII autophosphorylation appears essential in central synapse development, and particular cholinergic synapses are affected differentially, although they operate via the same nicotinic receptor.**

## Introduction

Studies in numerous species have revealed a critical role for calcium/calmodulin-dependent protein kinase II (CaMKII) in the regulation of structural and functional synaptic plasticity and the function of ion channels and receptors (Hudmon and Schulman, 2002b; Lisman et al., 2002; Griffith, 2004a,b; Yamauchi, 2005). $Ca^{2+}$/Calmodulin ($Ca^{2+}$/CaM) may be necessary for the initial activation of the enzyme, but autophosphorylation at conserved threonine 286 of the $\alpha$ isoform renders the kinase constitutively active (for review, see Hudmon and Schulman, 2002a). Similarly, the phosphomimic mutation T286D of the mammalian CaMKII renders the enzyme stably $Ca^{2+}$ independent. In contrast, the T286A mutation results in normal $Ca^{2+}$-dependent activity, which does not become $Ca^{2+}$ independent (Fong et al., 1989; Waldmann et al., 1990; Wang et al., 1998). Mutant CaMKIIs affect various aspects of neuronal development. For example, the constitutively active CaMKII slows dendritic growth in young optic tectal neurons (Wu and Cline, 1998) and retrogradely regulates the stabilization of retinal axons (Zou and Cline, 1996). T287D and T287A render the *Drosophila* kinase permanently calcium independent, mimicking autophosphorylation, or permanently calcium-dependent, respectively (Jin et al., 1998; Wang et al., 1998), and have been used to probe the role of $Ca^{2+}$ independence in cellular processes (Mehren and Griffith, 2004). Generally, pan-neuronal expression of CaMKII-T287D results in behavioral defects, failure of action potential propagation, and increases in the number of boutons in the larval neuromuscular junction (NMJ) without affecting the mechanics of neurotransmitter release (Park et al., 2002). Postsynaptic expression of CaMKII-T287D promotes strengthening of excitatory synaptic currents and expansion of presynaptic terminals in the developing NMJs (Kazama et al., 2003, 2007), but in older larvae (Morimoto-Tanifuji et al., 2004) it alters the density of glutamate receptors without changing the amplitude of excitatory junc-

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tional potentials (Haghighi et al., 2003; Morimoto et al., 2010). Finally, transgenic flies expressing the *ala* peptide inhibitors of CaMKII exhibit altered synaptic transmission along with abnormal NMJ terminal sprouting (Wang et al., 1994). Furthermore, CaMKII inhibition resulted in associative learning and defects in adult flies (Griffith et al., 1994).

*In vitro* studies of *Drosophila* neurons revealed an inhibitory role of CaMKII on neurite branching (Broughton et al., 1996). In contrast, CaMKII expression in the dendritic arbors of sensory neurons increased the dynamic nature and formation of filopodia during development (Andersen et al., 2005).

In contrast to the extensive effects of CaMKII in developmental plasticity of central glutamatergic synapses (Wu and Cline, 1998), its role in central cholinergic synapse development and function remains elusive. Here, we employed the giant fiber system (GFS), a well characterized circuit in *Drosophila* (Allen and Murphey, 2007), as a model to express different mutant forms of CaMKII using the Gal4-UAS system in apparently distinct components of the GFS. Considering that the circuit uses D$\alpha$7 nAChR for excitatory neurotransmission (Fayyazuddin et al., 2006), we found that driving constitutively active CaMKII postsynaptically during synapse formation and stabilization precipitated differential defects in synaptic transmission at different central cholinergic synapses, and this likely underlies observed effects in associated behaviors.

## Materials and Methods

*Drosophila strains and culture*. Flies were raised on standard corn flour-yeast-agar medium at 24°C in a humidified incubator. The following fly strains were used:

(1) $w^{1118}$ as a genetic background line. Internal control lines were obtained by crossing $w^{1118}$ flies with the corresponding Gal4 or UAS line.

(2) Gal4 driver strains: the pan-neuronally expressed c155 ElavGal4 (Luo et al., 1994); the OK307Gal4, which is preferentially expressed in GFS neurons (Phelan et al., 1996; Allen et al., 1998); the D42Gal4 expressed in motoneurons (Yeh et al., 1995; Sanyal, 2009), and the cholinergic neuron-specific chaGal4 (Salvaterra and Kitamoto, 2001). The c17Gal4, which is preferentially expressed in the GF neurons, was a gift from M. Allen, University of Warwick, Coventry, UK (Allen et al., 1999). c380Gal4, which is preferentially expressed in motoneurons, was obtained from S. Sanyal, Emory University, Atlanta, GA (Budnik et al., 1996; Sanyal et al., 2003; Sanyal, 2009) The muscle-specific MefGal4 (Ranganayakulu et al., 1996) and 24BGal4 (Luo et al., 1994) were gifts from C. Zervas, Biomedical Research Foundation, Academy of Athens, Athens, Greece, while P103.3Gal4, expressed in the adult indirect flight muscle motoneurons among other cells, has been described previously (Consoulas et al., 2002). The c17;Gal80$^{ts}$ was constructed by standard genetic crosses.

UAS-CaMKII-T287D, which has a T287→D287 point mutation that renders the kinase calcium independent, UAS-CaMKII-T287A, which has a T287→A287 point mutation that renders the kinase incapable of becoming calcium independent (calcium-sensitive), and the wild-type (isoform R3) UAS-CaMKII-R3 (Jin et al., 1998; Wang et al., 1998) were gifts of L. C. Griffith, Brandeis University, Waltham, MA.

Adult 3- to 10-day-old males and females were used in our experiments.

UAS-CaMKII-RNAi, a partial CaMKII knock down (as Western blot analysis indicated), was obtained from Vienna Drosophila RNA Center (Vienna, Austria). UAS-ala2, an inhibitory CaMKII peptide, was obtained from Bloomington Stock Center (Indiana University, Bloomington, IN).

*The giant fiber system*. A schematic diagram of the GFS is shown in Figure 1*A*. Visual input evokes the sequential activation of most likely cholinergic neurons in lamina, medulla, and lobula. A bundle of columnar lobula neurons, the ColA interneurons, output to the bilaterally symmetrical pair of giant fiber (GF) interneurons. (Hausen and Strausfeld, 1980; Fischbach and Dittrich, 1989; Gilbert and Strausfeld, 1991). The cell bodies of these interneurons are in the protocerebrum and possess large diameter (~8 $\mu$m) axons that descend to the mesothoracic neuromere to output to follower neurons (King and Wyman, 1980; Koto et al., 1981; Allen et al., 1998). GF follower neurons are the tergotrochanteral motoneurons (TTMns) that innervates the tergotrochanteral muscle (TTM, jump muscle) and the peripherally synapsing interneuron (PSI), which outputs to five dorsolongitudinal muscle motoneurons (DLMns). DLMns supply the large indirect dorsal longitudinal muscles (DLMs, wing depressors) (King and Wyman, 1980; Ikeda and Koenig, 1988; Sun and Wyman, 1997; Koenig and Ikeda, 2005).

The escape (jump and flight) response is plastic, presenting characteristics of habituation. The locus of this habituation response has not been defined, but it has to be searched at pathways afferent to GF (Engel and Wu, 1996, 1998; Engel et al., 2000). In contrast, the pathway efferent to GF (PSIs, motoneurons, and target muscles) delivers the signals stereotypically due to its unique architecture and synaptic connections (Tanouye and Wyman, 1980; Gorczyca and Hall, 1984).

There are two important issues that have to be taken into consideration when studying GFS physiology. First, both GF/PSI and GF/TTMn neuronal pairs communicate via a mixture of electrical and cholinergic synapses (Fig. 1*A*). However, genetically blocking gap junctions demonstrated that only the GF/TTMn chemical synapses participate in signal transmission (Thomas and Wyman, 1984; Baird et al., 1990; Allen and Murphey, 2007). Second, the two GF cells are interconnected at the dendritic level by a group of interneurons (giant commissural interneurons) as well as at the level of their medial axonal collateral branches. The PSIs couple to one another, and TTMn couples to the ipsilateral PSI (King and Wyman, 1980; Phelan et al., 1996; Jacobs et al., 2000). Thus, when the visual input is strong enough to initiate action potentials in GFs, then the architecture and type of connections of the pathway efferent to GF guarantees the transmission of signal to the muscles. Therefore, muscle recordings can be used as readouts of GF action potentials.

*Electrophysiological preparation and recordings*. Flies were anesthetized briefly with $CO_2$ and glued to a thin metal wire attached to the neck with cyanoacrylate adhesive. Flies were allowed to recover from anesthesia for 30 min. To stimulate electrically the GF neurons or thoracic motoneurons, a pair of uninsulated tungsten electrodes was used to penetrate the eyes or thorax, respectively. A similar electrode was used to record from the DLM (fibers 5 and 6) or TTM. A fourth tungsten reference electrode was placed into the scutellum or the abdomen (Tanouye and Wyman, 1980; Engel and Wu, 1992; Allen et al., 2000). Experiments involving the sensory to GF pathway, which is known to be amenable to habituation and more vulnerable to anesthesia, were carried out with flies that had recovered for at least 1 h (Engel and Wu, 1996).

Brain or thoracic stimulation was performed by delivering stimuli (0.15 ms in duration) with a Grass S88 stimulator (Grass Technologies), while DLM or TTM muscle action potentials were acquired in the frequency range (300 Hz to 10 KHz) and amplified 100× by a differential AC amplifier (model 1700; A-M Systems). Data were digitized with an analog-to-digital converter (Digidata 1200; Molecular Devices) without filtering and were analyzed and displayed with Clampex 8.1 version software (Molecular Devices).

The function of the pathway efferent to GFs can be studied satisfactorily by estimating three parameters. (1) Short latency response (SLR), an estimation of the time required for the signal, is initiated by electrical stimulation in the GFs to activate the target muscle. Five recordings were acquired to estimate the average response latency of each fly. To assure that giant fiber neurons were stimulated directly and not synaptically, the stimulus intensity was set to 15–20 V, sufficiently higher than the short latency response threshold. (2) Refractory period (RP) is a measure of the minimum time interval between a pair of stimuli that can successfully generate corresponding muscle responses. (3) Following frequency 50% ($FF_{50}$), a measure of GFS synaptic fidelity, was tested in this case by recording the number of evoked muscle action potentials in response to three high-frequency stimulation episodes (Tanouye and Wyman, 1980; Engel and Wu, 1992). For long-latency responses (LLRs), GF neurons were activated synaptically (indirect GF stimulation). Their activation was achieved by low strength (7–9 V) electrical pulses (Engel and Wu, 1996) delivered through electrodes positioned in more external eye lay-

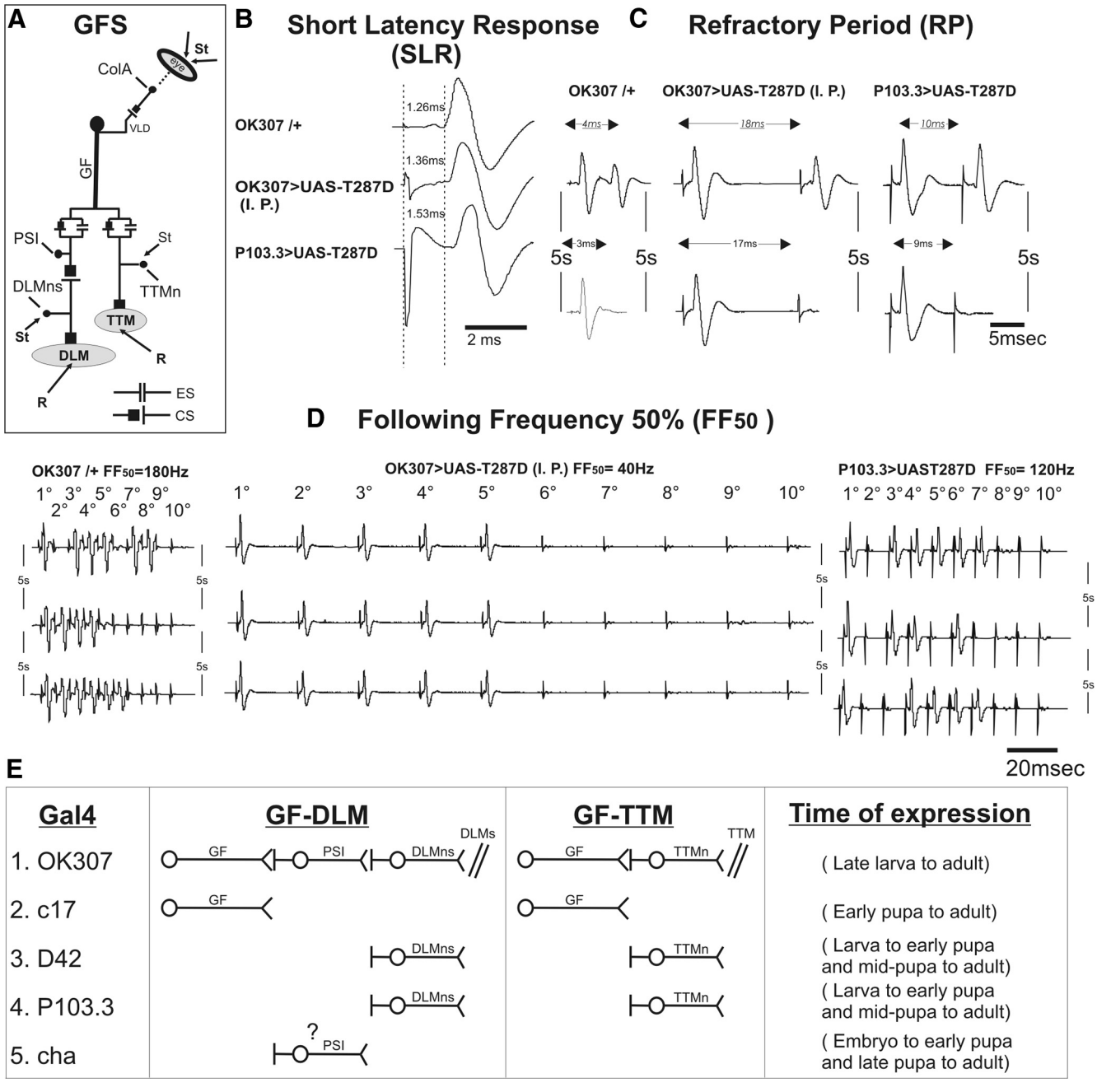


**Figure 1.** ***A***, GFS schematic depiction (for details, see Materials and Methods). ***B***, Representative DLM action potentials after GF stimulation with a 0.15 ms voltage pulse across the brain. The short latency response is the interval between the stimulus artifact and the onset of the initial phase of the muscle potential, as indicated by dotted lines. ***C***, ***D***, Refractory period (***C***) and following frequency of 50% (***D***) were measured in control flies and in flies expressing CaMKII-T287D. ***C***, The RP is the shortest interstimulus interval time for which at least one double response occurred. ***D***, The $FF_{50}$ was estimated by applying 3 trains of 10 stimuli each at a given frequency, with 5 s interval between trains, and taking the frequency value at which 50% of responses were successful (15 successful responses out of 30 stimuli). ***E***, Schematic representation of spatial and temporal Gal4 expression in identified efferent GFS neurons. cha-Gal4 driver is presumably expressed in PSI (question mark). CS, Chemical synapse; ES, electrical synapse; GF, giant fiber; R, recording site; St, stimulation site.

ers. The long-latency response pathway was estimated as the mean average of five LLR measurements for each fly. To avoid habituation of the response, recording trials were separated by 5 min intervals.

*Confocal microscopy*. Brain and ventral nerve cord were dissected and fixed in 2.5% paraformaldehyde in PBS, pH 7.3, for 2–3 h at room temperature in the dark. Preparations were washed in PBS and mounted in Vectashield. Digital images were captured on a Leica TCS SP5 (Leica Mikrosysteme Vertrieb) laser scanning confocal microscope using Leica Application Suite AF (Leica Microsystems Confocal Imaging) image acquisition software suite. Preparations were scanned with an argon laser line with an excitation maximum at 488 nm (GFP). Images were assembled using Corel Draw 12 software (Corel).

*Behavioral assay*. Flies were tested for light-induced escape response in a custom-made apparatus according to Fayyazuddin et al. (2006). Flies were individually aspirated into a 9 × 2 cm Petri dish illuminated by four green light-emitting diodes (LEDs; peak wavelength, 572 nm) placed at 90° to each other. The lights-off stimuli were provided by turning off the LEDs for 20 ms using a custom circuit with an interrupter.

## Results

To study the role of CaMKII in GFS development and function, we used the Gal4-UAS system (Brand and Perrimon, 1993) to express CaMKII mutant transgenes under the control of neuro-

nal and muscle drivers. As for the mammalian CNS, CaMKII is distributed widely in the larval and adult *Drosophila* CNS also. The expression pattern of $Ca^{2+}$/calmodulin-dependent protein kinase II gene in the CNS of *Drosophila* is highly dynamic throughout development (Rachidi et al.,1999) and appears present in areas where GFS neurons are expected. Therefore, although we have not experimentally determined whether CaMKII is endogenously present within components of the GFS, its widespread expression pattern within the fly CNS suggests that this is highly likely. Because our data are derived from targeted expression of transgenes in the GFS, they do not necessarily reflect the native levels and distribution of CaMKII in the system, thus representing overexpression of the kinase. In addition and although unlikely based on the evidence above, our data may represent ectopic expression in particular components of the system.

CaMKII can exist in several activity states that depend on autophosphorylation. In its basal state, the enzyme is tightly regulated by $Ca^{2+}$/CaM, being inactive in its absence. After activation, the enzyme is autophosphorylated at T286 (or T287 for *Drosophila* CaMKII), becomes independent of $Ca^{2+}$/CaM, and maintains as much as 80% of its maximal $Ca^{2+}$-stimulated activity in the absence of $Ca^{2+}$/CaM (Hudmon and Schulman, 2002a,b). The switch between $Ca^{2+}$-dependent and $Ca^{2+}$-independent activity has been proposed to be an important early event in synaptic plasticity in a number of systems (Lisman et al., 2002). Mutating T287 of *Drosophila* CaMKII or T286 of the mammalian enzyme to aspartate (T287D for fly kinase), mimics autophosphorylation and results in a $Ca^{2+}$/CaM-independent, constitutively active kinase (Fong et al., 1989; Waldmann et al., 1990; Wang et al., 1998). In contrast, the T287 to alanine mutation results in a kinase that cannot be autophosphorylated and thus maintains normal $Ca^{2+}$-dependent but not regulator-independent activity (Mehren and Griffith, 2004).

We are targeting the GFS circuit with a number of Gal4 lines expressed in these neurons. We have verified, with one exception—the PSI neuron—that these drivers are expressed in the cells we are targeting (data not shown), but they are also likely expressed at lower or similar levels elsewhere in the CNS. Since we are specifically recording from the GFS, Gal4-mediated expression of the CaMKII transgenes outside these neurons should have minimal if any effect on the neurophysiological results, but it may have general effects such as embryonic or pupal lethality. CaMKII is a protein kinase with a broad spectrum of action, and potentially many common targets are shared among different cell types. Thus, we cannot exclude the possibility that GFS neurons are not indirectly affected by ectopic expression of CaMKII.

Interestingly, expression of the calcium-independent CaMKII-T287D (Jin et al., 1998; Wang et al., 1998), among the majority of the neuronal drivers used, yielded adults with deflated wings (infantile phenotype per Park et al., 2002). In these pharate-like animals the wings appear properly developed but remain deflated, suggesting that hemolymph was not actually pumped in wing veins, perhaps due to failure of the bursicon-induced pumping motor behavior (Peabody et al., 2009). In contrast, CaMKII-T287D expression in the musculature postsynaptic to motoneurons with two different Gal4 drivers resulted in lethality at the early pupal stage (Table 1).

**Table 1. Targeted expression of mutant CaMKII transgenes**

| Targeted expression | Wing phenotype |
|---|---|
| Expression in GFS | |
| *OK307(Gal4)>UAS-T287D* | Infantile/few wings + |
| *OK307(Gal4)>UAS-T287A* | Wings + |
| *OK307(Gal4)>UAS-CaMKII-R3* | Wings + |
| *OK307(Gal4)>UAS-CaMKII-RNAi* | Wings + |
| *OK307(Gal4)>UAS-ala2* | Wings + |
| *c17(Gal4)>UAS-T287D* | Wings + |
| Pan-neural expression | |
| *Elav(Gal4)>UAS-T287D* | Infantile |
| Expression in motoneurons | |
| *D42(Gal4)>UAS-T287D* | Infantile |
| *C380(Gal4)>UAS-T287D* | Infantile |
| *P103.3(Gal4)>UAS-T287D* | Wings + |
| Expression in dopaminergic cells | |
| *Ple(Gal4).F>UAS-T287D* | Curly infantile |
| Expression in dopaminergic and serotonergic neurons | |
| *Ddc(Gal4)4.3D>UAS-T287D* | Infantile |
| *Ddc(Gal4)4.36>UAS-T287D* | Infantile |
| Expression in cholinergic neurons | |
| *cha(Gal4)>UAS-T287D* | Wings +/few infantile |
| *cha(Gal4)>UAS-T287A* | Wings + |
| *cha(Gal4)>UAS-CaMKII-R3* | Wings + |
| Expression in GABAergic neurons | |
| *GAD(Gal4)>UAS-T287D* | Wings + |
| Expression in glutamatergic neurons | |
| *OK371(Gal4)>UAS-T287D* | Wings + |
| Expression in the muscular system | |
| *mef2(Gal4)>UAS-T287D* | No adults |
| *24B(Gal4)>UAS-T287D* | No adults |

### Calcium-independent CaMKII impairs cholinergic synaptic transmission in the motor part of the GFS circuit

To examine the role of CaMKII in the GF and neurons efferent to it, the OK307Gal4 driver was crossed to five CaMKII transgenes. The OK307 driver was chosen because it is expressed in the CNS, GFs, and all neurons efferent to GF (GF, PSI, GFS motoneurons), as well as weakly in the target muscles (Allen et al., 1998; Fig. 1*E*). Thus, it is a useful tool to screen for transmission defects throughout both GF-DLM and GF-TTM pathways. The first indication of an effect of the $Ca^{2+}$-independent form of CaMKII was derived by the manifestation of a wing defect. The majority of the OK307>CaMKII-T287D flies exhibited the infantile phenotype (unfolded wings) in contrast to all genotypes expressing the other transgenes, which displayed normal wings (Table 1; see Materials and Methods). We examined the function of GF-DLM and GF-TTM pathways in these transgenic animals by electrically stimulating the GF neurons in the brain and recording from the target muscles (distal DLM fibers 5 and 6 and TTM, respectively; Fig. 1*A*; Coggshall, 1978; Tanouye and Wyman, 1980; Ikeda and Koenig, 1988; Engel and Wu, 1992; Sun and Wyman, 1997). Because we were systematically recording from the distal DLM fibers 5 and 6, which are innervated by a single motoneuron (MN5; Consoulas et al., 2002), muscle recordings reflect the output of two identified motoneurons, MN5 and TTMn.

OK307>CaMKII-T287D animals with infantile phenotype exhibited defects in the function of the GF-DLM and GF-TTM pathways, especially under high-frequency operation. Direct (nonsynaptic) stimulation of GF neurons caused a ~0.14 ms delay in the generation of the action potential from the DLM (Figs. 1*B*, 2*A*), a threefold increase in RP (Figs. 1*C*, 2*B*) and a threefold decrease in the ability of neurons in this pathway to respond to repetitive high-frequency stimulation (Figs. 1*D*, 2*C*). The few OK307>CaMKII-T287D flies with normal wings exhibited the same defects as flies with infantile phenotype (Fig. 2*A–C*) with comparably altered RP and $FF_{50}$. Flies examined for GF-TTM pathway function exhibited defects in RP and $FF_{50}$ only (Fig. 2*G–I*).

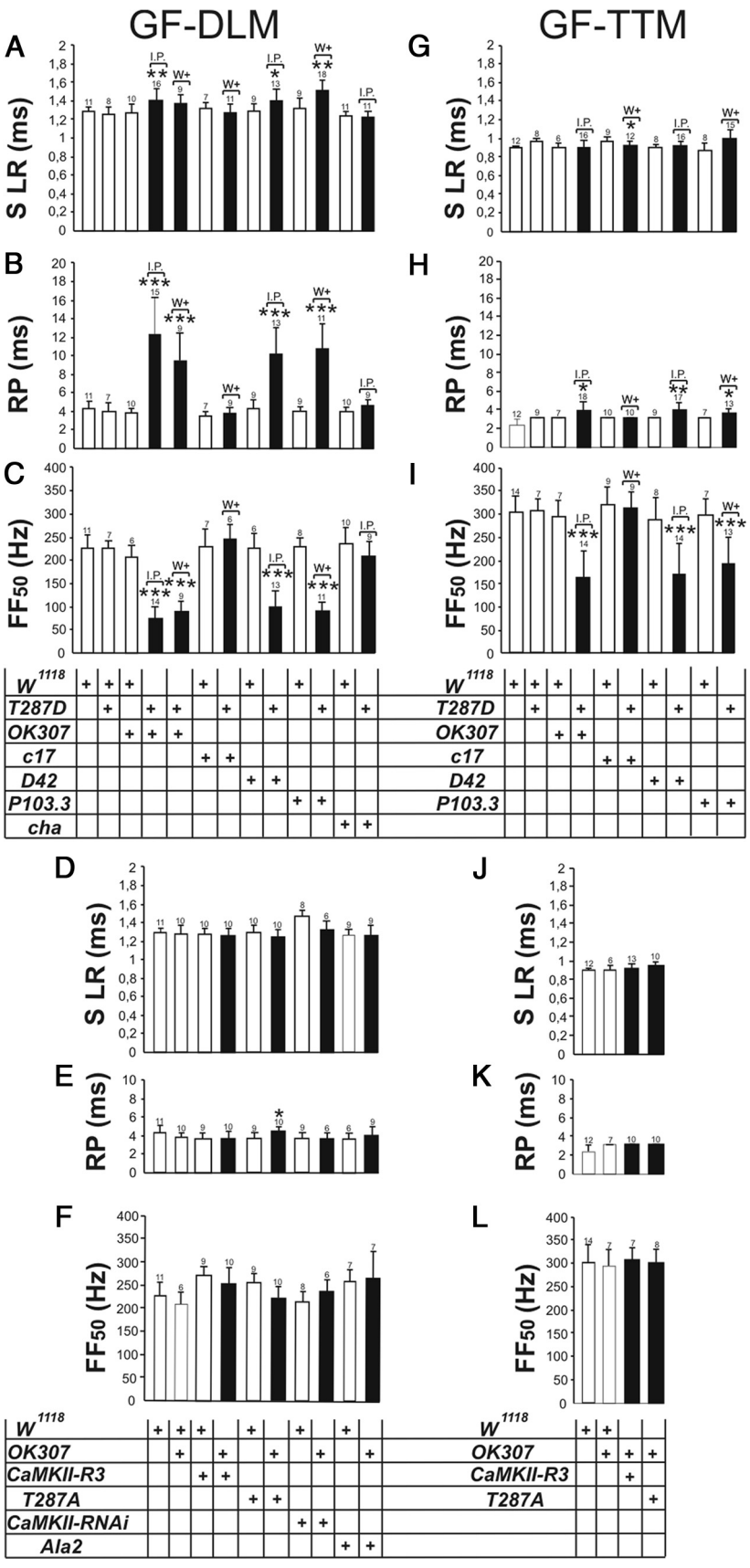


**Figure 2.** The roles of different mutant CaMKII transgenes on the three GFS electrophysiological parameters were examined for the GF-DLM (stimulation of GF and recording from DLM; ***A–F***) and the GF-TTM (stimulation of GF and recording from TTM; ***G–L***) pathways. Note that in the GF-DLM path, the constitutively active CaMKII-T287D expressed in all GFS neurons (OK307; black bars) or solely in GFS motoneurons (D42, P103.3; black bars) increases the SLR (***A***) and the RP (***B***), but decreases the $FF_{50}$ (***C***), while in the GF-TTM pathway only the last two parameters are affected (***H***, ***I***). On the contrary, the expression of the other transgenes does not affect the GFS parameters. White bars, Controls. Data are shown as mean ± SD. Numbers above the bars indicate the number of preparations tested and asterisks indicate *p* values from *t* test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) compared to the controls. I. P., Infantile phenotype, W+, normal wings.

Defects in $FF_{50}$ reflect synaptic dysfunction (Allen et al., 2007). Higher RP and lower SLR values can be explained by defects in axonal excitability or/and synaptic function. Thus, the transgenic $Ca^{2+}$/CaM-independent T287D-CaMKII may result either in synaptic or action potential propagation defects in both pathways. However, because changes in SLR in the GF-TTM pathway were not observed, the data suggest that the propagation was normal and the phenotype was likely due to synaptic delays.

In contrast to these findings, expression of the $Ca^{2+}$-dependent form CaMKII-T287A, which remains responsive to changes in intracellular $Ca^{2+}$ concentration, or the wild-type CaMKII-R3 in the GFS neurons did not affect GF-DLM and GF-TTM function (Fig. 2*D–F*, *J–L*). Similarly, no effects on GF-DLM and GF-TTM function were observed after partial abrogation of CaMKII by a RNAi-mediating transgene or block of CaMKII function with the synthetic competitive inhibitor ala2 peptide-encoding transgene (Fig. 2*D–F*). The lack of effects upon expression of the RNAi-mediating and *ala* transgenes could indicate lack of the kinase from the circuit. Importantly, the level of protein abrogation upon pan-neuronal expression of the particular RNAi-mediating transgenes was rather inefficient (data not shown), consistent with the lack of measurable effects. However, even if the RNAi or ala peptides did affect levels or activity of endogenous CaMKII, this could have resulted in improved functionality of the circuit, a situation we did not observe. Because excess protein that remains responsive to $Ca^{2+}$, either wild type or CaMKII-T287A, did not precipitate phenotypes, we suggest that even if ectopic within the circuit, over-accumulation of the $Ca^{2+}$-regulated kinase is not sufficient to perturb it functionally. Hence, we interpret these data as indication that $Ca^{2+}$-independent constitutive activation of CaMKII affects the function of the pathways efferent to GF. The locus of the defect could be at any of the chemical central cholinergic or/and peripheral glutamatergic (NMJ) synapses.

To identify the particular presynaptic or/and postsynaptic sites of CaMKII-

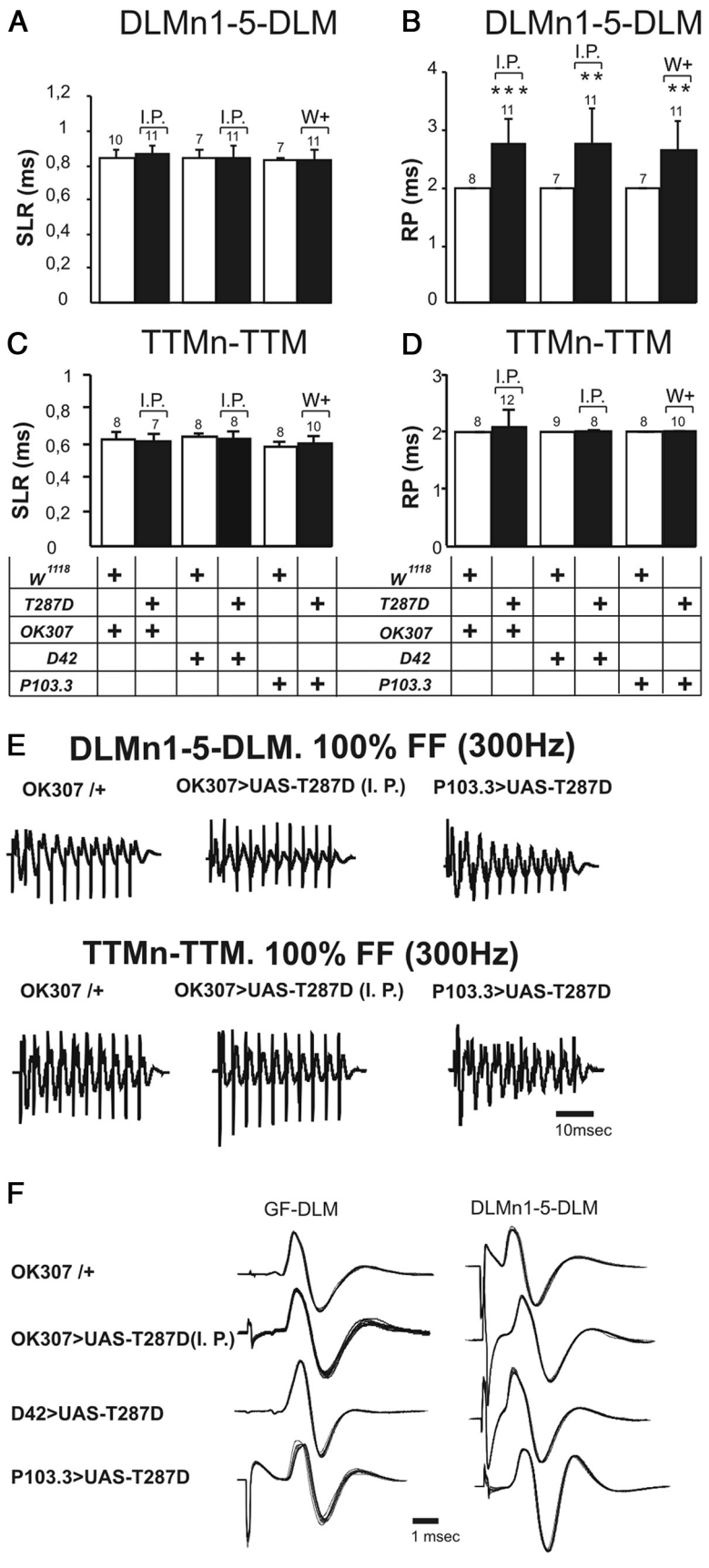


**Figure 3.** ***A–D***, The effect of CaMKII-T287D transgene on the SLR (***A***, ***C***) and RP (***B***, ***D***), was tested for the GFS motoneurons (DLMn1-5 or TTMn) and their neuromuscular synapses with the DLM or TTM, respectively (stimulation of the DLMn1-5 or TTMn in the thoracic ganglion and recording from DLM or TTM, correspondingly). The T287D mutation does not affect the SLR in both pathways. There is an increase of the RP in the flies expressing T287D compared to the controls for the DLMn1-5-DLM pathway (***C***). In the TTMn-TTM pathway, the RP value of the flies expressing T287D remains at the level of the controls (***D***). ***E***, The DLMn1-5 or TTMn and their neuromuscular synapses with the DLM or TTM, correspondingly, of control and flies expressing T287D exhibit a 100% following at 300 Hz frequency stimulation. ***F***, Overlapping evoked action potentials (10 traces) recorded from DLMs after direct 1 Hz stimulation of the GF or the motoneurons from control (OK307/+) and mutant (T287D expressed under the control of the drivers OK307, D42, and P103.3. In both control and mutant animals the responses were always time locked to the stimulation without showing failures or variable delays between stimulus and initial rise. White bars, Controls. Data are shown as mean ± SD. Numbers above the bars indicate the number of preparations tested and asterisks indicate $p$ values from $t$ test (**$p < 0.01$, ***$p < 0.001$) compared to the controls. I. P., Infantile phenotype, W+, normal wings.

T287D action in animals carrying the OK307 driver, we used a process of eliminating candidate sites by crossing the CaMKII transgene with four Gal4 drivers expressed in particular efferent GFS neurons (Fig. 1*E*). The c17Gal4 driver, for example, is expressed only in the GF neurons of the circuit among other cells located in distant brain areas (Fig. 1*E*). The electrophysiological data from these combinations of transgenic animals yielded the following results.

First, direct stimulation of the GFS motoneurons (including MN5 and TTMn) through the thorax and recordings from DLM or TTM allowed examination of response latencies and NMJ function. It has to be noted that direct stimulation eliminates the possibility of driving the motoneurons synaptically. Therefore, non-GFS cells in which these Gal4 drivers may be expressed nonspecifically should not influence the motor output. Animals expressing T287D in all GFS neurons (OK307>UAS-T287D) or in GFS motoneurons (P103.3>UAS-T287D) revealed no defects in SLR, and NMJs responded normally to >300 Hz frequency stimulation in both pathways (Fig. 3*A*,*C*,*E*). There is a statistically significant increase only in RP of CaMKII-T287D-expressing flies (from 2 ms in controls to ~2.7 ms; Fig. 3*B*) that is negligible compared to the RP increase in the GF-DLM pathway (from ~4 ms in controls to ~10–12 ms) (Fig. 2*B*). The lack of change in response latency (SLR) does not support defects in conduction velocity in the axons of GFS motoneurons and altered synaptic delays at NMJs. The NMJs themselves responded with high fidelity at frequencies much higher than the central synapses. Thus, the parts of the GF-DLM and GF-TTM pathways from the postsynaptic sites of motoneurons to target muscles, including the NMJs, do not appear to be affected by CaMKII-T287D.

Second, direct GF stimulation and recordings from DLM or TTM in animals expressing CaMKII-T287D exclusively in the GF neurons of the circuit (c17>UAS-T287D; Fig. 1*E*) did not reveal defects in either pathway (Fig. 2*G–I*). Hence, presynaptic expression of the $Ca^{2+}$-independent CaMKII does not affect GF/TTMn function, consistent with the notion that such a molecule would not play a presynaptic functional role at that synapse.

Third, expression of CaMKII-T287D under the D42 and P103.3 motoneuron drivers (Fig. 1*E*; Yeh et al., 1995; Consoulas et al., 2002) resulted in flies exhibiting longer SLR, increased RP, and decreased $FF_{50}$ in the GF-DLM pathway. Significantly, the values of these three parameters were similar to those from flies expressing CaMKII-T287D in all GFS neurons (Figs. 1*A*, 2*A–C*). RP and $FF_{50}$ were similarly affected in the GF-TTM pathway (Fig. 2*H*,*I*). Considering that NMJs are unaffected in these animals and lack presynaptic defects in both pathways, we conclude that the apparent locus of action of $Ca^{2+}$-independent CaMKII are the postsynaptic sites of the PSI/DLMns and GF/TTMn central cholinergic synapses.

We have shown that there are no SLR defects in DLMn-DLM pathway and excluded the possibility that SLR defects in GF-DLM could be attributed to changes in signal propagation in the GF or/and PSI axons. This is because evoked action potentials recorded from DLMs after direct 1 Hz stimulation of the GFs or the motoneurons (DLMns) were always time locked to the stimulation without exhibiting failures or variable delays between stimulus and initial rise of the muscle potential (Fig. 3*F*).

Finally, although animals expressing CaMKII-T287D under both motoneuron drivers exhibited severe RP and $FF_{50}$ electrophysiological deficits, only D42>CaMKII-T287D animals displayed the infantile wing phenotype. This clearly indicates that the wing defect is independent of the electrophysiological phenotypes. Therefore, the absence of normal, functional wings is not causally related to the defects in the GF-DLM pathway. Rather, aberrant function of the GF-DLM pathway was precipitated from accumulation of $Ca^{2+}$-independent CaMKII in the GFS neurons.

Although we did not estimate conduction velocity in GFS axons per se, delays and failures in evoked responses reported for CaMKII-T287D-expressing larval motor axons (Park et al., 2002) were not evident in the axons of PSI, GF, and GFS motoneurons. Because $FF_{50}$ and, at least in part, the RP deficits reflect synaptic dysfunction, the response delays and failures are likely consequent of increased synaptic delays and failures in cholinergic synapses rather than impairments in action potential generation and propagation mechanisms.

Collectively, the defects revealed in the GF-DLM and GF-TTM pathways are related to changes occurring in the postsynaptic region of the two central cholinergic synapses, the one between PSI and DLMn, and the second between GF and TTMn. Considering the temporal expression pattern of Gal4 drivers, the effect on synaptic function is either of developmental origin and/or reflects recent changes in synaptic function in an activity-dependent fashion.

### Postsynaptic expression of CaMKII-T287D causes signal transmission failure from ColA to GF neurons

It has been estimated that before reaching the ventral lateral dendrites (VLDs) of the GF neurons (Bacon and Strausfeld, 1986, Douglass and Strausfeld, 2003), the visual input has to cross four synaptic levels created by bundles of most likely cholinergic interneurons (Salvaterra and Kitamoto, 2001). In support of this contention, the D$\alpha$7 nicotinic acetylcholine receptor is enriched in the GF dendrites (Fayyazuddin et al., 2006). Low-voltage stimuli delivered at the outer layers of the visual neuropiles (indirect GF stimulation) activate neurons of the sensory part of the GFS circuit (photoreceptors-GF neurons), and long latency responses (LLRs) instead of short latency responses (SLRs) were recorded from the DLMs.

LLRs of flies expressing CaMKII-T287D in the GF neurons (c17Gal4) or in all identified GFS neurons (OK307Gal4) failed almost completely (Fig. 4*A*,*D*). Few flies responded to indirect GF stimulation: 2/10 infantile OK307>CaMKII-T287D; 4/20 normal-winged OK307>CaMKII-T287D; and 1/10 c17>CaMKII-T287D. These presented longer LLRs and dramatically increased the long latency response-refractory period, (L)RP (Fig. 4*B*, C). Because in the same preparations direct stimulation of the GF resulted in successful DLM responses, the GF-DLM branch is unlikely responsible for the LLR failure. This is consistent with a postsynaptic effect of CaMKII-T287D in PSI/DLMn and GF/TTM synapses and suggestive of a possible role of a similar endogenous molecule. Similarly, expression of CaMKII-T287D caused postsynaptic defects at the ColA/GF synapses. In contrast, expression of CaMKII-T287A and CaMKII-R3 in GF and other downstream neurons revealed only mild defects. OK307>CaMKII-T287A flies exhibited LLR and (L)RP values similar to those of their respective controls, while in OK307>CaMKII-R3 a significant decrease in LLR and RP was revealed (Fig. 4*F*,*G*) that is suggestive of improved synaptic function. However, inhibition of the kinase with CaMKII-RNAi or ala2 did not change the two parameters detectably (Fig. 4*F*,*G*). Hence, reducing levels or activity of the putatively endogenous CaMKII in this circuit appeared to not precipitate consequences. Given the broad expression of the kinase in the CNS, we do not interpret this as lack of CaMKII from these neurons but rather, as expected, tightly regulated $Ca^{2+}$-dependent activity. Furthermore, kinase abrogation would be expected to have the opposite effect of that described for CaMKII-T287D, that is, an increase in synaptic efficiency above wild-type levels. Although we think this is unlikely, even if it occurred it would again be probably below detection in our system.

To assess the effect of the five CaMKII transgenic proteins in neurons presynaptic to GF, we used the cholinergic driver cha-Gal4. In contrast to the motoneuron and GFS-preferentially expressed drivers, the cha-Gal4 driver is expressed by the end of the pupal stage [>85 h after puparium formation (APF)] and throughout adulthood (Fig. 1*E*). Considering that signal transmission from GF to PSI is mediated via electrical synapses, first we asked whether the defects in OK307>CaMKII-T287D flies were caused by presynaptic expression of CaMKII-T287D in the PSI itself by expressing CaMKII-T287D in cholinergic neurons (Fig. 1*E*). It has to be emphasized that cha-Gal4 is not expressed or is expressed at undetectable levels in GF neurons (Salvaterra and Kitamoto, 2001; Allen and Murphey, 2007; or data and data not shown), but we assumed that it is normally expressed in PSI neurons because they are enriched in cholinergic terminals (Gorczyca and Hall, 1984). Flies expressing CaMKII-T287D under cha-Gal4 (cha>CaMKII-T287D) exhibited the infantile phenotype, yet they did not show defects in the three parameters tested (Fig. 2*A–C*). Inasmuch as the cha-Gal4 is expressed in the PSI, these results excluded a presynaptic effect of the $Ca^{2+}$-independent CaMKII in PSI/DLMn1-5 synaptic transmission.

Our results indicate that cha>CaMKII-T287D (infantile wings) flies did not present statistically significant differences in LLR and (L)RP compared to their controls. On the other hand, expression of the $Ca^{2+}$-dependent CaMKII-T287A in cholinergic neurons resulted in significant improvement in LLR and (L)RP, while the wild-type CaMKII decreased only the LLR (Fig. 4*F*,*G*).

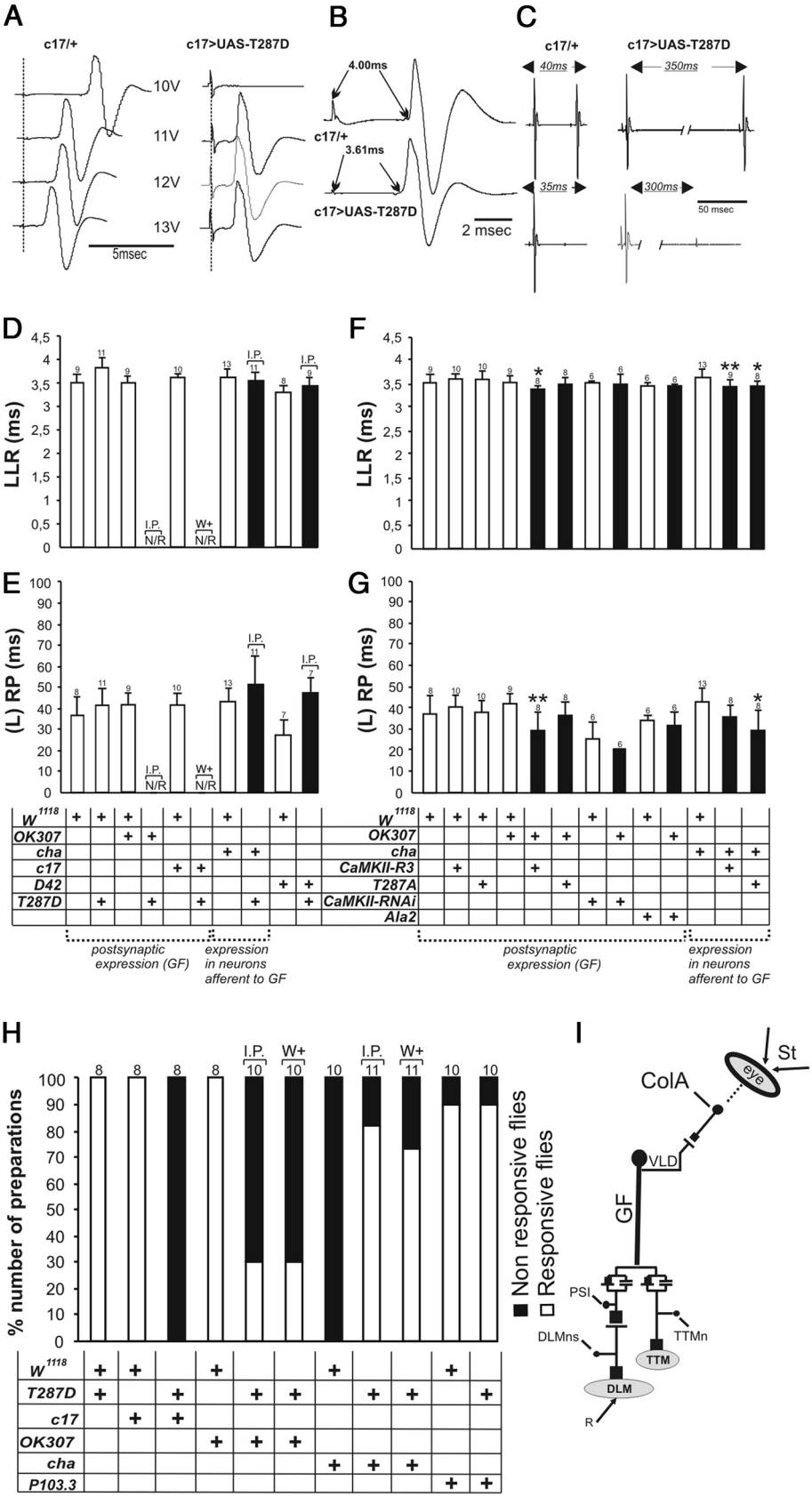


**Figure 4.** Central cholinergic transmission block. The expression of the T287D transgene, specifically in the GF, blockades the transmission in the ColA/GF synapse (see diagram in ***I***). ***A***, Left, Control flies (c17/+) respond to low voltage stimuli (10 V, indirect stimulation of the GF) by giving long-latency DLM responses, LLRs. Short latency responses, SLRs, are obtained by the DLMs after higher voltage stimulation (13 V, direct stimulation of the GF). Stimulation at 11–12 V evokes intermediate latency responses. ***A***, Right, c17>UAS-T287D flies do not respond to low voltage stimuli (10 V, indirect stimulation of the GF), indicating the blocked transmission. ***B***, ***C***, The excludable c17>UAS-T287D fly (1/10 flies tested), where the ColA/GF synapse is not blocked, exhibits longer LLR (***B***) and much more increased long-latency RP (***C***) than the corresponding control flies. ***D***, ***E***, Measurements of LLR (***D***) and long-latency RP (***E***) in flies expressing (*Figure legend continues*.)

Collectively, the data indicate that the postsynaptic expression of the $Ca^{2+}$-independent CaMKII, but not that of any of the other transgenes, blocks signal transmission in the synapses between GF and its presynaptic partner, the ColA neuron. Hence, it appears that the $Ca^{2+}$-independent CaMKII affects the ColA/GF more severely than the stereotypic cholinergic synapses (PSI/DLMn, GF/TTMn).

### Expression of CaMKII-T287D in GF neurons disrupts light-induced escape behavior

To examine whether the synaptic changes in the GFS pathway resulting from these genetic manipulations of CaMKII precipitated relevant behavioral effects in the freely moving animal, we investigated light-induced escape behavior. In *Drosophila*, this startle reaction can be activated experimentally by a sudden change in ambient light level (lights off for 20 ms).

Flies expressing CaMKII-T287D in GFS neurons (c17Gal4 or OK307Gal4) failed almost completely to respond (Fig. 4*H*). In contrast, flies expressing CaMKII-T287A or the wild-type CaMKII-R3 in GF neurons and flies expressing CaMKII-T287D in cholinergic neurons (chaGal4) and motoneurons (P103.3-Gal4) showed robust responses (Fig. 4*H*). It has been suggested that this type of escape behavior can be induced in white-eyed mutants only (Wyman et al., 1984; Bacon and Strausfeld, 1986; Milde and Strausfeld, 1990; Fayyazuddin et al., 2006). In contrast, our experiments with transgenic animals expressing CaMKII in GFS or in cholinergic neurons show that it is not the eye color (red in our case), but probably the genetic background ($w^{1118}$) that is the decisive factor in eliciting this escape behavior. These results provide independent behavioral confirmation of our conclusions regarding the consequences of CaMKII manipulations in GF neurons.

### Postsynaptic expression of CaMKII-T287D during development causes transmission failure at the ColA/GF synapse

Given that postsynaptic expression of CaMKII-T287D in GF neurons interrupts signal transmission at the GF/ColA cholinergic synapse, we wanted to determine whether this resulted from acute, activity-dependent disruption of the sensory input to the GF dendrites or more permanent developmentally mediated alterations in the ColA/GF synapse. This question was addressed using the TARGET (Temporal And Regional Gene Expression Targeting) system. TARGET permits spatiotemporal control of transgene expression manipulated by altering the temperature of the culture (McGuire et al., 2003). With this system,

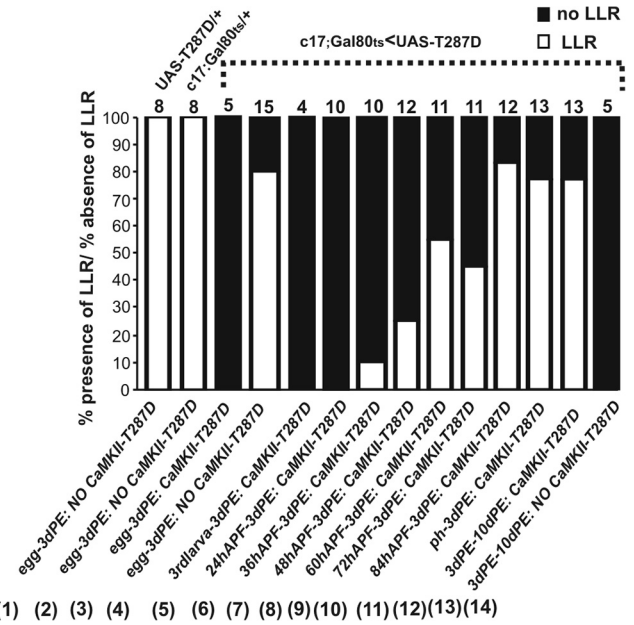


**Figure 5.** Postsynaptic expression of CaMKII-T287D during development interrupts signal transmission in the ColA/GF cholinergic synapse. The TARGET approach was used for spatial (in the GF with the c17-Gal4) and temporal (according to the temperature) expression of the mutant transgene T287D. At 31°C the expression of the T287D is permitted, while at 18°C it is prevented. The functionality of the synapse ColA/GF was tested by stimulating indirectly the GF and recording the response from the DLMs. Black indicates the percentage of flies that do not present LLR, while white indicates the percentage of flies that exhibit normal transmission (LLR present) in the ColA/GF synapse. Note that expression of the CaMKII-T287D after the critical point of 84 h APF fails to induce synaptic transmission block (bar 11). Above the bars are indicated the genotypes and the numbers of preparations. Below the bars are shown the intervals of time (numbered) that the expression of the mutant transgene T287D was induced or not. dPE, Days posteclosion, ph, pharate, hAPF, hours after puparium formation.

c17>Gal80$^{ts}$/ CaMKII-T287D flies kept at 18°C do not express the transgene, but they do so if shifted to 31°C. Indeed, as indicated in Figure 5, all c17>Gal80$^{ts}$/CaMKII-T287D animals maintained at 31°C from the embryonic stage to 3 d posteclosion (PE) exhibited completely blocked GF/ColA synapses (Fig. 5, bar 3). On the other hand, continuous maintenance of animals at 18°C until adulthood resulted in normal synaptic transmission in 80% of the flies, suggesting "leaky" expression and partial Gal4 production (Fig. 5, bar 4) even at this low culture temperature and provides a measure of "experimental background" levels for the parameters measured. The two control lines raised at 31°C did not show defects (Fig. 5, bars 1, 2), indicating that the synaptic phenotype (Fig. 5, bar 3) is not caused by high temperature, but rather it is a consequence of CaMKII-T287D expression.

Preventing CaMKII-T287D expression until the end of the pupal stage or the third day PE and then allowing it for 3 or 7 d (3 d pharate and 10 d PE, respectively) resulted in normal synaptic transmission phenotypes for nearly 80% ("background levels") of c17>Gal80$^{ts}$/CaMKII-T287D flies (Fig. 5, bars 12, 13). Conversely, when CaMKII-T287D was expressed throughout development until the third day of PE but then its expression was blocked for the next 7 d (3–10 d PE), all flies exhibited transmission failure at the GF/ColA synapse (Fig. 5, bar 14). Therefore, the CaMKII-T287D-induced defect at this synapse has developmental origin.

Consequently, we defined the critical developmental stage when CaMKII-T287D expression alters the GF/ColA synapse, resulting in transmission failure. Onset of transgene expression by the end of the third larval instar or during the first half of

←

(*Figure legend continued.*) CaMKII-T287D under the control of GFS and cholinergic Gal4 drivers. Note the lack of response (N/R, no response) in OK307>CaMKII-T287D and c17>CaMKII-T287D flies. ***F***, ***G***, Measurements of LLR (***F***) and long-latency RP (***G***) in flies expressing the other four CaMKII transgenes (T287A, CaMKII-R3, CaMKII-RNAi, Ala2) under the control of the Gal4 drivers. Flies expressing the $Ca^{2+}$-sensitive CaMKII-T287A or overexpressing the wild-type protein (CaMKII-R3) exhibit a significant improvement in the function of the synapse between GF and its presynaptic interneuron. ***H***, Light off-induced escape behavior. Flies expressing the transgene T287D under the control of a series of Gal4-driver were tested for their ability to escape (jump and occasionally flight) in response to a rapid dimming of light. Note that expression of the CaMKII-T287D protein, specifically in the GF, prevents the manifestation of the visually mediated escape behavior, verifying the electrophysiological findings. ***I***, Schematic diagram of the GFS. In ***D***–***H***, numbers above the bars indicate the number of preparations tested, and asterisks in ***D***–***G***, indicate *p* values from *t* test (*$p < 0.05$, **$p < 0.01$) compared to the controls. Genotypes. Data are shown as mean ± SD. I. P., Infantile phenotype; W+, normal wings; R, recording site; St, stimulation site.

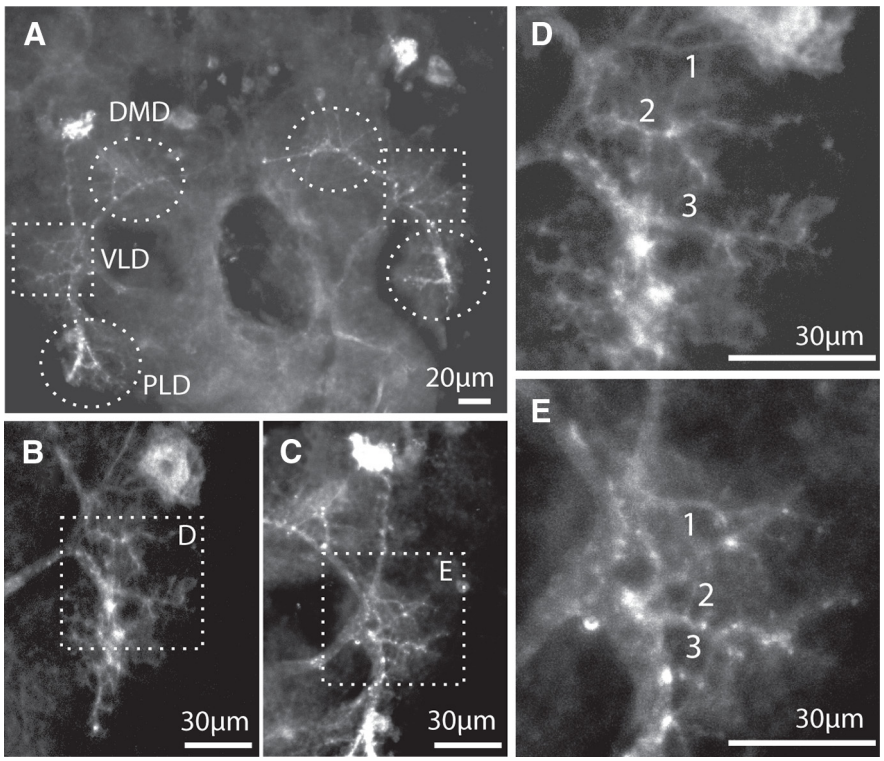


**Figure 6.** Confocal images of OK307mCD8GFP>UAS-T287D (***A***, ***C***, ***E***) and appropriate genetic control OK307mCD8GFP/+ (***B***, ***D***). Note that the three primary dendritic VLD branches (1, 2, 3) are apparent in both mutant and control GF neurons.

metamorphosis (24, 36, 48 h APF) also resulted in adults with blocked ColA/GF synapses (Fig. 5, bars 5, 6, 7, 8). Permitting transgene expression from 60 to 72 h APF yielded GF/ColA electrophysiological deficits in about half of the flies (Fig. 5, bars 9, 10). Expression onset 12 h later (84 h APF) resulted in nearly 80% of the adults with fully responsive ColA/GF synapses (Fig. 5, bar 11) similar to c17>Gal80$^{ts}$/CaMKII-T287D raised at 18°C (Fig. 5, bar 4). Therefore, our data suggest that unregulated $Ca^{2+}$-independent CaMKII activity during development of the circuit affects mechanisms of synaptogenesis, or alternatively, synaptic defects could be the result of the failure of GF dendritic arbors to grow into their receptive area. Confocal imaging of GF neurons coexpressing CaMKII-T287D and GFP revealed no gross morphological changes in GF dendritic morphology (Fig. 6). In agreement, high-magnification images did not show differences in the VLD dendritic pattern in comparison to the controls (Fig. 6*B–E*). Furthermore, axonal branching of GF neurons in the mesothoracic neuromere appeared to occur normally (data not shown). Hence, expression of the $Ca^{2+}$-independent, constitutively active CaMKII during metamorphosis after GF axon pathfinding, bending, and initial contact with the PSI and TTMn, but before 84 h APF, did not result in obvious morphological dendritic and axonal changes but in functionally compromised adult ColA/GF synapses.

## Discussion

This study provides evidence that postsynaptic accumulation of transgenically derived, $Ca^{2+}$-independent, constitutively active CaMKII in developing *Drosophila* GFs and motoneurons permanently impairs, but with different severity, the function of central cholinergic synapses in GF and motoneuron dendrites, while the neuromuscular junctions seem unaffected. Although not supported by our morphological data, we cannot otherwise exclude the possibility that the physiological phenotypes we describe arise from undetectable wiring changes (e.g., additional, or different interneuron(s) incorporated into the circuit) and not from altered synaptic functions in an otherwise normal circuit.

Our forced expression data do not necessarily emulate the native CaMKII distribution or activity state in these neurons. In addition, our manipulations and perturbations of the system may induce homeostatic regulation, especially during development when plasticity is prominent. This suggests that some of the observed effects may be the consequence of CaMKII accumulation in non-GFS cells. However, we do not favor the latter explanation based on previous work on giant neurons in culture (Yao and Wu, 2001) that respond to CaMKII antagonists, and our own results with the constitutively active CaMKII-T287D suggest that these neurons and relevant synapses of the GFS contain the molecular machinery to respond to the elevated kinase activity. This, along with the abundant neuronal expression of the kinase, suggest that it is normally present in the circuit, although its unequivocal demonstration (Schmitt et al., 2004; Burkert and Duch, 2006) is not trivial and is the subject of ongoing work.

Our data indicate that the electrophysiological phenotypes result from sustained CaMKII activity during development of cholinergic synapses. In contrast, expression of the CaMKII-R3 and T287A transgenes did not alter cholinergic transmission. We suggest that CaMKII elevation per se did not appear to result in altered neurotransmission, but rather it resulted from sustained $Ca^{2+}$/CaM-independent activity during a critical developmental period when low or no activity is required. This is consistent with the lack of effects due to CaMKII-R3 expression that, although it elevated wild-type protein in the circuit, was nevertheless dependent on $Ca^{2+}$/CaM and consequent autophosphorylation to exert its effects. Similarly, the $Ca^{2+}$-sensitive mutant CaMKII-T287A, which cannot sustain the activated state and mimic CaMKII-T287D (Wang et al., 1998), did not precipitate defects. Moreover, if the deficits are a consequence of constitutive CaMKII activity in developmental periods when it should either be inactive or at least $Ca^{2+}$/CaM-dependent, then reducing the endogenous kinase with RNAi or its activity with ala even with the highest efficiency would likely have no adverse consequences. However, in contrast to our findings, synaptic transmission and growth in larval NMJs and $K^+$ currents and membrane excitability in GF neurons in culture are altered by CaMKII antagonists or/and ala peptide expression (Wang et al., 1994; Yao and Wu, 2001). It is formally possible that this reflects differences in functional properties between larval abdominal NMJs and adult DLM, TTM NMJs. In addition, differences in the membrane properties between the cultured GF and adult GF *in situ*, may explain the differential response in CaMKII abrogation.

Characteristics of synaptic improvement were detected upon expression of CaMKII-T287D and CaMKII-R3 with chaGal4 in GFS neurons afferent to the GF. This improvement in GFS synaptic function is either of developmental origin or reflects an

activity-dependent mechanism since the chaGal4 driver is expressed by the end of synapse formation and then strongly in the adult. Unfortunately, the locus of the phenotype cannot be defined due to the lack of specific drivers for particular groups of neurons of the sensory part of the GFS.

Our data indicate that presynaptic forced expression of CaMKII-T287D and the other forms of the kinase in GFs and motoneurons does not impair synaptic transmission in GF/TTMn synapses, probably in PSI/DLMn synapses and the NMJs. In contrast, presynaptic expression of CaMKII-T287D in cholinergic chordotonal (sensory) neurons eliminates habituation of a reflex controlling the leg position (Jin et al., 1998), while at the larval glutamatergic NMJs, presynaptic expression in the motoneurons increases the number of boutons without affecting neurotransmitter release and alters potassium conductance in motoneuron axons (Koh et al., 1999; Park et al., 2002) Thus, both GFS central cholinergic and peripheral glutamatergic NMJs in the adult appear to be nonresponsive to kinase alterations. Consistent with this, a recent study provides evidence that in mammalian cultured cortical neurons, expression of constitutively active CaMKII$\alpha$ has no effect on presynaptic release (Pang et al., 2010).

### $Ca^{2+}$-independent CaMKII acts during the development of the GFS

Expression of CaMKII-T287D in GF neurons during metamorphosis could affect each or a combination of axon pathfinding (larval stages, 24 h APF), target recognition and initial synapse formation (24–55 h APF), or synapse formation, stabilization, and maintenance (55–100 h APF) (Allen et al., 2006). We report that the constitutively active CaMKII exerts its effects during a 60 h window (24–84 h APF) in the pupa. The end of this time window seems to coincide with completion of synapse formation and stabilization. In congruence, GFS synapses have been reported susceptible to permanent disruptions if endocytosis is temporarily blocked, but only before 85 h APF (Hummon and Costello, 1987; Murphey et al., 2003). Interestingly, even if CaMKII-T287D activity is continuously present in the circuit when the TARGET system was not used, the effect was still specific to the ColA/GF synapse. This suggests at least temporal specificity in the effects of $Ca^{2+}$-independent CaMKII. Nevertheless, in affected neurons the dendrites appear normal, and their axons reach the mesothoracic neuromere and show their characteristic bends and functional electrochemical contacts with PSI and TTMn neurons. Therefore, the initial phase of axon pathfinding (larval stages, 24 h APF) can be excluded from the effect of $Ca^{2+}$-independent CaMKII on GF development. Thus, the effects of CaMKII-T287D seem specific to synapse formation and stabilization of central cholinergic neurons rather than on dendritic and axonal growth. This may be distinct from similar processes in central glutamatergic neurons (Wu and Cline, 1998; Zou and Cline, 1996), and we will explore this in the future.

### Potential role of the $Ca^{2+}$-independent CaMKII during development of GFS cholinergic synapses

How does constitutive CaMKII activation affect cholinergic synapses? The cholinergic (Gorczyca and Hall, 1984; Allen and Murphey, 2007) GFS central synapses operate, mainly if not exclusively, through the D$\alpha$7 nicotinic acetylcholine receptor, and GFS-mediated escape response is abolished in nAChR receptor mutant animals (Fayyazuddin et al., 2006). Because the locus of the CaMKII-T287D defect is postsynaptic and CaMKII participates in the suppression of nAChR gene expression in mammalian muscles (Macpherson et al., 2002), one likely explanation for the observed GFS phenotypes is a decreased number of nAChRs. In the class of interneurons known as Kenyon cells, activation of nAChRs causes rapid and reversible intracellular calcium increase that depends on indirect calcium influx through voltage-gated calcium channels directly via nAChRs (Campusano et al., 2007). In accord with these studies, we propose that CaMKII may act as a negative feedback element regulating nAChR density during normal development.

An alternative explanation could be the abnormal function or localization of the nAChRs in the postsynaptic area due to defective formation and/or stabilization of the synapses. The constitutively active CaMKII-T287D is known to cause structural abnormalities in the larval NMJ by phosphorylating Discs Large (DLG) and increasing its extrasynaptic localization (Koh et al., 1999). Dephosphorylated DLG associates with the synaptic complex and clusters the cell adhesion molecule Fasciclin II (Fas II) and the Shaker $K^+$ channels (Tejedor et al., 1997; Thomas et al., 1997; Zito et al., 1997). Importantly, FasII is essential for synapse maintenance and plasticity (Schuster et al., 1996a, 1996b; Davis and Goodman, 1998). Thus, either the homolog of PSD93 in mammalian central cholinergic synapses, DLG (Parker et al., 2004), or another scaffolding protein regulated by CaMKII could participate in a similar mechanism for the correct localization of the nAChRs in the postsynaptic area of GFS neurons.

Finally, a third possible explanation for the CaMKII-T287D phenotypes could be impaired potassium conductance in GFS neurons. Indeed, Shaker $K^+$ channels mutations (*Sh*) cause longer RP and lower $FF_{50}$ values in the GFS of the mutant animals (Engel and Wu, 1992), and shaker knock down also strongly affects MN5 motoneuron firing responses (Ryglewski and Duch, 2009). Furthermore, eag (ether-à-go-go) potassium channels are known to interact with CaMKII (Griffith et al., 1994; Wang et al., 2002), and DLMn5 motoneuron firing behavior is altered upon expression of an eag dominant negative isoform (Duch et al., 2008).

GF/TTMn, PSI/DLMn and ColA/GF cholinergic synapses of the GFS exhibit similar malfunctions, but postsynaptic, constitutively active CaMKII is differentially detrimental for the ColA/GF synapses. Distinctions in the severity of the synaptic phenotypes may reflect functional differences between the stereotypic GF/TTMn, PSI/DLMn, and the weak-plastic ColA/GF synapses. Synapses in GFS motoneuron dendrites (GF/TTMn) and axons (PSI/DLMn) receive strong synaptic input and must generate a single action potential only per GF activation (Allen et al., 1998). These synaptic sites can operate at activation frequencies above 150 Hz and are not designed to summate postsynaptic potentials. Thus, synaptic transmission is unlikely to be affected by subtle postsynaptic alterations. In contrast, synapses in GF dendrites integrate sensory input and operate at a maximum of 30 Hz, and small postsynaptic defects uncovered by CaMKII activation likely result in failure of action potential generation and light-induced escape response. If the constitutively active kinase downregulates the cholinergic receptor or compromises synaptic stabilization and/or neurotransmitter release, then the lower number of active synapses may be sufficient for GFS motoneurons, but not for the GF neurons to reach threshold for action potential generation.

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