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

Mauro A. Zordan,[2] Michele Massironi,[1] Maria Giovanna Ducato,[1] Geertruy te Kronnie,[1] Rodolfo Costa,[2] Carlo Reggiani,[1] Carine Chagneau,[3] Jean-René Martin,[3] and Aram Megighian[1]
[1]*Departments of Human Anatomy and Physiology and* [2]*Biology, University of Padova, Padova, Italy; and* [3]*Equipe Action Thématique et Initiatíve sur Programme et Equipe: Bases Neurales du Mouvement chez la Drosophile, Neurobiologie de l'Apprendissage, de la Mémozie et de la Communication, Centre National de la Recherche Scientifique, Unité Mixte de Recherche 8620, Université Paris Sud, Orsay, France*

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Zordan, Mauro A., Michele Massironi, Maria Giovanna Ducato, Geertruy te Kronnie, Rodolfo Costa, Carlo Reggiani, Carine Chagneau, Jean-René Martin, and Aram Megighian. *Drosophila* CAKI/CMG protein, a homolog of human CASK, is essential for regulation of neurotransmitter vesicle release. *J Neurophysiol* 94: 1074–1083, 2005. First published May 4, 2005; doi:10.1152/jn.00954.2004. Vertebrate CASK is a member of the membrane-associated guanylate kinase (MAGUK) family of proteins. CASK is present in the nervous system where it binds to neurexin, a transmembrane protein localized in the presynaptic membrane. The *Drosophila* homologue of CASK is CAKI or CAMGUK. CAKI is expressed in the nervous system of larvae and adult flies. In adult flies, the expression of *caki* is particularly evident in the visual brain regions. To elucidate the functional role of CASK, we employed a *caki* null mutant in the model organism *Drosophila melanogaster*. By means of electrophysiological methods, we analyzed, in adult flies, the spontaneous and evoked neurotransmitter release at the neuromuscular junction (NMJ) as well as the functional status of the giant fiber pathway and of the visual system. We found that in *caki* mutants, when synaptic activity is modified, the spontaneous neurotransmitter release of the indirect flight muscle NMJ was increased, the response of the giant fiber pathway to continuous stimulation was impaired, and electroretinographic responses to single and continuous repetitive stimuli were altered and optomotor behavior was abnormal. These results support the involvement of CAKI in neurotransmitter release and nervous system function.

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, glycophorins (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 (Bachmann et al. 2004; MacMullin et al. 2001). CASK 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). CASK 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 *Drosophil*a 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

Address for reprint requests and other correspondence: A. Megighian, Dept. of Human Anatomy and Physiology, Section of Physiology, University of Padua, Italy Via Marzolo 3, 35131, Padova, Italy (E-mail: aram.megighian@unipd.it).

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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′) *Eco*RI-*Xho*I (3′) restriction sites. The *Eco*RI site is located 474 bp upstream of the ATG and the *Xho*I 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 constantly 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 (Gotz 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/m^2. 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), fed to a signal conditioner (CyberAmp, Axon 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 (PClamp 6.04, 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

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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.

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

The GF pathway is a well defined neuronal circuit responsible of a stereotyped "jump and flight" escape response in *Drosophila* (Engel and Wu 1996, 1998; Engel et al. 2000; Levine and Wyman 1973; Tanouye and Wyman 1980; Thomas and Wyman 1984; Trimarchi and Schneiderman 1993, 1995).

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; Engel 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; Engel et al. 2000; 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). Each stimulation session consisted in 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 imbedding 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 electronmicroscope.

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 χ^2 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.

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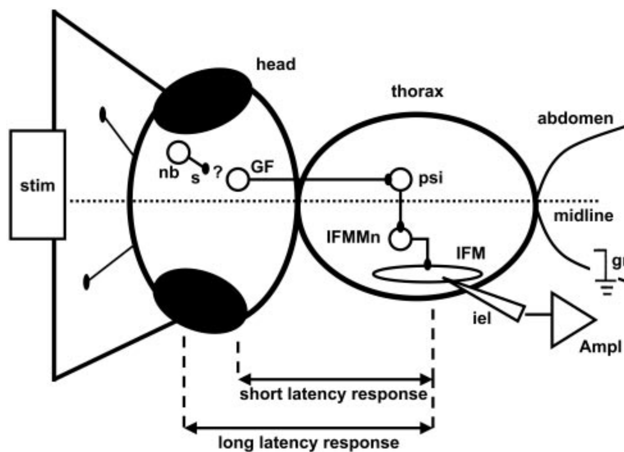

FIG. 1. Diagram of giant fiber pathway circuit in an adult fly. stim, stimulator; iel, intracellular microelectrode; ampl, amplifier; gr, ground electrode; nb, neuron cell body; s, synapse; GF, giant fiber neuron; ?, unknown number of synapses in the afferent pathway to the GF; psi, peripherally synapsing interneuron; IFM, indirect flight muscle; IFMMn, IFM motoneuron

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 (Gorczyca and Hall 1984; Tanouye and Wyman 1980). The IFM action potentials thus evoked, show a dual behavior: a short-latency (SL) response with stimuli >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 ≥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 (~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 ≥120 responses obtained from 40 flies). After the onset of habituation, dishabituation was obtained 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 *caki/camguk* 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 –70 mV or less were

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

Genotype	No. Flies	No. Responses	SL	LL
Wild type	45	170	1.6 ± 0.3	3.8 ± 0.5
Caki	40	123	1.8 ± 0.5*	4.4 ± 0.7*

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).

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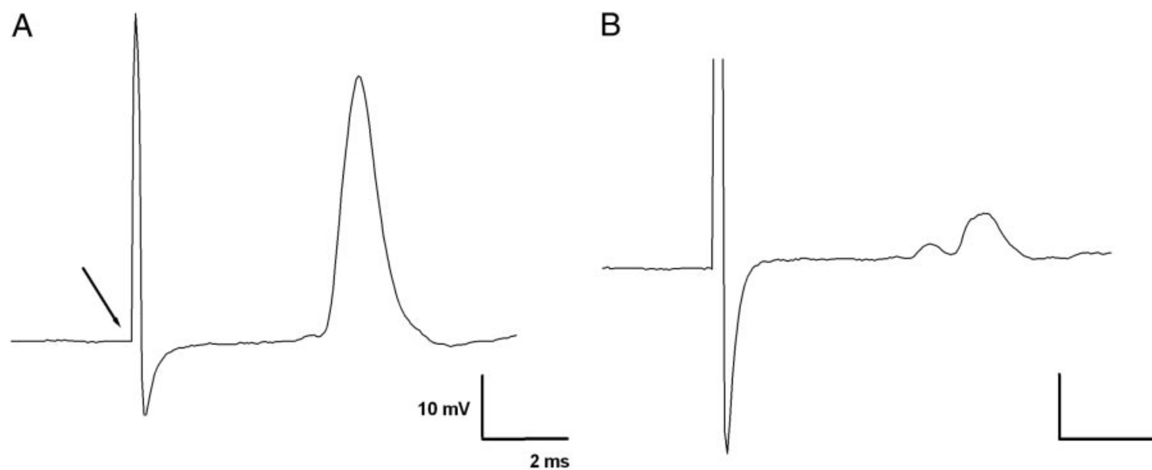

FIG. 2. Intracellular recording of IFM responses evoked by brain stimulation of the giant fiber pathway. *A*: an example of a typical spike observed in wild-type (WT) individuals; *B*: 1 case in which a lack of response was observed in a *caki* mutant (see text). Calibration bar units, as reported in *A* are the same in both figures. Arrow indicates the onset of the stimulus artifact.

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* mutants (124.21 ± 0.42 μV, $n = 22,205$ in 10 flies) than in WT (76.47 ± 0.74 μV, $n = 4,268$ 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 μV, 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).

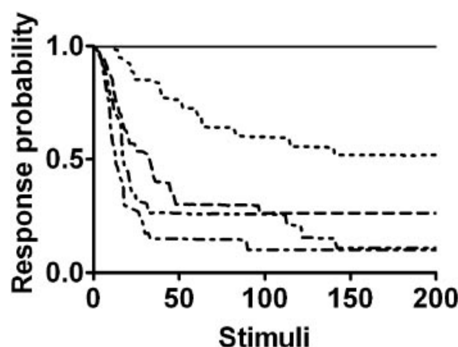

FIG. 3. Kinetics of the short-latency GFr pathway response. Frequency-dependent decrement of response probability is plotted using the mean of pooled responses from 16 WT, 23 *caki* mutants, and 7 *caki* "rescued" individuals. In WT and "rescued" flies, short-latency responses did not show any failure at any of the stimulation frequencies tested. In contrast *caki* mutants showed a clear response decrement even at low stimulation frequencies (0.5, 1, 2, 3 Hz). —, WT and *caki* rescued; *caki* mutant: · · · , 0.5 Hz; - - -, 1 Hz; · · -, 2 Hz; · - · , 3 Hz.

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.

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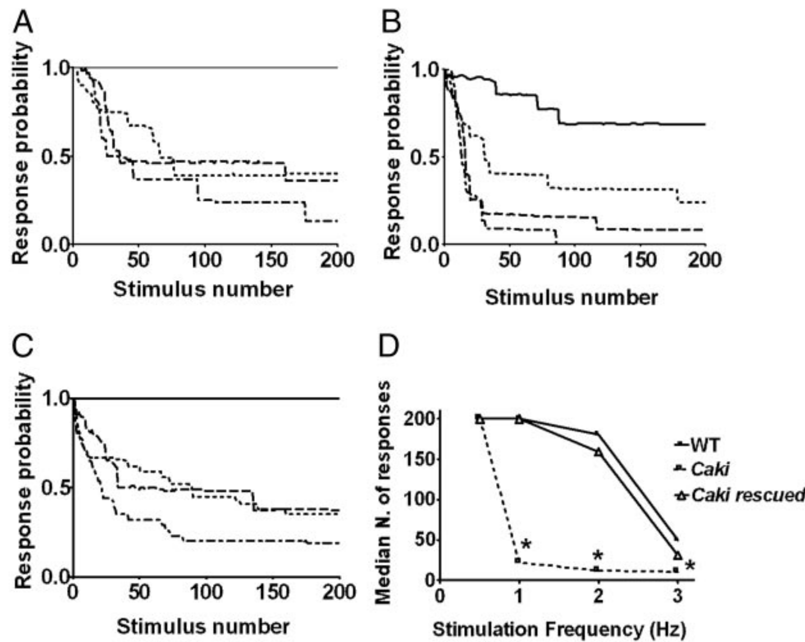

FIG. 4. Kinetics of habituation of the long-latency giant fiber pathway response. Frequency-dependent decrement of response probability. Mean of pooled responses from 18 WT (*A*), 30 *caki* mutants (*B*), and 7 *caki* "rescued" individuals (*C*). In *A–C*: —, 0.5 Hz; · · · , 1 Hz; - - -, 2 Hz; · - · , 3 Hz. Comparison of *A–C* shows that *caki* flies habituate consistently earlier than WT and rescued individuals at all stimulation frequencies as is clearly summarized in *D,* which shows the median number of responses at each stimulation frequency. In *D,* * indicates significant ($P < 0.05$, median test) differences between *caki* mutant and WT.

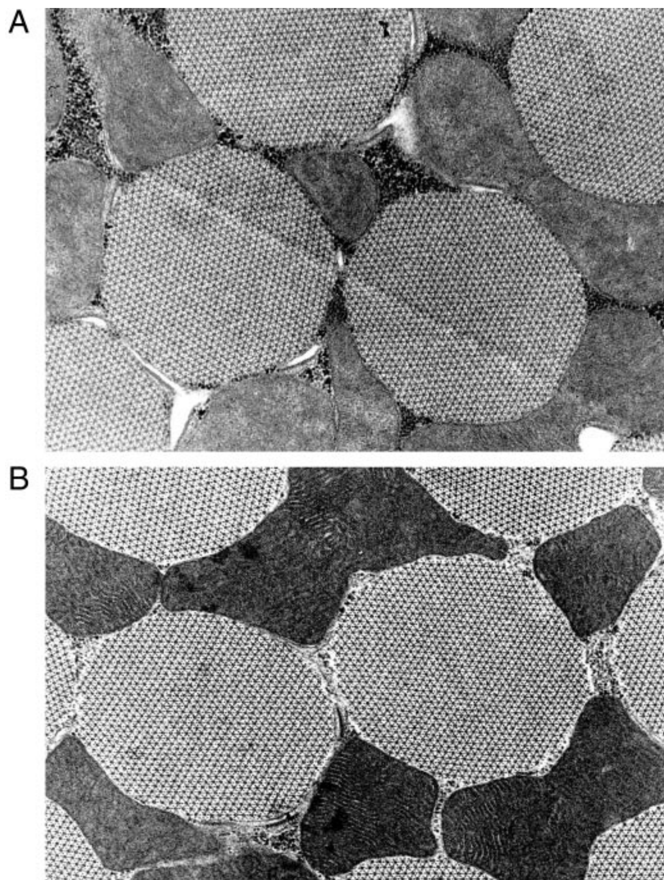

FIG. 5. Electron microscopy ultrastructure of myofibrils in WT (*A*) and *caki* mutant (*B*) IFM. Electron microscopic examination of muscle fibers shows no alteration in ultrastructural organization of the myofibrils in *caki* IFM muscles compared with WT. Cal bar: 0.5 μm.

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*;;X-313; individuals to *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, 7 IFM 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. 6*C*). In addition, as shown in Figs. 3*C* and 4*C*, 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

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FIG. 6. Normalized distribution of miniature end-plate potentials (MEPPs) amplitudes recorded from IFM fibers of WT (*A*), *caki* mutants (*B*), and *caki* "rescued" individuals (*C*). Amplitude histograms show a wider distribution for *caki* IFM MEPPs; lines show the multipeak nature of MEPPs distributions.

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 neurotrasmitter 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

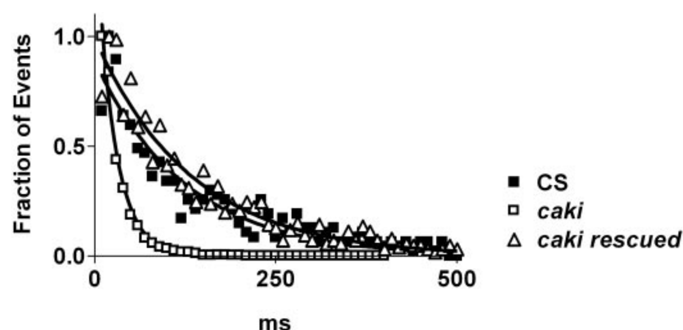

FIG. 7. Normalized distribution of MEPP latencies. Curve fitting assuming a mono-exponential decay. Spontaneous neurotransmitter release is a random process and as such can be described by a Poisson model. Latency values obtained in *caki* mutants in normal saline fit the exponential equation predicted by the Poisson model well, thus confirming that under all experimental conditions the random nature of spontaneous release is conserved. However, as expected on the basis of the higher frequency of MEPPs (see RESULTS), the latency values were much shorter in *caki* mutants in normal saline than in WT and "rescued" flies.

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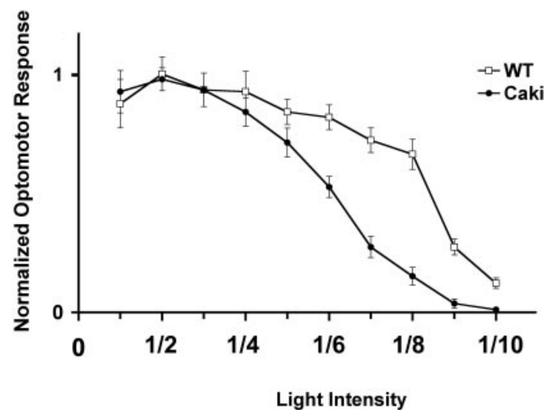

FIG. 8. *A*: optomotor response on the styrofoam ball, determined at different light intensity. The graph represents the normalized turning tendency of the fly rev.R/revF: (expressed as a value between 0 and 1) as function of the light-intensity. In WT flies, the turning tendency slowly decreases as light intensity decreases and in particular presents an abrupt decrement at a light intensity of ~8/10. In *caki* mutants the turning tendency is not different from WT at the 3 highest light intensities (10/10-6/10); however, this response rapidly decreases in parallel to the light intensity but more steeply than in WT flies. For WT flies, the data are from 4 individuals for a total of 57 runs. For *caki* mutants, the data are from 4 flies for a total of 73 runs.

(Spafford and Zamponi 2003; Spafford et al. 2003; Zamponi 2003). Moreover, functional studies of the in *Lymnea stagnalis* Cav2 homologue (which corresponds to vertebrate N- and P/Q-type Ca^{2+} channels) show that it behaves like an N-type Ca^{2+} channel (Spafford et al. 2003). It has also been proposed that CASK and/or MINT colocalize with the synaptic release machinery and may contribute to the appropriate targeting of presynaptic Ca^{2+} channels (Spafford and Zamponi 2003). Ca^{2+} channels and Ca^{2+} influx are, in fact, important regulators of evoked and spontaneous neurotransmitter release (Rizo and Sudhof 2002) in vertebrates and invertebrates (Fatt and Katz 1952; Grinnell et al. 1989; Kidokoro and Nishikawa 1994; Muchnik et al. 1975; Sweeney et al. 1995). Finally, MINT-1 interacts with MUNC-18, which plays an important role in neurotransmitter release, because *Munc-18* KO mice showed a complete block of spontaneous and evoked neuro-

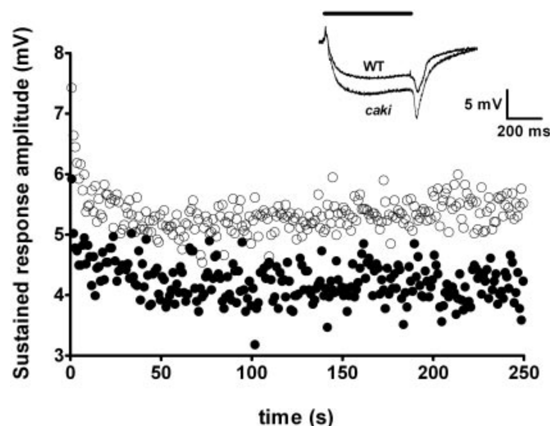

FIG. 9. kinetics of electroretinogram (ERG) sustained responses during 1-Hz continuous light stimulation. ●,WT; ○, *caki* mutants. Comparison of the 2 plots shows that the amplitude of the sustained response remains significantly higher ($P < 0.001$; Kolmogorov-Smirnov test) in *caki* flies during the entire stimulation period. *Inset*: representative ERG extracellular recordings after light stimulation of WT and *caki* mutant eyes. Comparison of the 2 traces shows that the amplitudes of the sustained response and OFF transient are larger in *caki* flies. Top horizontal bar represents the duration of light stimulation.

TABLE 2. *ERGs parameters after light stimulation*

Genotype	*n*	ON	Sustained Response	OFF
Wild type	28	1.6 ± 0.3	7.0 ± 1.4	4.2 ± 1.0
Caki	26	1.3 ± 0.5	10.5 ± 1.1*	6.0 ± 1.3*

Electroretinographic (ERG) response parameters extracellularly recorded after light stimulation of *Drosophila* eye. ON and OFF are the transient responses at the onset and offset of light stimulation, respectively, and they are due to the synaptic activation of second-order neurons in the visual pathway. The sustained response measures light-induced depolarization of photoreceptors (the sustained response of ERG) Values are mean ± SD (units are mV). *n* is number of animals. $*P < 0.05$ (Student's for unpaired *t*-test).

transmitter release at normally developed NMJs (Verhage et al. 2000).

Considering the structural framework described in the preceding text, the results of the present work suggest that CAKI is involved in the control of exocytosis: loss of CAKI causes an increased frequency of MEPPs, and is not involved in determining vesicle size: loss of CAKI does not modify the amplitude of single MEPPs but causes multiquantal release. Crucially, presynaptic rescue of CAKI expression, restores WT MEPP frequency. Our results further provide evidence that the impaired control of neurotransmitter release leads to the depletion of presynaptic vesicle stores and consequently to synaptic failure. The electrophysiological characterizations reported in this paper rest on the observation of motor and learning defects displayed by *caki* mutants (Lu et al. 2003; Martin and Ollo 1996) and by the impairment in flight ability, even in the presence of structurally normal IFM fibers (see Fig. 1) observed in the present research. Therefore we conducted a detailed neurophysiological analysis of the GF pathway, which is a well-defined neuronal circuit responsible of 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.

Habituation

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.

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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 *quantitative* 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 produces a depletion of presynaptic neurotransmitter stores, resulting 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 synapsis.

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