Drosophila CAKI/CMG Protein, a Homolog of Human CASK, Is Essential for Regulation of Neurotransmitter Vesicle Release

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INTRODUCTION

Recently there has been a growing interest concerning pre- and postsynaptic membrane scaffolding proteins. A major function of these proteins is the recruitment, to specific regions of the cell membrane, of ionic channels, receptors and other structures involved in the pre and postsynaptic machinery, allowing the precise alignment and organization of the pre- and postsynaptic elements that are involved in neurotransmitter release and signal transduction (Sudhof 2001).

The membrane-associated guanylate kinases (MAGUK) constitute one class of such scaffolding proteins. MAGUKs are characterized by various evolutionarily conserved domains (PDZ, SH3, HOOK, and GUK) involved in protein–protein interactions (Anderson 1996; Hata et al. 1996).

Vertebrate CASK is a MAGUK protein. Its central PDZ domain interacts with other membrane proteins such as neurexins, syndecan NG2, glycoporphins (Hata et al. 1998; Hsueh et al. 1998; Martinez-Estrada et al. 2001). CASK also has a catalytically inactive N-terminal CaM-kinase domain (Hata et al. 1996) and forms an evolutionarily conserved molecular complex with two other PDZ proteins, VELIS and MINT-1. The C. elegans homologs of CASK, VELIS and MINT-1 are LIN2, LIN7, and LIN10, respectively. The Drosophila homologue of CASK is CAKI or CAMGUK (Drummond et al. 1991; Martin and Ollo 1996) and the putative homologs of VELIS and MINT-1 have also been identified (Buchmann et al. 2004; MacMullin et al. 2001). CAKI is present in the nervous system where it binds presynaptically to neurexin (Butz et al. 1998). Neurexin interacts postsynaptically with neuroligin, leading to the correct alignment of the pre- and postsynaptic machinery (Butz et al. 1998; Tabuchi et al. 2002). CAKI is also present outside the nervous system, where it is involved in tight junction formation and maintenance (Irie et al. 1999). In C. elegans, mutations of Lin2, Lin7, or Lin10 cause alterations in vulval epithelial cells, leading to vulva malformations (Hoskins et al. 1996; Kaech et al. 1998). In mice, mutations of Cask cause palatal cleft malformations (Caruana and Bernstein 2001). CASK has also been proposed as a co-activator of TRB1, a transcription factor involved in brain development through the activation of genes with T-element-containing promotors (Hsueh et al. 2000). In addition, interaction of CASK with the molecular motor KIF17 has also been shown (Mok et al. 2002).

In the nervous system, the interaction of CASK with the other components of the tripartite complex suggests a role in neurotransmitter release (Butz et al. 1998). In this context, the role of CASK remains to be elucidated. Martin and Ollo (1996) cloned the Drosophila homologue of mammalian Cask, caki/camguk, and obtained a viable mutant by P-element excision. Adult caki mutants were characterized by a reduced locomotor behavior (Martin and Ollo 1996). These mutants also show altered courtship conditioning, a test that evaluates associative and nonassociative memory formation (Lu et al. 2003). In Drosophila adults, the most evident expression of caki was in the visual brain regions (Martin and Ollo 1996). In the larval CNS, caki is expressed in synaptic regions of the ventral...
ganglion and brain lobes (Lu et al. 2003; Martin and Ollo 1996) and in the pre- and postsynaptic region of the neuromuscular junction (NMJ) (Lu et al. 2003). Moreover, similarly to the association of CASK with VELI, it has recently been shown that CAKI interacts with the Drosophila homologue of VELI in the yeast two-hybrid assay (Bachmann et al. 2004).

In this study, we employed electrophysiological methods to analyze the spontaneous and evoked neurotransmitter release at the NMJ and to explore the functional status of the giant fiber pathway and of the visual system. Our results suggest the involvement of CAKI in neurotransmitter release and nervous system function.

**Methods**

**Fly stocks**

Flies were maintained on standard medium at room temperature (22°C). Canton S strain was used as the wild-type (WT) reference strain. caki mutants were transheterozygous for two overlapping deletions and were obtained by crossing two lines (X-313 and X-307), each carrying a recessively lethal partial deletion of the caki gene (Martin and Ollo 1996).

In genetic rescue experiments, we also employed a UAS-caki transgenic line bearing a single X-chromosome insertion of the transgene coding for the complete caki CDNA. The transgene consisted in a 3,200-bp caki CDNA fragment included between the (5') EcoRI-XhoI (3') restriction sites. The EcoRI site is located 474 bp upstream of the ATG and the XhoI site is 1,016 bp downstream of the TAG (stop codon). The fragment was thus cloned into the pUAST transgenesis vector. To activate the expression of the UAS-caki transgene in a caki mutant background, crosses were set up to transfer the transgene into a X-313 background. Parallel crosses were arranged to transfer an elav-GAL4 transgene (originally present in strain C155; Bloomington) into the X-307 background. Finally by crossing UAS-caki;X-313; individuals to elav-GAL4;X-307 flies, a progeny was obtained that consisted of caki mutant females, in which the expression of the UAS-caki transgene was also activated and caki mutant males in which the production of the wild-type caki transgene was not active.

**Behavioral tests**

**FLIGHT TEST.** A flight test was carried out as described by Drummond et al. (1991). Flies were allowed to fly inside a transparent perspex box (20 × 20 × 40 cm) toward a light source placed above the box. Flies flying upward (toward the light) were scored as UP, whereas if they flew down but reached the opposite side of the box, they were scored as DOWN. Flies falling inside a petri dish placed on the bottom of the box were scored as NULL.

**WALKING OPTOMOTOR RESPONSE.** The optomotor response was tested following the method described by Burnet (1968). Three- to 8 days-old flies were dark-adapted for 4 h in food vials. Each fly was tested individually for its turning behavior in a moving visual field. The latter was created using a rotating perspex drum (diameter: 8 cm; height: 9 cm) the inner surface of which was painted with alternating black and white vertical stripes. The stripes subtended an angle of 12.4°. The drum was continuously rotated at 30 rpm. Each fly was placed in the middle arm of a T-shaped glass tube. The middle arm was painted black so that the fly was forced to walk out toward the light into a choice point where it could turn into the transparent right or left arm of the T. To test whether the turning behavior corresponded to the moving environment, the tube was placed in the middle of the rotating drum. A fly produced a correct response every time it chose to turn in the same direction as the rotating stripes. For each genotype, ≥10 individuals were tested. Each fly was given 10 trials, and each time the direction of rotation of the striped drum was inverted. A lamp (60 W) was placed above the drum to provide a uniform illumination of the central part of the cylinder. All tests were performed at room temperature (22°C).

**OPTOMOTOR RESPONSE SENSITIVITY.** A tethered fly (attached by the dorsal part of the head and the thorax to a manipulator) was allowed to walk on top of a Styrofoam ball in the center of a rotating drum with the inner surface painted in black and white stripes (Gottz 1970). The ball was supported by a gentle stream of air and is easily rotated by the fly. The rotations of the ball were recorded optoelectronically in four separate channels for forward/backward and left/right movements (Buchner et al. 1978). The number of revolutions of the Styrofoam ball around the vertical axis, following and against the pattern of movement was counted. After 50 counts, obtained by monitoring the revolutions of the ball around its transverse horizontal axis (forward walking; named a run), a new measurement (run) was started. The stripes subtended an angle of 24° and the rotations of the drum were calibrated to obtain a 3-Hz contrast frequency, an experimental condition known to elicit maximal responses. The quotient of the numbers of revolutions of the ball (rev/R/rev.F) as defined by the recording technique represents the turning tendency of the fly (Buchner et al. 1978). The average luminance (I) of the pattern was in the order of 300cd/m². Each condition was repeated with a gradual decrement of the normalized light intensity ranging from 1 to 1/10.

**Electrophysiological analyses**

**ELECTRORETINOGRAm (ERG).** The ERG is an extracellular recording from the Drosophila eye that measures light-induced depolarization of photoreceptors (the sustained response) and synaptic activation of second-order neurons in the visual pathway (Heisenberg 1971; Hotta and Benzer 1969; Pak et al. 1969). The latter synaptic events occur at the onset and termination of a light pulse and are represented by the ON and OFF transients.

Cold-anesthetized flies were immobilized in dental wax (Bellevue); one glass microelectrode (the reference electrode) was inserted in the median head region between the eyes, and the other (recording electrode) was inserted into one eye just below the cornea (Heisenberg 1971; Sandrelli et al. 2001). After a 10-min recovery period, flies were dark adapted for 5 min and then submitted to several 2- to 5-s light pulse stimuli using a DC-powered LED mounted close to the head. Recorded signals were amplified with an intracellular amplifier (705, WPI Instruments), low-pass filtered (3 kHz), and then sent to a PC through an A/D converter (Digidata 1200, Axon Instruments). The output signals from the signal conditioner were also displayed on a digital oscilloscope (Tektronix) for on-line evaluation. The amplitude of ON and OFF transient responses and of the sustained response were measured using appropriate software (PCclamp 6.0, Axon Instruments).

**LATENCY MEASUREMENT IN THE GIANT FIBER PATHWAY.** Flies anesthetized on ice, were immobilized in a slab placed on the bottom of a perspex recording chamber and then covered, with the exception of the dorsal thorax, with dental wax (Bellevue). The flies were then submerged with adult fly physiological saline (Kawasaki et al. 1998). A piece of polyethylene tubing (1.0 mm ID, 1.5 mm OD, Clay Adams) connected to a hole in the bottom of the slab, continuously delivered fresh air to fly thorax spiracles, maintaining a constant physiological oxygen afflux to indirect flight muscle (IFM) fibers (Ikeda and Koenig 1988). Experiments were carried out at room temperature (22°C). After allowing the flies to recover from anesthesia for 15 min, an intracellular glass recording microelectrode (1.2 mm OD, 0.9 mm ID , tip diameter: 0.5 μm, 5–10 MΩ resistance, Hingelborg) was inserted into IFM fibers through a hole made in the
scutellum. Two tungsten stimulating electrodes (150-μm diam, WPI) inserted into the fly eyes, just underneath the cornea (1 electrode for each eye), were used to stimulate the fly brain with square pulse stimuli (0.2-ms duration, 4–15 V intensity) generated by a stimulator (Grass S88) connected to a stimulus isolation unit (Grass, SIU5). An Ag/AgCl ground electrode was placed in the bathing solution.

Synchronous transmission along the giant fiber (GF) pathway was explored by measuring the evoked action potentials in IFM following brain stimulation.


Usually, stimuli >7–9 V elicited short-latency IFM evoked action potentials, while stimuli <7–9 V evoked long-latency IFM action potentials. According to Engel and Wu (Engel and Wu 1996, 1998; Engle et al. 2000), short-latency responses are a consequence of direct giant neuron stimulation, whereas long-latency responses are due to the stimulation of giant neuron afferents. Signals from the recording electrode were amplified using an intracellular current-voltage-clamp amplifier (NPI Turbo Tec), fed to an A/D interface (Digidata 1200, Axon Instruments) digitized and recorded on a computer. Digitized data were analyzed using PClamp v.6 (Axon Instruments) and Mini v.5 (Synaptosoft). Measurements were made on 100–150 evoked responses from three to five fibers per fly. Latencies were calculated from the onset of the stimulus artifact to the beginning of the evoked response at the muscle membrane (Engel and Wu 1992).

**Giant fiber response to continuous stimulation**

Analysis of giant fiber response habituation was carried out as previously described (Engel and Wu 1996, 1998; Megighian et al. 2001) using intracellular recording techniques as described in the preceding text. Briefly, after continuous stimulation, the giant fiber IFM long-latency (LL) response habituates according to the criteria described by Thompson and Spencer (1966). According to Engel and Wu (1996), the appearance of habituation was recognized when five consecutive failures in the response to brain stimulation were recorded. The time of onset of habituation was established to correspond to the last stimulus that was followed by a response before the five consecutive failures. Intracellularly amplified IFM action potentials were fed to a window discriminator (WPI), which detected both stimulus artifact and action potential peak, generating a corresponding square wave as the output signal. The discriminator output was fed to an A/D converter (Digidata 1200, Axon) and recorded on a PC. Recorded signals were then analyzed using pClamp v.6 software (Axon Instruments, San Diego, CA). Each stimulation session consisted of 200 continuous stimuli delivered at a constant frequency. Four different stimulation frequencies (0.5, 1, 2, 3 Hz) were tested for each habituation analysis. Dishabituation was tested by directing an air puff to the fly head after having attained the five-failure criterion for the onset of habituation. The same protocol, based on 200 consecutive stimuli at the same frequencies used for habituation analysis, was used to study the short-latency giant fiber pathway response.

**Intracellular recording of miniature end-plate potentials (MEPPs)**

MEPPs were intracellularly recorded from the IFM fibers. Flies were prepared in the recording chamber as described in the preceding text. In this experimental protocol, however, the temperature of the extracellular saline was maintained at 19°C by means of a Peltier device. This temperature was chosen in view of the high frequency of MEPPs registered in IFM fibers at room temperature: the lower temperature was previously shown to reduce the MEPPs frequency, permitting a clearer discrimination of the single events (Ikeda and Koenig 1988). After allowing the flies to recover from anesthesia for 15 min, an intracellular glass recording microelectrode (1.2 mm OD, 0.9 mm ID, tip diameter: 0.5 μm, Hingelborg) was inserted into IFM fibers through a hole made in the scutellum. Signals from the recording electrode were amplified with a low noise voltage-clamp amplifier (NPI, Turbo Tec), fed to an A/D interface (Digidata 1200, Axon Instruments) and stored in a PC. Recorded signals were subsequently analyzed using the Mini Analysis software (Synaptosoft) or custom software written using the Python scripting language (www.python.org). MEPPs were recorded from two to three IFM fibers per fly. Each recording session consisted of 60 s of continuous recording. Particular care was taken to record MEPPs only from fibers with a clear, stable and normal resting membrane potential. Fibers with unstable resting membrane potential at the beginning or during the recording session were discarded.

**Electron microscopic analysis of IFM fibers**

For transmission electron microscopy, thoraces of caki mutants and WT flies were divided in half along the midline and immediately transferred to ice-cold fixation solution. The fixation solution contained 3% paraformaldehyde, 2% glutaraldehyde, 100 mM sucrose, and 2 mM EGTA in 0.1 M sodium phosphate buffer at pH 7.2. Pieces were fixed by immersion for 6 h and washed overnight at 4°C in 0.1 M phosphate buffer, pH 7.2. The next day, postfixation of specimens was carried out in 1% OsO4, followed by dehydration and embedding in Epon. Ultra-thin cross sections of IFM muscles were cut with a diamond knife and stained for 20 min in 2% aqueous uranyl acetate followed by 30 s of lead citrate. Sections were examined and photographed with a Philips 200 electron microscope.

**Statistical analysis**

All comparisons between mutant and WT flies were performed using a Welch-corrected t-test with Graphpad 3.0a for Macintosh (Graphpad Software, San Diego, CA), unless otherwise noted. The significance of the difference between genotypes in walking optomotor tests was determined by ANOVA and post hoc Tukey-Kramer tests. Flight test data were analyzed by means of the χ² test.

**RESULTS**

**Behavior**

Locomotor performance of adult caki flies had been previously tested using the Buridan paradigm (Martin and Ollo 1996) and a significant motor impairment was reported. To confirm and integrate this observation, we performed flight tests on the caki mutants (Drummond et al. 1991), and the results showed that the proportion of flies classified as “down,” i.e., flies that show impairments in their ability to fly (see METHODS), was significantly increased in caki mutants compared with WT flies (34.7% in caki vs. 17.7% in WT, respectively; n = 121 in caki and 119 in WT; P < 0.05).

**Giant fiber pathway response**

Given the alterations in locomotor and flight performance, we conducted a detailed neurophysiological analysis of the GF pathway, which constitutes the jump-and-escape response neuronal circuit in the adult fly (Fig. 1). Furthermore, the GF is an example of a complete and relatively simple neuronal circuit, which is amenable to an in situ electrophysiological analysis in whole-animal preparations.
GF neurons send long axons through the cervical connective to the thoracic ganglia, where they indirectly (via an interneuron) stimulate the IFM motoneurons. GF activity evokes an action potential in the IFM, and this can be recorded extra- or intracellularly (Engel and Wu 1996; Tanouye and Wyman 1980). The GF pathway can also be activated by direct brain stimulation using a pair of stimulating electrodes placed in the eyes (Gorzynski and Hall 1984; Tanouye and Wyman 1980). The IFM action potentials thus evoked, show a dual behavior: a short-latency (SL) response with stimuli $\geq 6$–8 V and LL responses with lower intensity stimuli. The SL response is attributed to the direct stimulation of the GF neurons or the GF itself, whereas the LL response results from the stimulation of GF afferents with the interposition of $\geq 1$ chemical synapse (Engel and Wu 1996). With repeated stimuli, LL response probability diminishes, showing all the characteristics of habituation (Engel and Wu 1996, 1998; Engel et al. 2000; Megighian et al. 2001; Thompson and Spencer 1966). Habituation is a form of non associative short-term learning due to mechanisms such as homosynaptic- or extrinsic-inhibition (Castellucci et al. 1970; Krasne and Teshiba 1995), with the functional significance of fine tuning the gain and sensitivity of a behavioral response (Engel and Hoy 1999; Fisher et al. 1997). SL responses do not show habituation (Engel and Wu 1996).

In caki mutants both SLs and LLs were significantly longer than in WT (Table 1). Furthermore, in caki flies, in some IFMs ($\sim 5\%$) displaying a normal resting membrane potential, the stimulation of the GF pathway failed to evoke a clear response, which instead appeared as a small depolarization; an example is shown in Fig. 2. This lack of response remained unchanged with time as well as with increasing stimulus voltage or duration, suggesting that synaptic transmission was impaired in these fibers. We further established that in caki individuals, the ability of the GF pathway to follow continuous stimulation was also impaired. In WT flies, SL responses did not show any failure up to stimulation frequencies as high as 150 Hz. By contrast in caki mutants, SL responses showed a significant response decrement even at low stimulation frequencies (0.5, 1, 2, 3 Hz). In the range of these test frequencies, no response failures, during continuous stimulation, were observed in WT flies, as already reported by other authors (Engel and Wu 1996) (Fig. 3). On the other hand, LL responses showed the typical phenomenon of habituation (Engel and Wu 1996): during continuous stimulation, the probability of LL responses diminishes with a time course which depends on the stimulus frequencies (Fig. 4). At stimulation frequencies between (1 and 3 Hz) the onset of habituation occurred significantly earlier in caki than in WT flies (see Fig. 4, A, B, and D). Considering the individuals which displayed a LL response after stimulation of the GF pathway, the percentage of caki flies which then showed habituation was lower than in the case of the WT controls (71.4 vs. 90%, respectively, determined from $\geq 120$ responses obtained from 40 flies). After the onset of habituation, dishabituation was observed by delivering a different stimulus (air puff). If dishabituation was not observed, the responses that attained the five consecutive failure criteria were discarded from analysis. However, as previously observed (Engel and Wu 1996), the efficacy of air puffs to achieve dishabituation varied in the sense that in some cases, one puff was not sufficient to obtain the desired effect. As a rough index of this efficacy, we analyzed the number of flies displaying dishabituation after the first air puff, and we observed that caki flies were less responsive than controls (40 vs. 61%, respectively).

### MEPPs

As a corollary to the electrophysiological analysis at the GF neuronal circuit level, and on the basis of the evidence of the association of CASK and its invertebrate homologue, with presynaptic membrane proteins (see INTRODUCTION), suggesting the possible involvement of cakilcamguk in neurotransmitter release, we examined spontaneous neurotransmitter release at the NMJ of the IFM. First of all, the structural and functional preservation of IFM in caki mutant flies was assessed respectively by electron microscopy (see Fig. 5) and by measurement of the resting membrane potential, which is a sensitive indicator of fiber membrane integrity. The resting membrane potential of IFM fibers of caki mutant flies was not significantly different from WT (79.1 ± 1.6 vs. 79.4 ± 2.6, respectively, data determined from 4 IFMs for each of 10 flies) suggesting, together with the electron-microscopy observation, that the caki mutation does not cause overt damage to IFM fibers. Spontaneous neurotransmitter release was monitored by intracellularly recording MEPPs in IFM fibers. Only fibers displaying a resting membrane potential of $\sim 70$ mV or less were

### TABLE 1. Short (SL) and long latencies (LL) of IFM responses following giant fiber pathway stimulation

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. Flies</th>
<th>No. Responses</th>
<th>SL</th>
<th>LL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>45</td>
<td>170</td>
<td>1.6 ± 0.3</td>
<td>3.8 ± 0.5</td>
</tr>
<tr>
<td>Caki</td>
<td>40</td>
<td>123</td>
<td>1.8 ± 0.5*</td>
<td>4.4 ± 0.7*</td>
</tr>
</tbody>
</table>

Measured short and long latencies of the action potentials intracellularly recorded in indirect flight muscle (IFM) fibers after brain stimulation of the giant fiber pathway. Latencies were calculated from the onset of the stimulus artifact to the beginning of the evoked response at the muscle membrane. Values are means ± SD (units are ms). Sampling frequency of 25 KHz allows a true sensitivity in the reported range. *$P < 0.001$ (Student’s t-test for unpaired data).
considered. Fibers with an oscillating or unstable resting membrane potential were discarded. Average MEPPs frequency in caki flies was significantly higher (23.8 ± 1 Hz, \( n = 22,205 \) in 10 flies) than in WT (5.8 ± 1 Hz, \( n = 4,268 \) in 10 flies; \( P < 0.05 \)). Furthermore average MEPPs amplitude was greater in caki flies (124.21 ± 0.42 \( \mu \text{V}, n = 22,205 \) in 10 flies) than in WT (76.47 ± 0.74 \( \mu \text{V}, n = 4,268 \) in 10 flies). Event amplitudes were compared by constructing normalized cumulative amplitude histograms and testing whether the observed differences were statistically significant using a Kolmogorov-Smirnov test. In addition, the broader distribution of the caki MEPPs amplitude suggests the frequent occurrence of multi-quantal neurotransmitter release (Fig. 6, A and B). Peaks corresponding to single and multi-quantal release were detected: they corresponded to multiples of 60 \( \mu \text{V} \), suggesting that the amplitude of a single “quantum” remains unchanged in caki mutants with respect to WT flies (Fig. 6, A and B). Spontaneous neurotransmitter release is a random process and as such can be described by a Poisson model. MEPPs latencies (i.e., intervals between successive MEPPs) obtained in caki mutants fit the exponential equation predicted by the Poisson model well, thus confirming that in all experimental conditions the random nature of spontaneous release is conserved. However, as expected on the basis of the higher frequency of MEPPs (see preceding text), the latency values were much shorter in caki mutants than in WT (Fig. 7).

Visual function: optomotor test and ERG

Finally, in view of the high expression of CAKI in the optic lobes of adult flies (Martin and Ollo 1996) and also to ascertain whether nervous function was impaired in other districts, we performed an analysis of the behavioral responses of the visual system by means of the walking optomotor test. The frequency of correct turns (see METHODS) was significantly lower in caki (66 ± 13\%, \( n = 10 \)) than in WT flies (83 ± 13\%, \( n = 10, P < 0.05 \)). Furthermore we also assayed the sensitivity of the optomotor response to light intensity. This was measured by recording the ability of a fly, which is kept suspended with its legs resting on a freely rotating Styrofoam ball connected to a tracking system, to follow a rotating pattern. In this paradigm, the responses of both caki mutants and WT flies decreased with decreasing light intensity (Fig. 8). However, whereas at high light intensity (points 10/10, 8/10, and 7/10 on Fig. 7), the response of caki flies is similar to WT, the response of caki was significantly worse than WT at light intensities below intermediate levels, suggesting an impairment of visual function at low light intensity in caki flies. To ascertain visual function directly, we performed electroretinogram (ERG) analyses in caki flies. ERG transient (on and off) and sustained responses were recorded in caki and WT flies (see Fig. 9, inset). As shown in Table 2, the sustained ERG response was higher in caki than in WT individuals. Moreover, although the on transient amplitude was similar in both WT and caki flies, off transients were significantly higher in caki than in WT individuals. These differences remained even when a high-intensity light stimulus was used instead of a low-intensity stimulus (data not shown). Adaptation of the sustained response, induced by continuous 1-Hz stimulation, was qualitatively similar in both caki and WT flies (Fig. 9), whereas the amplitudes of the ERG sustained responses were significantly higher in caki flies during the whole stimulation period.
Caki mutant rescue

In genetic rescue experiments, we employed a UAS-caki transgenic line bearing a single X-chromosome insertion of the transgene coding for the complete caki cDNA. Rescue was obtained by crossing UAS-caki; elav-GAL4; X-307 flies, the progeny of which consisted of caki mutant females in which the expression of the UAS-caki transgene was activated and caki mutant males in which the production of the WT caki transgene was not active. We thus evaluated the capacity of the panneuronal expression (presynaptically at the level of the NMJ) of the caki transgene to rescue the key phenotypical aspects displayed by the caki mutant.

In particular, female progeny of the preceding cross showed a practically fully rescued phenotype (not significantly different from WT) as regards the frequency of NMJ MEPPs as well as the giant fiber habituation response. In these individuals, IFM resting membrane potential was 83.4 ± 1.6 mV from seven flies. Average NMJ MEPPs frequency was 4.61 ± 1 Hz, n = 1,099 in 7 flies, whereas the average NMJ MEPPs amplitude was 87.57 ± 1.50 μV, n = 1,099, in 7 flies. The distribution of NMJ MEPPs amplitudes in caki “rescued” flies was restored to the WT values (Fig. 6C). In addition, as shown in Figs. 3C and 4C, the kinetics of SL and LL giant fiber responses to continuous stimulation was also clearly restored to near WT values.

On the contrary, male progeny from the same cross (in which the rescuing caki transgene is not expressed) displayed a clearly mutant phenotype for these key aspects (data not shown).

**Discussion**

The main result of this study is that neurotransmitter release is altered in caki mutants: a four times increase in spontaneous neurotransmitter release at the NMJ was revealed by the intracellular analysis of MEPPs in IFMs. MEPPs were more
frequent and also showed an increased mean amplitude. Furthermore distinct peaks on the MEPPs amplitude distribution curve suggest multi-quantal release in the presence of conserved quantal amplitude. Thus the absence of CAKI protein determines an impaired vesicle release control, leading to frequent multiple vesicular release. However, conserved quantal amplitude indicates that vesicle size remains unaltered, suggesting that CAKI is involved in the regulation of neurotransmitter release at the synaptic level. Importantly, the synaptic localization of CAKI has been previously shown (Bachmann et al. 2004; Lu et al. 2003).

As mentioned in the introduction, CASK is part of a presynaptic complex with VELIS and MINT-1 (Butz et al. 1998). CASK binds to the cytoplasmic tail of neurexin and, via protein 4.1, is linked to the actin cytoskeleton (Hata et al. 1996). CASK also binds N-type Ca^{2+} channels through its SH3 domains (Maximov and Bezprozvanny 2002). Such Ca^{2+} channels also bind MINT-1, which in turn is tightly connected to CASK (Ho et al. 2003; Sudhof 2001). Invertebrates express a single representative of the Cav2 Ca^{2+} channel family...
shows that the amplitudes of the sustained response and transient are larger after light stimulation of WT and stimulation period. Moreover, functional studies of the in vertebrate N- and 2003). Furthermore, electrophysiological characterization of the in present research. Therefore we conducted a detailed neurophysiological analysis of the GF pathway, which is a well-defined neuronal circuit responsible for a stereotyped "jump-and-flight" escape response in Drosophila. In caki mutants, GF pathway stimulation evoked an IFM response in 95% of IFM fibers. The remaining 5% of IFM fibers did not respond at all, suggesting the complete impairment of synaptic transmission in these IFM.

**Habitation**

Continuous stimulation of the GF pathway, even at low frequencies (0.5–3 Hz), caused transmission failure in both SL and LL responses. In WT flies, SL responses show no habituation but only fatigue at very high stimulation frequencies (100–150 Hz) (Engel and Wu 1992), thus the loss of response at low frequency (0.5–3 Hz) observed in caki mutants is suggestive of synaptic failure. In view of the preceding model for CAKI function, the transmission failure in caki mutants might be attributed to vesicular depletion, which in turn would be determined by uncontrolled spontaneous vesicle release. GF LL responses in WT flies show habituation even at low-frequency stimulation. The impaired LL response observed in caki mutants was reversible (as expected in the case of true habituation) by an alternative stimulation in ~70% of the cases, while in the remaining 30% it was not. The latter may be due to the depletion of neurotransmitter stores as proposed for SL responses, whereas in the former it could be due to precocious habituation of these flies. Precocious habituation in caki mutants might be explained by assuming that the mechanisms for short-term plasticity are activated earlier in 70% of the mutants than in WT flies.
ERGs and optomotor response

The “visual” part of the WT brain (optic lobes) shows high levels of CAKI (Martin and Ollo 1996): interestingly, caki mutants, in which CAKI is lacking, show quantititative ERG alterations and an impaired optomotor response. ERG alterations in caki flies are characterized by a significant increase (with respect to WT controls) in the sustained response (10.5 ± 1.1 vs. 7.0 ± 1.4 mV) and off transients (6.0 ± 1.3 vs. 4.2 ± 1.0 mV). The preceding measurements were conducted on 28 WT flies and 26 caki mutants. On the other hand, the presence of a qualitatively normal ERG response in caki flies confirms that these flies are receptive to visual stimuli. In fact Martin and Ollo (1996) had previously ascribed the altered Buridan test in caki flies to locomotor impairments and not to visual defects. It seems that photoreceptor sensitivity of caki flies to light stimuli is normal at standard light-intensities, but, as revealed by the optomotor test, photoreceptor sensitivity is decreased at low light intensity. Because the optomotor response is the result of neural processing of visual field movement (Bausenwein et al. 1986; Gotz and Buchner 1978), the impaired optomotor response in caki individuals suggests the presence of visual system alterations that interfere with the physiological flow and integration of the visual stimuli.

Conclusions

The results presented here provide strong support for a role of CAKI within the tripartite complex as a regulator of vesicle release. However, CAKI does not seem to be involved in determining vesicle size. Instead, the lack of CAKI likely results in an increased frequency of synaptic transmission failures. This hypothesis finds support in the observation that targeted presynaptic expression of CAKI, in a null CAKI background, abolishes this defect. As an alternative hypothesis, based on the expression of CAKI also at the postsynaptic level, Lu et al. (2003) suggested that CAKI could regulate postsynaptic CaMKII T306 autophosphorylation and thus modulate synaptic plasticity. Our data indicate a major role of CAKI in regulating the function of presynaptic elements, although we cannot exclude a postsynaptic role. Furthermore, because CAKI is highly evolutionarily conserved, it is likely that vertebrate CASK plays a similar role in the mammalian synapse.

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