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Central Regulation of Locomotor Behavior of *Drosophila melanogaster* Depends on a CASK Isoform Containing CaMK-Like and L27 Domains

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ABSTRACT

Genetic causes for disturbances of locomotor behavior can be due to muscle, peripheral neuron, or central nervous system pathologies. The *Drosophila melanogaster* homolog of human CASK (also known as caki or camguk) is a molecular scaffold that has been postulated to have roles in both locomotion and plasticity. These conclusions are based on studies using overlapping deficiencies that largely eliminate the entire *CASK* locus, but contain additional chromosomal aberrations as well. More importantly, analysis of the sequenced Drosophila genome suggests the existence of multiple protein variants from the *CASK* locus, further complicating the interpretation of experiments using deficiency strains. In this study, we generated small deletions within the *CASK* gene that eliminate gene products containing the CaMK-like and L27 domains (CASK-β), but do not affect transcripts encoding the smaller forms (CASK-α), which are structurally homologous to vertebrate MPP1. These mutants have normal olfactory habituation, but exhibit a striking array of locomotor problems that includes both initiation and motor maintenance defects. Previous studies had suggested that presynaptic release defects at the neuromuscular junction in the multigene deficiency strain were the likely basis of its locomotor phenotype. The locomotor phenotype of the CASK-β mutant, however, cannot be rescued by expression of a *CASK*-β transgene in motor neurons. Expression in a subset of central neurons that does not include the ellipsoid body, a well-known pre-motor neuropil, provides complete rescue. Full-length CASK-β, while widely expressed in the nervous system, appears to have a unique role within central circuits that control motor output.

MOVEMENT disorders are characterized as any neurological condition affecting the speed, frequency, fluency, or ease of motion. Recent years have seen an explosion in the identification of susceptibility genes for these disorders, but far less is known about the mechanisms through which these genes contribute to proper locomotion (Scholz and Singleton 2008). A prevailing theory is that motor dysfunction may be the result of abnormal neural plasticity within specific central brain circuits (Pisani *et al.* 2005; Peterson *et al.* 2010). For this reason, synaptic proteins serve as attractive candidates for facilitating this plasticity, and a better understanding of these proteins could provide the link between genes and mechanism in movement disorders.

*M*embrane-*a*ssociated *gu*anylate *k*inase (or MAGUK) proteins are a family of proteins thought to act as anchors for multi-protein complexes. MAGUK proteins are characterized by having PDZ, SH3, and guanylate kinase (GUK) domains at their C termini. CASK (also known in Drosophila as camguk or caki) is a member of this group and has an N-terminal CaMK-like domain and two L27 domains (Dimitratos *et al.* 1997; Funke *et al.* 2005) upstream of the canonical PDZ, SH3, and GUK domains. The most recent release of the annotated Drosophila genome (version 5.3) has suggested the existence of a second transcriptional start site farther downstream in the *CASK* locus, encoding smaller proteins with a unique N-terminal region of unknown function in place of the CaMK-like and L27 domains (Tweedie *et al.* 2009). Little work, however, has been done toward characterizing the small isoforms, which we designate as CASK-α (curated as CASK-PA, -PD, -PE, and -PG in FlyBase) to differentiate them from the canonical CASK homologs (CASK-PB and -PF), which we call CASK-β. The addition of CaMK-like and L27 domains to the MAGUK core would be expected to give CASK-β additional unique functionality compared with the shorter proteins. In particular, CASK-β has previously been shown to regulate the autophosphorylation of calcium/calmodulin-dependent protein kinase II (CaMKII) in a calcium-dependent manner via an

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interaction with the CaMK-like domain (Lu *et al.* 2003; Hodge *et al.* 2006).

Recent work has implicated the disruption of the *CASK* gene in a number of behavioral phenotypes. One such phenotype is a defect in synaptic plasticity; flies lacking *CASK* were defective in courtship habituation, which is thought to be mediated via interaction with CaMKII (Lu *et al.* 2003). Loss of *CASK* also produces a gross locomotor deficit (Martin and Ollo 1996; Sun *et al.* 2009), but the cellular circuitry affected by loss of *CASK* has not yet been identified. Flies missing the *CASK* gene also show abnormally long responses to stimulation of the giant fiber pathway, the multisynaptic behavioral circuit that underlies the adult escape response (Zordan *et al.* 2005). While the molecular cause of the locomotor deficit following the disruption of *CASK* expression is still unclear, interaction via the PDZ domain with Drosophila *neurexin* (or *dnrx*) at the presynaptic terminals of the neuromuscular junction has been recently suggested as a candidate mechanism for the larval locomotor defect (Sun *et al.* 2009).

Despite the large number of studies that have previously attempted to elucidate the behavioral role of CASK proteins, only limited conclusions can be drawn from these experiments. The reason for this lies in the nature of the CASK null model used in these studies: CASK null flies were produced by crossing together two overlapping deletions [*Df(3R)x307* and *Df(3R)x313*] (Martin and Ollo 1996). The resulting trans-heterozygote flies are unhealthy, infertile, and contain additional heterozygous gene disruptions due either to extension of the deletion into neighboring genes (Martin and Ollo 1996) or to linked lethals outside the *CASK* region (Dimitratos 1999). An additional complication is that these overlapping deficiencies would be predicted to eliminate both the CASK-β and CASK-α isoforms, making it impossible to assign function to a specific form of the protein. To date, however, this null model has been the best available, as disrupting CASK in other species (*i.e.*, mammals) appears to be lethal (Atasoy *et al.* 2007).

To investigate the molecular and cellular role of CASK in behavior, we generated a new set of *CASK* mutants using imprecise *P*-element excision mutagenesis. Here we provide evidence for the adult expression of *CASK* transcripts encoding small isoforms and show that this new set of *CASK* mutants harbor deletions affecting only the well-characterized CASK-β forms. Using a courtship habituation assay, we show that, although these flies do have a higher-than-normal latency to initiate courtship (likely stemming from locomotor deficits), they habituate normally. To further characterize the locomotor defects, we use high-resolution locomotor tracking (Slawson *et al.* 2009) to identify specific parameters of locomotion that are defective in the mutant. These data demonstrate that the locomotor defect in CASK-β null flies is very complex and appears to affect multiple aspects of locomotion, including motor initiation, maintenance, speed, and acceleration. Although similar defects were seen in the trans-heterozygote null model of *CASK*, the magnitude of many of the defects in the deficiency flies appears to be more severe, likely owing to the loss of both known classes of *CASK* transcripts. We then used the Gal4/UAS binary expression system to perform tissue-specific rescue with CASK-β. We find that the motor deficits stem from loss of CASK-β expression in the central nervous system, but not in motor neurons as previously hypothesized. Surprisingly, locomotor behavior in the mutant can also be rescued without expression in the ellipsoid body, which is a well-characterized center for motor control in the insect brain.

MATERIALS AND METHODS

Fly strains: For all experiments, fly strains were maintained on standard cornmeal-dextrose agar media at 25° under a 12 hr:12 hr light:dark (LD) cycle unless stated otherwise. For the *P*-element excision screen, line EY07081 was generated as part of the Berkeley Drosophila Genome Project. This line contains a P{EPgy2} insertion in the first intron of the *CASK* gene at cytological position 3R(93F12) (Spradling *et al.* 1999; Bellen *et al.* 2004). The CASK deficiency lines *Df(3R)x307* and *Df(3R)x313* (Martin and Ollo 1996) were maintained over *TM6Tb-UbGFP* and crossed together to generate the trans-heterozygote null fly *307/313* (as verified by absence of markers and GFP). *Df(3R)exel6187* (Parks *et al.* 2004) was maintained over *TM6bTb* and verified by the absence of the humoral marker. For rescue experiments, the Gal4 driver lines *C155-Gal4* (Lin *et al.* 1994), *C164-Gal4* (Torroja *et al.* 1999), and *OK371-Gal4* (Mahr and Aberle 2006) were first crossed into the CASK-β null background. Expression of CASK-β in this background was accomplished by crossing these driver lines with the *UAS-CASK* 10.20 transgenic line, which was also first crossed into the CASK-β null background. *UAS-mCD8GFP* flies (Lee and Luo 1999) were crossed with each Gal4 strain to verify the presence of the drivers in the CASK-β null background. The validation of these lines was based on the presence of GFP in the resulting progeny. All lines were obtained from the Bloomington Drosophila Stock Center (Bloomington, IN), except *UAS-CASK* 10.20 (the cDNA used corresponds to the CASK-RB mRNA in FlyBase), which was provided by Peter Bryant (University of California, Irvine), and *C164-Gal4*, which was provided by Vivian Budnik (University of Massachusetts Medical School, Worcester).

***P*-element mutagenesis:** The *P*-element excision screen was performed using line EY07081, which harbors a *P* element inserted 1751 bp upstream of the *CASK* translational start site for the CASK-β transcript (Figure 2A). Excision of the *P* element was achieved using standard genetic methods (Greenspan 1997). Briefly, EY07081 flies were crossed with *w; +/+; MKRSΔ2-3Sb/TM2UbxΔ2-3* flies. The resulting F_1 female progeny were crossed with third chromosome double balancer flies (*w; +/+; TM3Sb/TM6B*), and the F_2 progeny from this cross were selected for *P*-element mobilization (determined by loss of eye color).

Antibodies: The polyclonal antibody used to visualize CASK-β was a kind gift from Gisela Wilson (University of Wisconsin, Madison). This antibody was raised in guinea pigs immunized with GST-$CMG_{152\text{-}897}$ as previously described (Marble *et al.* 2005). A monoclonal antibody was used for actin normaliza-

tion (Millipore). Both primary antibodies were used at a concentration of 1:1000. Both HRP-conjugated anti-mouse (GE Healthcare) and guinea pig (Jackson Laboratories) secondary antibodies were used at a concentration of 1:5000. The anti-CASK antibody was found to interact most strongly with epitopes in the CaMK-like and L27 domains by immunoblotting of CASK full-length and deletion proteins produced in transfected COS cells (data not shown). Immunoblots of wild-type adult head and body extracts failed to show CASK-α-size proteins over background, indicating either that this antibody is specific for CASK-β or that CASK-α is expressed at extremely low levels in adult flies (data not shown).

Immunoblots: Male and female flies were frozen and decapitated by vortexing. Heads were collected manually and homogenized in 1× SDS buffer. Samples were separated by 8% SDS-PAGE, transferred to nitrocellulose, and visualized on immunoblots. Bound secondary antibody was detected via enzymatic assay using ECL detection reagents (Amersham) and visualized with film using a Kodak X-OMAT 2000A Developer.

PCR deletion mapping: Genomic DNA was extracted from whole flies using the Puregene Core Kit A (Qiagen). Custom-designed primer pairs were each used to selectively amplify genomic regions of ~1 kb immediately upstream and downstream of the *P*-element insertion site (see supporting information, Table S1, for primers). PCR reactions were performed using a PTC-100 thermocycler (MJ Research) with Taq Polymerase PCR Master Mix (Promega). Deletions were identified by an absence of, or a reduction in size of, a PCR band. Chromosomal aberrations were confirmed by DNA sequencing (Genewiz).

RNA quantification and identification: RNA was extracted from equal numbers of anesthetized whole flies using Tri Reagent (Molecular Research Center). RNA samples were then denatured at 65° for 5 min and chilled on ice. RNA (5 μl) from each genotype was reverse-transcribed using the Superscript III First Strand kit (Invitrogen). Quantitative real-time PCR was performed using a Rotor-Gene 3000 Thermocycler (Corbett Research) and custom-designed primers specific for the *CASK* gene (Table S1). Quantification of the ribosomal gene *rp49* was used for normalization, which was done using primers provided by Michael Rosbash (Brandeis University). PCR amplification was accomplished using Platinum Taq Polymerase (Invitrogen) and visualized/quantified using SYBR green I dye (Invitrogen). For identification of alternative *CASK* transcripts, custom primers were designed to amplify from the conserved 3′ region to either the CaMKII-like 5′ region of CASK-β or the unique 5′ region of transcripts encoding CASK-α (Table S1).

Courtship habituation: Male flies were collected within 6 h of eclosion under anesthesia and sorted individually into test tubes containing yeast-free media. At 5 days old, a male was placed with a decapitated immature male in a mating chamber (8 mm in diameter, 3 mm in depth) and tested for courtship response. The courtship index (CI) is the percentage of time that the male spent in courtship activity during a 10-min observation period. Courtship latency is the time lag between pairing and initiation of the first courtship behavior. If a male did not start courtship during the 10-min observation period, a score of 600 was given. For the habituation assay, a male was paired either with a decapitated immature male "trainer" or with immature male pheromones over fine nylon mesh (Tetko, 3-180/43) for 60 min. Immediately after training, the male was transferred into a clean chamber and paired with a decapitated immature "tester" male and tested for courtship responses. As a sham, the males were kept alone in the chamber for the first 60 min and paired with a tester for 10 min. The habituation index was calculated by dividing the test CI by the mean of sham CIs. When the habituation index is 1, this indicates that there has been no courtship habituation because the courtship level of trained males is equivalent to that of sham trained males. Twenty or more males were tested for each condition. All courtship experiments were performed under dim red lights (>700 nm) in a controlled-environment room (25°, 70% humidity). For these experiments, lines from the *P*-element screen were backcrossed for five generations into the *white Berlin* (*WB*) background, and absence of CASK-β was verified by immunoblot (Figure S1).

Locomotor analysis: High-resolution video tracking for locomotion was performed as described previously (Slawson *et al.* 2009). Briefly, male flies aged 1–3 days were sorted into groups of 10 under anesthesia. Following a 2-day recovery period at 25°, flies were gently knocked into a square observation chamber based on a previously designed apparatus (Wolf *et al.* 2002). Acclimation to the chamber was allowed for 30 min. Following a brief pulse of air, fly movement was video-recorded for a 30-sec trial period. Ten seconds prior to the administration of the air pulse, the chambers were given five gentle taps on a padded surface to wake the flies up for testing. Locomotor analysis on the videos was performed using DIAS 3.2 software (Soll 1995; Soll *et al.* 2001). Instantaneous speed was calculated and consequently smoothed twice using a "5,15,60,15,5" Tukey smoothing window. The resulting data were then processed using a Matlab script to output a variety of locomotor parameters (percentage of inactivity, initial pause length, average bout length, average pause length, average speed, average maximum speed, average peak speed, average acceleration, maximum acceleration, and average deceleration). Data points from each parameter were analyzed individually to compute an average value for each fly within a given genotype. Individual means were then averaged together to produce a population mean, which is shown in each bar graph. For analysis of locomotor rescue experiments (Figure 6, Figure S3), all UAS- or Gal4-containing lines were normalized to control flies by dividing each individual average by the mean of control flies for a given parameter. Control performance for each parameter is denoted by a dotted black line in the bar graphs. At least eight trials over ≥3 days were performed for each genotype (unless otherwise noted). Some traces were broken or distorted due to collisions between flies in the arena. To avoid sampling errors caused by counting broken traces as multiple objects, traces consisting of <18 sec were excluded for all parameters, with the exception of the initial pause length parameter. For this parameter, all trials with at least 5 sec of uninterrupted tracking from time point 0 were included. All flies for this behavioral manipulation were raised and maintained on yeast-free media, and all experiments were performed in a controlled-environment room at 25° with 70% humidity.

Circadian rhythm analysis: Male flies aged 1–2 days were individually sorted into 65- × 5-mm glass tubes, each containing 5% agarose with 2% sucrose at one end. Following a 3-day entrainment period in a 12 hr:12 hr LD cycle, the activity of these flies was monitored for 5 days in LD using Drosophila Activity Monitors (Trikinetics, Waltham, MA). The flies were then subjected to 5 days in a 24-h dark cycle (DD) and analyzed in the same manner. Analyses were performed using a Matlab-based signal-processing toolbox (Levine *et al.* 2002). Within this program, autocorrelation and spectral analysis were used to determine period length. All experiments were performed at 25°. At least 60 flies were loaded into the activity monitors per genotype for each trial.

Imaging: For confocal imaging of adult brains, *mCD8-GFP;C164-Gal4* animals were dissected in phosphate buffered saline, fixed for 15 min in 4% paraformaldehyde, and mounted using Vectashield. Images of the brains were taken on a Leica

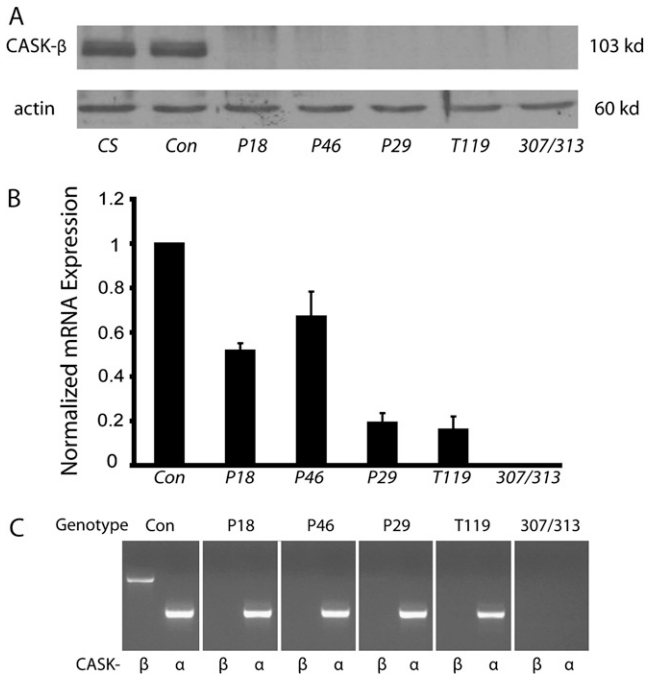

Figure 1.—Characterization of *CASK* mutants. (A) Immunoblot of candidate *P*-element excision lines shows that lines P18, P29, P46, and T119 appear to be null for CASK-β protein, while the precise excision control line (labeled as *Con*) has wild-type (*CS*) levels of the protein, consistent with a precise excision of the *P* element. *307/313* is shown as a negative control, and actin normalization was used as a loading control for all samples. (B) Quantitative real-time PCR with primers specific to the distal part of the *CASK* transcript shows relative mRNA expression (normalized to RP49). A clear reduction of mRNA levels can be seen in all four candidate mutants, while no mRNA is seen in *307/313* flies. (C) RT-PCR was performed on cDNA using primers specific for either the CASK-α or the CASK-β isoform. Lines P18, P46, and T119 have no expression of CASK-β, while line P29 has a small amount of transcript (compared with control levels). All four mutants express the CASK-α mRNA.

TCS SP5 confocal microscope at ×20 magnification. For fluorescent imaging of the peripheral sensory cells, pictures of live *mCD8-GFP;C164-Gal4* flies were taken under anesthesia with a Nikon D100 camera mounted on a Leica MZFLIII fluorescent dissecting microscope.

Statistics: Data from all behavioral manipulations were analyzed using JMP 5.0.1.2 software for Macintosh (SAS Institute, Cary, NC). For courtship experiments, each CI was subjected to arcsine square-root transformation to effect an approximation of normal distribution. One-way analysis of variance (ANOVA) with each indicated condition as the main effect was performed on the transformed data. Post-hoc analysis was done using Fisher's PLSD test. For locomotor experiments, one-way ANOVA with each indicated parameter as the main effect was used. Post-hoc analysis was performed using a Tukey HSD test. Multivariate analysis of variance (MANOVA) with a discriminant function analysis was performed on all locomotor parameters and genotypes. This analysis is presented using a centroid plot, where each oval represents a 95% confidence interval about a centroid value for the location of each genotype in multivariate space. The amount of overlap between centroids indicates the degree of statistical significance of the aggregate phenotype between genotypes (*i.e.*, non-overlapping centroids are significantly different from each other). In all behavior figures, the bars in each graph represent means ± SEM with significant differences between groups indicated by different letters ($\alpha < 0.05$). *F*-values for all ANOVAs are listed in Table S2.

RESULTS

***P*-element excision eliminates the CASK-β isoform:** To target the *CASK* locus, we used a strain of flies from the Berkeley Drosophila Genome Project that contains a P{EPgy2} element within the first intron at the 5′ end of the gene (Spradling *et al.* 1999; Bellen *et al.* 2004). The *P* element was mobilized, and 259 candidate excision lines were identified by their lack of eye color (see materials and methods). These lines were screened by immunoblot to determine if they had alterations in CASK protein expression (Figure 1A). Membranes were probed with antibodies against CASK (top) and actin (bottom) for normalization. Although the CASK antibody used in this study was made against an almost full-length CASK-β protein, the strongest interaction is with the N-terminal CaMK-like and L27 domains (data not shown). For this reason, only *CASK* products containing these motifs (*i.e.*, CASK-β) can be visualized on immunoblots of fly tissue. Four lines showed apparent reductions in expression. One candidate that had a precise excision of the *P* element was maintained as a control line for genetic background. Protein levels in this line, which are indicated as "*Con*" in Figure 1A, are comparable to those of Canton-S (*CS*) wild type. As a positive control, this blot also shows the previously identified transheterozygous null [*Df(3)x307/Df(3)x313*, hereafter referred to as *307/313*], which lacks the majority of the *CASK* locus (Martin and Ollo 1996).

Deletions within the *CASK* locus reduce, but do not eliminate, *CASK* mRNA: To further characterize the candidates, quantitative real-time PCR was utilized to assay mRNA levels. Primers specific for the spliced 3′ end of the *CASK* cDNA-coding region were chosen to prevent genomic DNA contamination. These primers recognize mRNAs encoding both CASK-β and CASK-α. Results from the quantitative real-time PCR are shown in Figure 1B. Ribosomal protein rp49 mRNA was used as a normalization control. These data reveal a clear reduction in *CASK* mRNA transcript levels in all four mutant lines, as compared with the control line. There are two lines with substantially reduced message (P29 and T119), and two lines with a smaller reduction in mRNA levels (P18 and P46). As expected, *307/313* flies had no mRNA expression.

To address the issue of whether or not these mutants expressed CASK-α-encoding transcripts, cDNA from whole flies was amplified using the right-side real-time PCR primer (which recognizes mRNAs for both forms), coupled with an isoform-specific left primer. Left primers were specific for either the 5′ end of the CaMK-like region found in the N terminus of CASK-β

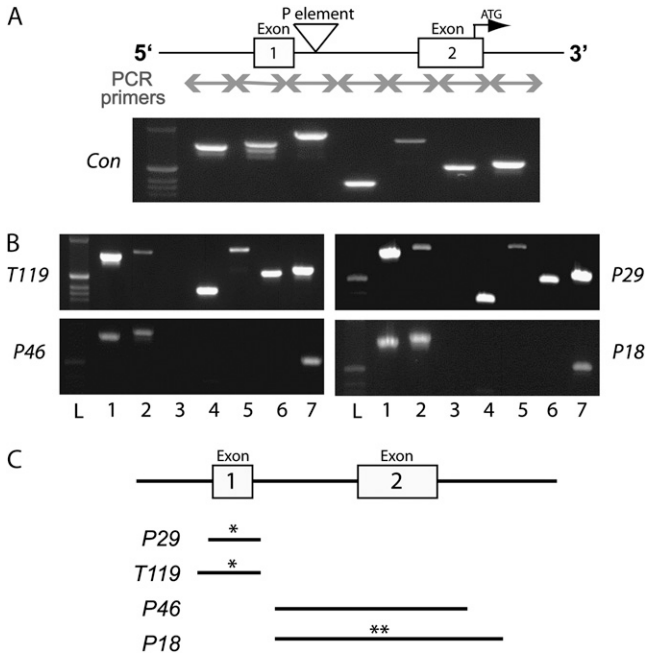

Figure 2.—Deletion mapping of *CASK*-β mutants. PCR across the genomic region surrounding the original *P*-element insertion site was used to map the candidate lines. (A) Schematic shows the genomic region, with arrows denoting the location of primer pairs. Each primer produces a uniquely sized band in wild type. The precise excision line (Con) is shown. Molecular weight marker is shown in the far left lane; the brightest ethidium band corresponds to 506 bp. (B) Deletion maps from the four candidate lines are shown. Loss of a band signifies deletion of the genomic region corresponding to one or both primers. Lines T119 and P29 are missing band 3, which corresponds to the region immediately surrounding the original insertion site. Lines P18 and P46 are missing band 3, as well as downstream bands 4–6. (C) Sequencing confirmed PCR deletion mapping and showed that P29 and T119 harbor deletions of exon 1 (UTR) and a small part of the promoter region. P18 and P46 harbor deletions of exon 2, which consists of both UTR and coding sequence. P29 and T119 both have small insertions elsewhere in the genome (denoted by a single asterisk), while P18 has an additional insertion at the deletion site (denoted by a double asterisk).

mRNA or the unique region presumed to lie in the N-terminal region of CASK-α (see Table S1 for primers). PCR products run on an agarose gel (Figure 1C) demonstrate that wild-type flies express both types of transcripts, while three of the lines (P18, P46, and T119) lack the full-length mRNA for CASK-β. The P29 strain appears to have a very faint band at this molecular weight, indicating that it is not a complete mRNA null and is likely a severe CASK-β hypomorph. *307/313* expresses neither mRNA. This implies that some of the residual *CASK* mRNA in the new mutants corresponds to the CASK-α transcript, although there are also truncated CASK-β mRNAs that can be detected with other primer sets (data not shown).

Mapping of genomic lesions: The specific genomic changes induced by *P*-element excision were probed by PCR of genomic DNA. Small chunks of the genomic region surrounding the original *P*-element insertion site were selectively amplified and subsequently run on an agarose gel. Failure of one or more regions to produce a band when visualized on the gel would indicate a deletion of DNA containing one or both of the primer sequences. Figure 2 shows PCR amplification of the genomic regions corresponding to the schematic (Figure 2A). As expected, the control line clearly has all seven adjacent genomic regions intact. Both line P29 and line T119 are missing band 3, which corresponds to the region surrounding the original *P*-element insertion site (Figure 2B). This area encompasses a large portion of the 5′ UTR of the long isoform transcript, as well as a small chunk of the promoter region. The coding region in these two mutants, however, remains intact. Sequencing of PCR products that span the deletions confirmed that P29 harbors a 650-bp deletion, while T119 has a 701-bp deletion, both upstream from the coding region. Sequencing also revealed that both these lines harbor a small duplication of the deleted region elsewhere in the genome (denoted by a single asterisk in Figure 2C), but these duplications do not appear to rescue CASK expression.

Both P18 and P46 lines are missing bands 3–6 (Figure 2B), which correspond to a large portion of the 5′ UTR and the entire first coding exon of the *CASK* gene. This exon contains the translational start and the first 20 codons of the open reading frame. If a protein were to be made in these mutants, it would most likely start within the CaMK-like domain at the next methionine (M68) and have a molecular weight of ~95 kDa. Because no such protein is seen in the immunoblots (Figure 1A), we believe that these mutants are null for isoforms containing CaMK-like and L27 domains. Sequencing of PCR products spanning the deletions indicates that P18 contains a 2624-bp deletion, while P46 contains a 2284-bp deletion. The P18 line, which is the largest deletion, also has a small piece of a roo transposon inserted at the deletion site, further lengthening the gap between the promoter region and the remaining coding exons (denoted by a double asterisk in Figure 2C).

While the P18 line has the most severe disruption of the *CASK* genomic region, it is fertile and significantly healthier than *307/313* flies (data not shown), potentially allowing for a more interpretable assessment of the roles of CASK-β in behavior. *307/313* animals lacking most of the *CASK* locus, as well as having lesions in other genes, have previously been shown to display motor deficits (Martin and Ollo 1996; Zordan *et al.* 2005; Sun *et al.* 2009) and difficulty habituating (Lu *et al.* 2003; Zordan *et al.* 2005). The causal role of mutation of *CASK* in these behavioral defects, as well as the role of the two gene products, had never been rigorously tested using a single-gene mutant or genetic rescue, nor had the cellular locus of CASK action been determined.

CASK-β is required for normal levels of courtship of immature males: Wild-type males will court both females and immature males that do not contain aversive mature-male pheromones (Gailey *et al.* 1982, 1986). When a male initiates courtship of any type of target, he evaluates the suitability of that target using multiple sensory modalities, including vision, chemosensation, and audition, and integrates that information with his previous experience to produce an appropriate behavioral response (reviewed in Griffith and Ejima 2009). Courtship of immature males is similar to courtship of females in that both are reproducibly vigorous behaviors, but these types of courtship differ in several ways, including the chemical nature of the stimulatory pheromone. As shown in Figure 3A, when *CASK*^{P18} males were tested for immature-male courtship, they showed a significantly lower overall level of courtship than the excision control ($P < 0.0001$). Furthermore, the courtship initiation latency of the mutant was also significantly longer than the control (Figure 3B, $P < 0.01$), indicating that the mutant had trouble locating and orienting to the immature male. Since all courtship experiments were performed in the dark, this phenotype cannot stem from differences in visual acuity. Under such conditions, initiation is driven by a combination of olfaction and mechanosensation (Ejima and Griffith 2008). Our data indicate that *CASK*^{P18} flies may have either reduced olfactory sensitivity to immature male pheromones or defective mechanosensation.

CASK-β mutants have normal ability to sense and habituate to immature-male pheromones: One of the most interesting differences between courtship of females and immature males is the fact that a mature male can habituate to immature male pheromone (Gailey *et al.* 1982). Previous experience with an immature male or exposure to an extract of immature male cuticle will reduce subsequent courtship of young males. *307/313* flies display a defect in this type of habituation (Lu *et al.* 2003). To test the ability of the CASK-β mutants to sense immature male pheromone, we performed the immature-male habituation assay on the *CASK*^{P18} males using either courtship of an immature male or exposure to pheromone extract as a habituating stimulus. Figure 3C shows a habituation index (ratio of courtship after exposure to mean courtship level of sham-habituated controls) for each genotype. A habituation index of 1.0 indicates that there was no courtship reduction after exposure, *i.e.*, no habituation. Among both control and *CASK*^{P18} males, exposure to immature male trainers resulted in a significant reduction of immature-male courtship (Figure 3C, left). This suggests that CASK-β is not essential for courtship habituation. It should be noted that *307/313* flies were omitted from this analysis because there was too little overall courtship to construct a reliable habituation index. In both control and CASK-β null mutants, exposure to immature male pheromone alone could effect courtship reduction

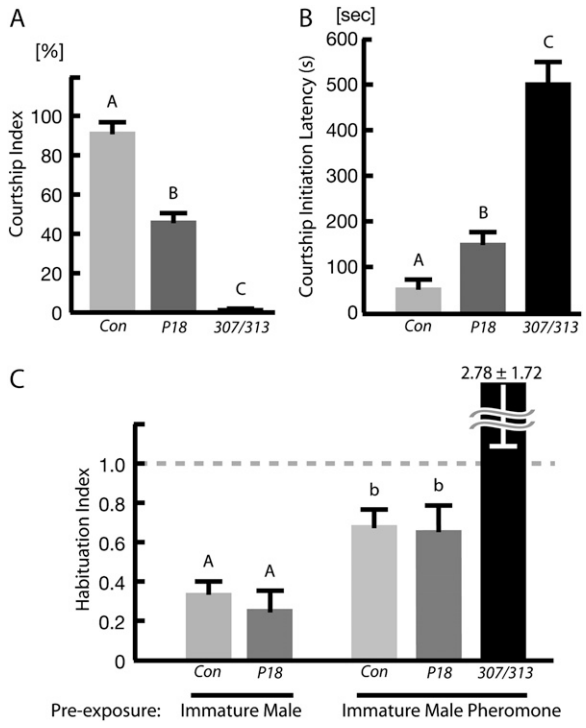

Figure 3.—*CASK*^{P18} mutants display reduced levels of courtship, but not a reduction of pheromone sensitivity. (A) Loss of CASK-β lowers the courtship index ($P < 0.0001$ for all pairwise comparisons) and (B) increases the latency to initiate courtship ($P < 0.01$ for all pairwise comparisons) in both *CASK*^{P18} and *307/313* flies. The behavioral changes in the *307/313* flies are more severe than in *CASK*^{P18} flies in both cases. Letters signify significant differences between groups. (C) Courtship habituation assays performed either with immature male exposure (left) or with direct exposure to isolated immature male pheromone (right) produced no plasticity defect in *CASK*^{P18} flies compared to control flies. The courtship index was so low with *307/313* flies that a valid habituation index could not be constructed for the exposure to the immature male condition, but these flies did demonstrate a significantly larger and more variable habituation index than either control or *CASK*^{P18} flies in the direct exposure condition ($P < 0.05$). Statistical significance is represented by capital letters for immature male habituation and small letters for pheromone exposure habituation because ANOVA and pairwise comparisons were performed separately for these two conditions.

(Figure 3C, right), indicating that the *CASK*^{P18} male is capable of sensing the immature male pheromones and habituating to them. In contrast, *307/313* male flies failed to habituate with pheromone exposure, as shown by the significantly larger and more variable habituation index, which is consistent with previous findings (Lu *et al.* 2003).

These data suggest that CASK-β is not required for non-associative olfactory learning, as had been previously suggested. The large difference seen in basal courtship behavior between *CASK*^{P18} and *307/313* may be due to the fact that *307/313* flies lack the CASK-α isoforms, while the new mutants still express them. It is also possible that the difference in phenotype is due to

the fact that *307/313* flies result from the combination of two deficiencies. These chromosomal aberrations either lack, or have mutations in, other genes in addition to *CASK* (Martin and Ollo 1996; Dimitratos 1999), some of which might genetically interact and be important for proper habituation. Another possibility is that the severe courtship initiation defect seen in these flies is due to an inability to get oriented toward the courtship target in the allotted time window. This idea is supported by previous reports that suggest that *307/313* flies have a severe motor defect (Martin and Ollo 1996; Zordan *et al.* 2005; Sun *et al.* 2009) and might have trouble initiating any kind of behavioral response involving movement. The courtship index of the *307/313* males to immature males was indeed extremely low (Figure 3A), and many of these males never initiated courtship during a 10-min observation, resulting in a very large average courtship initiation latency.

CASK-β mutants have a complex locomotor deficit: To elucidate the specific nature of the locomotor deficit in the $CASK^{P18}$ mutants, we used a high-resolution video tracking assay, which has been described previously (Slawson *et al.* 2009). The movement of a group of flies was videotaped in an open arena for 30 sec following a brief air pulse, which has been shown to initiate normal locomotion in this paradigm. Use of an air pulse has been used in other protocols to manipulate parameters of locomotion in similar ways (Yorozu *et al.* 2009). The Dynamic Image Analysis System (DIAS 3.2) tracking software was applied in conjunction with a Matlab analysis script to look at a variety of parameters of motion, including measures of speed, acceleration, activity, and bout structure.

Visual inspection of the unprocessed movement of flies during a 30-sec trial shows obvious differences between genotypes (Figure 4A). The percentage of inactivity (or time spent standing still) was calculated for each genotype (Figure 4B). $CASK^{P18}$ flies exhibit significantly less movement than control flies, while $CASK^{P18}$ heterozygote flies fall somewhere in the middle of the two groups ($P < 0.05$) (Figure 4B, left). This suggests that CASK-β plays a dose-dependent role in locomotion.

307/313 flies demonstrate a more severe increase in the percentage of inactivity, but $CASK^{P18}$/*Df(3)x313* flies show activity levels similar to $CASK^{P18}$ (Figure 4B, right; $P < 0.05$). Similar trends can also be seen with $CASK^{P18}$ in *trans* with other deficiencies, such as *Df(3)x307* and *Df(3)excel6187* (Figure S2). This implies that the enhanced severity of inactivity seen in *307/313* flies is due to some additional genetic aberration in these two deficiencies (including but not limited to loss of CASK-α) and is not simply a result of the loss of CASK-β. To characterize the nature of these behavioral differences, MANOVA was used to determine the contribution of specific parameters (such as speed, pause length, etc.) to the overall behavioral phenotype of each genotype. Figure 4C shows a centroid plot depicting this analysis, where each oval represents the location of each genotype in multivariate space. The relative locations of each centroid are placed according to how important the various locomotor parameters are in maximizing the differences between the groups, while minimizing the within-group variation. The length and direction of each line vector indicate how important each variable is to separating the centroids from each other. Whereas control, $CASK^{P18}$/+ heterozygote, and $CASK^{P18}$ homozygote flies appear to be separated only along a single axis (shown as vertical in the centroid depiction), *307/313* flies seem to be separated from other groups along two different axes (the vertical axis and a second axis shown as the horizontal plane). This indicates that *307/313* flies do not simply harbor more severe manifestations of the same locomotor deficits as $CASK^{P18}$ flies, as this would be depicted by the alignment of all centroids along the same multivariate plane. Since the vertical axis of multivariate space is largely determined by measurements of speed and acceleration, it can be assumed that these parameters define the largest differences between control and $CASK^{P18}$ flies. The horizontal plane of multivariate space, however, is dominated heavily by measurements of time spent in or out of motion, suggesting that although the *307/313* flies present a similar locomotor phenotype as $CASK^{P18}$ flies, they also suffer from additional behavioral deficits that further affect levels of activity. This indicates that the nature of the deficit in the *307/313* flies is in fact qualitatively different from that of $CASK^{P18}$ flies and may reflect a role for CASK-α proteins or possibly for variation in other genes on the deficiency chromosomes.

Figure 5 shows an individual breakdown of the nine additional parameters of locomotion depicted in Figure 4C (see figure legend for full parameter descriptions). Initial pause length and average pause length (Figure 5, top row) were consistent with each other and demonstrate that loss of the CASK-β protein appears to lengthen an animal's pause durations in a dose-dependent fashion. This parameter can also be thought of as a measure of motor initiation. The average bout length (Figure 5, top row) of these animals seems to be inversely correlated with the pause length. As the amount of CASK-β decreases, the length of each bout of activity also decreases, indicating an inability to maintain locomotion once initiated (motor maintenance). Average speed, average maximum speed, and average peak speed (Figure 5, middle row) all followed the same trend where loss of CASK-β expression caused a dose-dependent decrease in the resulting speed. Average acceleration, average deceleration, and maximum acceleration (Figure 5, bottom row) also behaved similarly; loss of CASK-β slowed acceleration and deceleration in a dose-dependent manner.

In all conditions, the performance of $CASK^{P18}$ flies was significantly different from that of genetic control

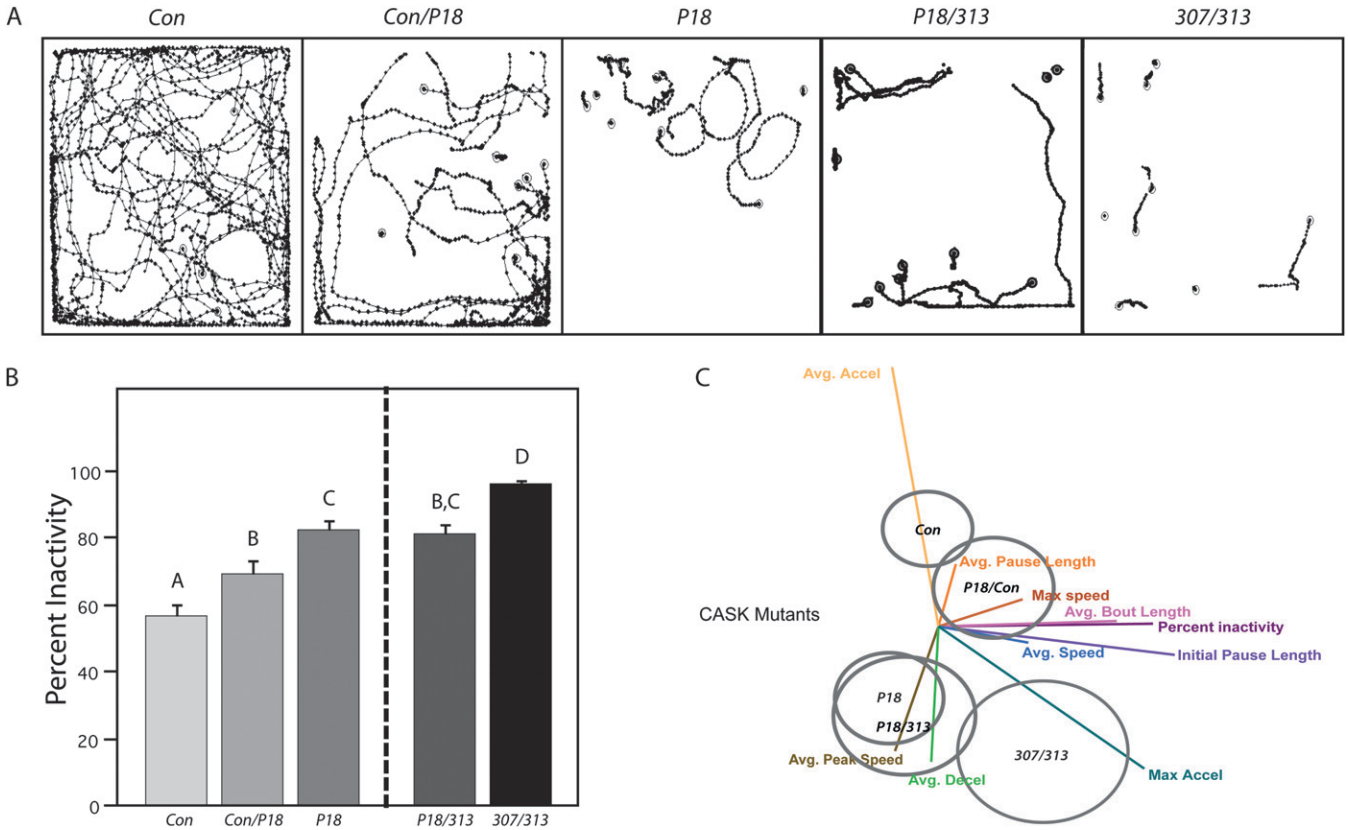

Figure 4.—*CASK*^{P18} mutants display a unique locomotor deficit. (A) Locomotor traces from a 30-sec trial are shown for five groups: *Con* (wild-type precise excision control), *Con/P18* (control/*CASK*^{P18}), *P18* (*CASK*^{P18}), *P18/313* [*CASK*^{P18}/*Df(3R)x313*], and *307/313* [*Df(3R)x307/Df(3R)x313*] flies. (B) Percentage of inactivity (total percentage of time standing still) is shown for the five genotypes and demonstrates a dose-dependent increase of inactivity with loss of CASK-β. *P18/313* flies have activity levels similar to those seen in *CASK*^{P18} and heterozygote flies, while the *307/313* flies appear to have a much more severe increase in inactivity, together suggesting that the overlapping deficiencies have additional elements contributing to the locomotor defect. Letters signify significant differences between groups ($P < 0.05$). (C) Centroid plot for MANOVA depicting the relative positions of each genotype (canonical centroids) in multivariate space. The combination of the length and direction of the vector lines indicates how strongly the behavioral parameters differentiate the genotypes.

behavior, with *CASK*^{P18}/+ heterozygote flies performing at an intermediate level. Furthermore, there were no significant differences between *CASK*^{P18} flies and *CASK*^{P18}/*Df(3R)x313* in any of the conditions ($P < 0.05$). Similar trends were also seen when comparing *CASK*^{P18} flies with other *CASK*^{P18}/Df flies (data not shown). This indicates that the additional mutations present in these deficiencies do not act dominantly to affect locomotion. *CASK*^{P18} flies appear to demonstrate a locomotor phenotype that manifests as a complex deficit involving problems with motor initiation, motor maintenance, speed, and acceleration. Along with this, however, all parameters involving inactivity (*i.e.*, initial pause length and average pause length) were significantly different between *307/313* and *CASK*^{P18} animals ($P < 0.05$), with all other parameters trending toward significance. This finding supports the multivariate analysis (Figure 4C) and further suggests that the phenotypes observed in previous studies using *307/313* animals that are not seen in *CASK*^{P18} likely result from loss of CASK-α, although we cannot rule out a genetic interaction between the additional mutations on these two deficiency chromosomes. It should be noted that, although *CASK*^{P18} is the only mutant presented here, other precise and imprecise *CASK* excision lines have been run in this assay and show consistent phenotypes (data not shown).

Locomotor defects can be rescued by CASK-β expression in the nervous system: To confirm that these motor phenotypes stem from loss of CASK-β, we assayed the ability of a CASK-β cDNA transgene (used previously in Hodge *et al.* 2006) to rescue locomotor performance using the UAS/Gal4 binary expression system (Fischer *et al.* 1988; Brand and Perrimon 1993; Phelps and Brand 1998). We found that expression of CASK-β with a weak pan-neuronal driver (*C155-Gal4*) partially rescued all parameters that were previously deficient in *CASK*^{P18} flies ($P < 0.05$). Figure 6 (top row) shows parameters representing motor maintenance, motor initiation, speed, and acceleration, with additional parameters in those categories shown in Figure S3. Because expression of *C155-Gal4* tends to be weak in

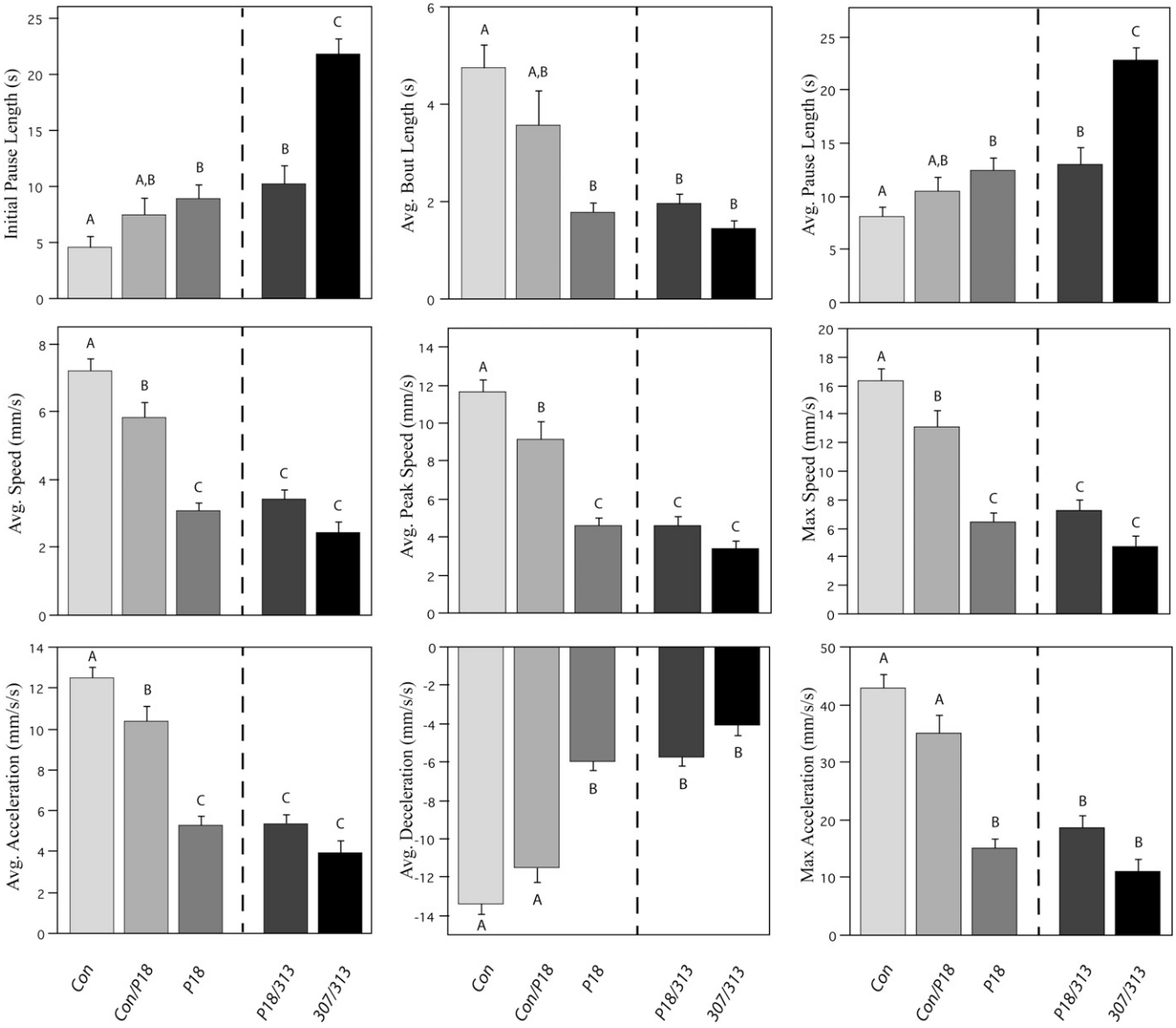

FIGURE 5.—Complete locomotor profile of *CASK*-β mutants. All parameters of locomotion from Figure 4C are analyzed individually. The nine parameters are initial pause length (time to resume movement following air pulse), average bout length (average length of all bouts per animal), average pause length (average length of all pauses per animal), average speed (mean of all speeds while in motion), max speed (mean of single maximum speed per animal), average peak speed (mean of all maximum speeds per bout), average acceleration (mean of all accelerations while in motion), average deceleration (mean of decelerations while in motion), and max acceleration (mean of single maximum acceleration per animal). Loss of CASK-β leads to dose-dependent increases in motor initiation time, decreases in ability to maintain active movement, and decreases in speed and acceleration. Letters signify significant differences between groups ($P < 0.05$).

adulthood, a partial rescue phenotype was not surprising. Reconstitution of CASK-β expression in a more limited population of neurons with a very strong, but more restricted, driver (*C164-Gal4*) showed not only a full rescue of the locomotor defects, but also an enhancement of locomotor activity above wild-type levels (Figure 6, middle row; $P < 0.05$), further supporting the idea that locomotor behavior is very sensitive to the absolute levels of CASK-β.

CASK-β is required outside the motor system: *C164-Gal4* is a well-known driver of motor neuron expression in both larval and adult flies (CHOI *et al.* 2004; ROMERO *et al.* 2008), but little is known about the expression pattern outside of these neurons. For this reason, we examined adult brains from flies containing this driver and a *UAS-mCD8GFP* transgene using confocal imaging (Figure 7, A and B). *C164-Gal4* appears to express in many cells in the central nervous system, including many larger, well-characterized neuropils such as the antennal lobes, the mushroom bodies, the subesophageal ganglion (SOG), and the pars intercerebralis. Interestingly, this driver does not appear to express in

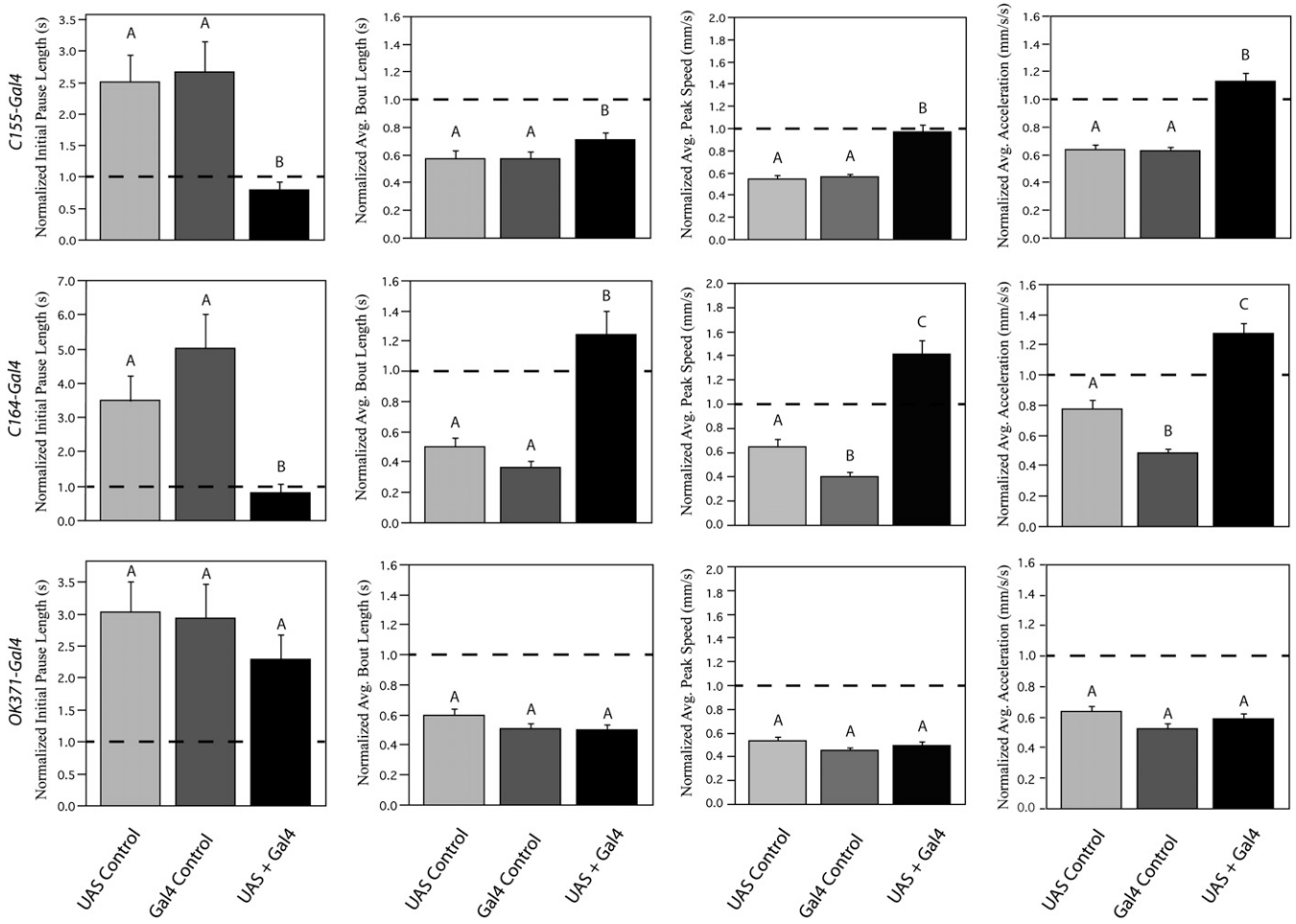

Figure 6.—Locomotor deficits can be rescued by expression of *CASK*-β in neurons. *CASK*-β cDNA was expressed in the nervous system using the UAS/Gal4 system. Data from all experimental genotypes are normalized to performance of control flies, and four representative parameters per rescue experiment are shown. Dashed lines represent control fly performance, which is always 1.0 due to normalization. For all parameters, the UAS and GAL4 controls each have one copy of the respective transgene (either UAS-CASK-β or a Gal4 driver) in a homozygous *CASK*-β null background. The UAS+Gal4 condition implies that flies contain one copy of both UAS-CASK-β and Gal4 driver, all in a homozygous *CASK*-β null background. (Top row) Pan-neuronal expression with the weakly expressing *C155-Gal4* driver either rescues or partially rescues all parameters compared to both the UAS and Gal4 controls. (Middle row) Spatially restricted expression in the motor neurons and subsets of the brain with the strongly expressing driver *C164-Gal4* rescues behavior in all four parameters, and in many conditions even enhances locomotor behavior beyond wild-type levels. (Bottom row) Specific expression in glutamatergic cells with the strongly expressing driver *OK371-Gal4*, however, did not rescue behavior in any of the parameters tested. Letters signify significant differences between groups ($P < 0.05$).

peripheral sensory neurons (Figure S4). Furthermore, the ellipsoid body, which is known to be a major locomotor control center of the insect brain (Strauss and Heisenberg 1993; Martin *et al.* 1999; Strauss 2002), appears to be devoid of any GFP expression, indicating that a full rescue of locomotor behavior with CASK-β is possible without involvement of this region.

To determine whether or not the previously reported larval motor neuron expression of *C164-Gal4* played a role in rescuing adult locomotor behavior, we expressed CASK-β in glutamatergic neurons using *OK371-Gal4*, an enhancer trap line that expresses strongly in both larval and adult motor neurons (Mahr and Aberle 2006). None of the representative parameters are rescued by expression of CASK-β with this driver (Figure 6, bottom row; $P < 0.05$). Preliminary results with *MHC-Gal4*, a driver that expresses only in muscle, also failed to rescue (data not shown). These results indicate that the neuromuscular junction is not the site of action for the large isoform of CASK in locomotion and that the central nervous system (central lobes or thoracic ganglion) is where the relevant neuronal population lies.

CASK-β mutants have normal circadian rhythms: It has been known for some time that, in Drosophila, circadian rhythms are intimately intertwined with both locomotion and courtship (Konopka and Benzer 1971; Kyriacou and Hall 1980) and their timing (Stanewsky 2003). For this reason, we determined whether or not the new *CASK* mutants had disruptions in their circadian control of locomotion. Analysis of activity in the Drosophila activity monitoring system indicated that

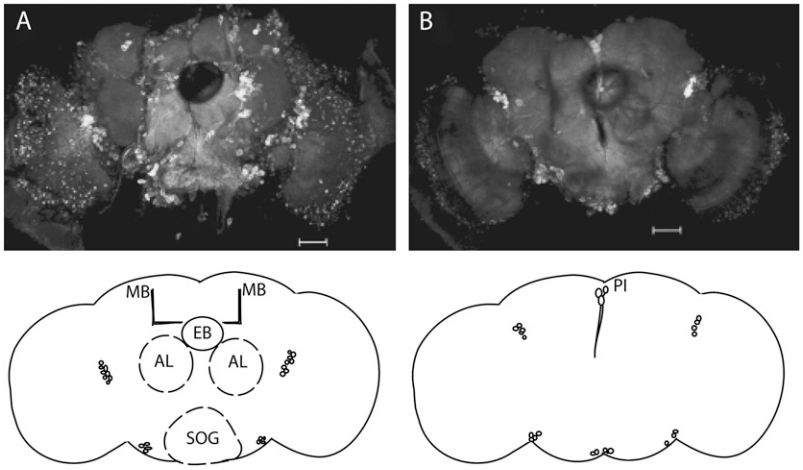

Figure 7.—*C164-Gal4* expresses in a subset of CNS neurons. Confocal imaging was used to map *C164-Gal4* expression in the brain of the adult fly with *UAS-mCD8GFP*. (A) A confocal stack of the anterior brain reveals low-level expression throughout much of the brain, with high expression in the antennal lobes (AL), mushroom bodies (MB), and subesophageal ganglion (SOG). Interestingly, the ellipsoid body (EB) is completely devoid of expression. There are also several other small unidentified clusters of cells that express strongly in the anterior brain (shown in schematic). (B) A confocal stack of the posterior brain appears relatively devoid of strong expression except in the pars intercerebralis (PI). Bar, 50 μm.

CASK^{P18}, *307/313*, and control flies all had normal behavior in LD and DD (data not shown), with free running periods of 24.5, 24.3, and 24.3 hr, respectively. This indicates that circadian rhythms and light responsiveness are not affected by loss of CASK-α or CASK-β, and alteration of these processes is therefore unlikely to be responsible for the CASK-β mutant's other behavioral phenotypes.

DISCUSSION

Previous work has implicated disruption of *CASK* in a suite of behavioral deficits. These studies, however, all suffered from the same limitation because the null animals used in these experiments had lost both of the proteins encoded at the *CASK* locus and also had disruptions of other third-chromosome genes. To address this, we generated a new set of isoform-specific mutants to better dissect the behavioral contribution of the CASK homolog in the fly. While these mutants shared similarities with the *307/313* flies used in previous studies, they were strikingly different in other ways.

***CASK* locus encodes two distinct MAGUKs:** CASK proteins have been defined as a subfamily of MAGUK proteins with a unique N-terminal CaMK-like domain in addition to the more typical L27, PDZ, SH3, and GUK domains. The CaMK-like domain has a constitutively active structure that grants it low levels of Ca^{2+}/calmodulin-independent activity against complexed substrate. Unlike all other known kinases, this activity is inhibited by Mg^{2+} (Mukherjee *et al.* 2008). This domain also participates in regulation of CaMKII autophosphorylation (Lu *et al.* 2003). CASK-β would therefore be expected to have properties different from other MAGUK proteins, and it represents the true ortholog of vertebrate CASK.

The Drosophila genome project annotation of the *CASK* locus predicts that, in addition to canonical CASK proteins (CASK-β), this locus has separately initiated transcripts that encode shorter proteins with a unique N-terminal region that is followed by PDZ, SH3, and GUK domains (CASK-α). These proteins are, in structure, more like the p55/MPP1-type MAGUKs than a true CASK. Phylogenetically, the MPP1 MAGUK group in vertebrates appears to be an offshoot of the CASK branch of the tree that arose from a gene duplication with subsequent loss of the CaMK-like and L27 domains (de Mendoza *et al.* 2010). Interestingly, Drosophila has no known MPP1 homolog, and it appears that the niche of this type of MAGUK has been filled by the short *CASK* gene product. It would therefore not be surprising if CASK-β and CASK-α had quite different roles. Indeed, the transcripts encoding these two proteins have different developmental profiles (Tweedie *et al.* 2009). Elucidation of the functions of the MPP1-like isoforms awaits the generation of CASK-α-specific mutants and antibodies, but it is tempting to speculate that the high expression in ovaries (Martin and Ollo 1996) might indicate that loss of CASK-α underlies the sterility phenotype of *307/313* flies.

Loss of the *CASK* isoform containing the CaMK-like and L27 domains underlies the *CASK* locomotor deficit: Mutants lacking CASK-β displayed an obvious motor defect (Figure 4A), which was further dissected using a high-resolution video tracking system (Figure 4B and Figure 5). This analysis revealed a very complex defect with deficits in four major areas: motor initiation, motor maintenance, speed, and acceleration. Furthermore, this defect is clearly dose-dependent, as the severity of the phenotype appears to change in a correlated fashion with the amount of CASK-β protein present in the animal, with *CASK*^{P18}/+ heterozygotes being more normal than *CASK*^{P18} homozygotes and with equivalent locomotor behaviors observed between these homozygous null flies and *CASK*^{P18}/*Df* for three independent deficiency lines. In addition, expression of CASK-β in a null fly rescues the behavioral deficit, also in a dose-dependent fashion; Gal4 lines with stronger expression can even make animals hyperactive (Figure 6). Taken together, these data indicate that the locomotor defect seen in these flies results from loss of CASK-β

in the nervous system, and not from extragenic mutations that arose as a result of the *P*-element excision.

The fact that mRNA encoding CASK-α, a *CASK* gene product that contains the PDZ, SH3, and GUK domains of CASK-β, is still expressed in the $CASK^{P18}$ mutant suggests that there may be unique functions for the CaMK-like and L27 domains of the CASK-β form. The CaMK-like domain has been shown to have both biochemical activity (Lu *et al.* 2003; Mukherjee *et al.* 2008) and specific binding partners, such as MINT1/Lin10 (Borg *et al.* 1998; Butz *et al.* 1998) and CaMKII (Lu *et al.* 2003). The L27 domains also have specific binding partners such as DLG/SAP97 (Sanford *et al.* 2004) and Veli/Lin7 (Borg *et al.* 1998; Butz *et al.* 1998). The inability of residual CASK-α to take over CASK-β function might also reflect a difference in localization of the two proteins, as CASK-α has a conserved palmitylation site at its very N terminus, whereas CASK-β does not have such a motif. This assumes, however, that both CASK-α and CASK-β are expressed in the same populations of neurons, which cannot be known for certain until better visualization tools for these proteins are developed.

CASK-β functions in a pre-motor circuit: Although CASK-β is expressed throughout much of the nervous system (Martin and Ollo 1996), its role in locomotor behavior is restricted to a limited number of cells. The *C164-Gal4* driver, which rescues locomotor behavior beyond wild-type levels (Figure 6), has strong expression in only a subset of central neurons, including the antennal lobes, mushroom bodies, SOG, pars intercerebralis, and parts of the central complex (fan-shaped body), while the periphery is completely devoid of expression (Figure 7, Figure S4). Interestingly, the ellipsoid body, which is known primarily for its role in locomotion (Strauss and Heisenberg 1993; Martin *et al.* 1999; Strauss 2002), is not a region where the Gal4 protein is expressed with this driver, suggesting that CASK is not acting in this population of cells to rescue behavior.

Strong CASK-β expression in glutamatergic cells with the *OK371-Gal4* driver did not rescue locomotor behavior (Figure 6). This is an important finding because insect motor neurons are primarily glutamatergic, implying that this subpopulation of cells within the central nervous system is also not the site of action for CASK-β in locomotion. This finding is at odds with the conclusions of recent work, which have suggested that alterations in the regulation of neurotransmitter release at the neuromuscular junction (NMJ) in *307/313* larvae and adults (Zordan *et al.* 2005; Sun *et al.* 2009) underlie the defective motor behavior of the null. Our experiments suggest that these NMJ defects (if they are indeed even present in the *CASK*-β-specific mutant) are not the basis of the locomotor problems demonstrated by $CASK^{P18}$ flies. Instead, the site of action is within a premotor population of neurons in the central nervous system that does not include ellipsoid body cells.

Judging by the expression pattern of *C164-Gal4*, the groups of neurons relevant for CASK-β action in locomotor behavior could include cells from the pars intercerebralis, mushroom bodies, thoracic ganglion interneurons (data not shown), or central complex structures such as the fan-shaped body or protocerebrum, all of which have been previously implicated in regulating insect motor activity (Strauss 2002; Matsui *et al.* 2009; Serway *et al.* 2009). These cells could also include populations of antennal lobe neurons involved in sensory processing or smaller groups of neurons (denoted in the schematic in Figure 7), but they are difficult to identify on the basis of morphology and location alone. Behavioral rescue experiments using Gal4 lines with more restricted expression patterns will be necessary to elucidate the cells relevant for CASK-β action in locomotion. In addition, the mechanisms behind proper subcellular localization of CASK-β within these cell populations will be of interest, as this could help determine potential binding partners and signaling cascades that interact with CASK-β.

Loss of CASK-β does not impair olfactory habituation: Mutants lacking CASK-β display a lower courtship index and a longer courtship latency than control flies (Figure 3, A and B). This indicates that CASK-β mutants are less adept at finding the target fly, which could be explained by a reduced sensitivity to pheromonal cues as previously suggested (Lu *et al.* 2003). Surprisingly, however, when $CASK^{P18}$ were tested for courtship habituation, which is a task requiring non-associative memory formation and olfactory processing, these flies performed similarly to control flies. This was seen when male $CASK^{P18}$ flies were trained with either a decapitated target immature male or direct exposure to immature male pheromone (Figure 3C). This finding suggests that both olfactory processing and plasticity remain intact in this assay following the loss of CASK-β. It should be noted that these results are specific to male–male courtship and that plasticity defects involving other pheromonal cues or sensory modalities remain to be examined.

***307/313* has additional chromosomal aberrations that affect behavior and fertility:** In all behavioral assays, *307/313* flies perform very differently from CASK-β mutants in addition to being sterile. This is not surprising since *307/313* flies are trans-heterozygous for two overlapping deficiencies. These deficiencies eliminate CASK-α as well as CASK-β and also contain mutations in genes in addition to *CASK* (Martin and Ollo 1996; Dimitratos 1999), which could have an effect on the resulting behavior of the flies. The low level of basal courtship observed in $CASK^{P18}$ flies, which is likely attributable to locomotor problems, is far less severe than the deficit seen in *307/313* flies (Figure 3, A and B). In addition, unlike the $CASK^{P18}$ mutants, *307/313* flies display an abnormally high and unusually variable habituation index (Figure 3C), consistent with previous work

(Lu *et al.* 2003). These additional problems of the *307/313* flies could reflect a reduction in olfactory sensitivity or a short-term plasticity defect, stemming from the loss of CASK-α or from heterozygosity at other genes.

Alternatively, these differences could also stem from the more severe courtship initiation defect observed in *307/313* flies, as a difficulty initiating any kind of movement could affect the reliability of training and testing. This idea is supported by the finding that *307/313* flies display a qualitatively different locomotor profile compared with $CASK^{P18}$ flies (Figure 4, B and C, and Figure 5). Importantly, multivariate analysis demonstrates that the individual parameters contributing to the qualitative difference between $CASK^{P18}$ and *307/313* are primarily initiation parameters. This suggests that the loss of the MPP1-like CASK-α (or potentially genetic interactions between haploinsufficient loci) in *307/313* flies may confer a unique locomotor deficit. For this reason, *307/313* is not a good model for loss of CASK-β, the CaMK-like/L27-containing MAGUK, as it pertains to behavior.

***CASK* and motor dysfunction:** Our work with CASK-β mutants shows that there is a clear motor phenotype resulting from loss of the Drosophila CASK homolog. These flies appear to suffer from problems with motor initiation, motor maintenance, speed, and acceleration. Such a complex deficit stemming from a higher-level region within the central nervous system suggests that CASK-β may work to allow the integration of multiple parameters of locomotion together into coordinated movement. Not surprisingly, this strong locomotor phenotype also appears to affect other behavioral tasks involving a motor response, such as courtship and habituation.

Many diseases such as Parkinson's disease and Huntington's disease are characterized by motor dysfunction that disrupts multiple motor parameters. Fly models for these movement disorders, as well as for many others, have been developed and characterized and show deficits similar to those of $CASK^{P18}$ flies (Feany and Bender 2000; Lee *et al.* 2004). Furthermore, recent work has suggested that molecular scaffolds like MAGUK family proteins, of which CASK is a member, interact directly or indirectly with many proteins thought to be associated with these diseases (reviewed in Gardoni 2008). Determining the role that scaffolds such as CASK play in such interactions may lead to a deeper understanding of motor disease and potentially provide a basis for development of novel therapeutics.

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GENETICS

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Central Regulation of Locomotor Behavior of *Drosophila melanogaster* Depends on a CASK Isoform Containing CaMK-Like and L27 Domains

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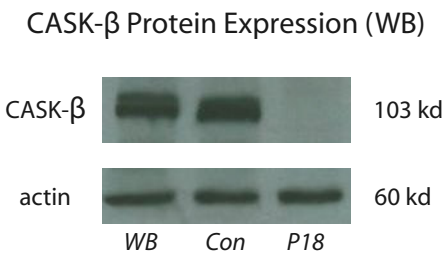

FIGURE S1.—Validation of *CASK*-β mutant after outcrossing for behavior. Immunoblot was used to confirm successful outcrossing of control and *CASK*^{P18} lines to the *white Berlin* (*WB*) background. *WB* wild-type flies were run as a positive control, and actin was used as a normalization control. As expected, *CASK*^{P18} flies in the WB background express no CASK-β protein, while control flies express comparable protein levels comparable to *WB* wild-type flies.

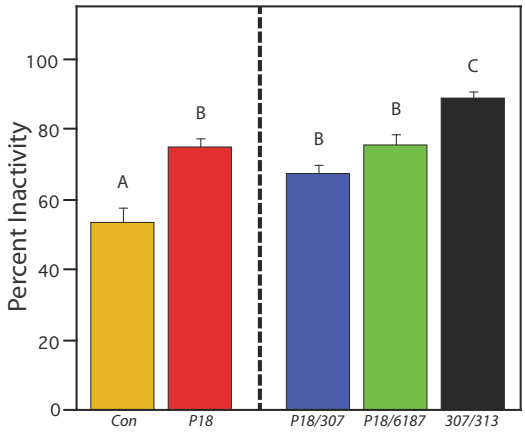

FIGURE S2.—Percent Inactivity is shown for *CASK^{P18}/Df(3R)x307* and *CASK^{P18}/Df(3R)exel6187*, compared with control, *CASK^{P18}*, and *307/313* flies. Consistent with Figure 4, levels of activity seen in these additional *CASK^{P18}/Df* flies are not significantly different from *CASK^{P18}*, but do show differences when compared with both control and *307/313* flies. In this dataset, only seven trials were run with *307/313* flies, due to difficulties keeping them alive for proper age-matching.

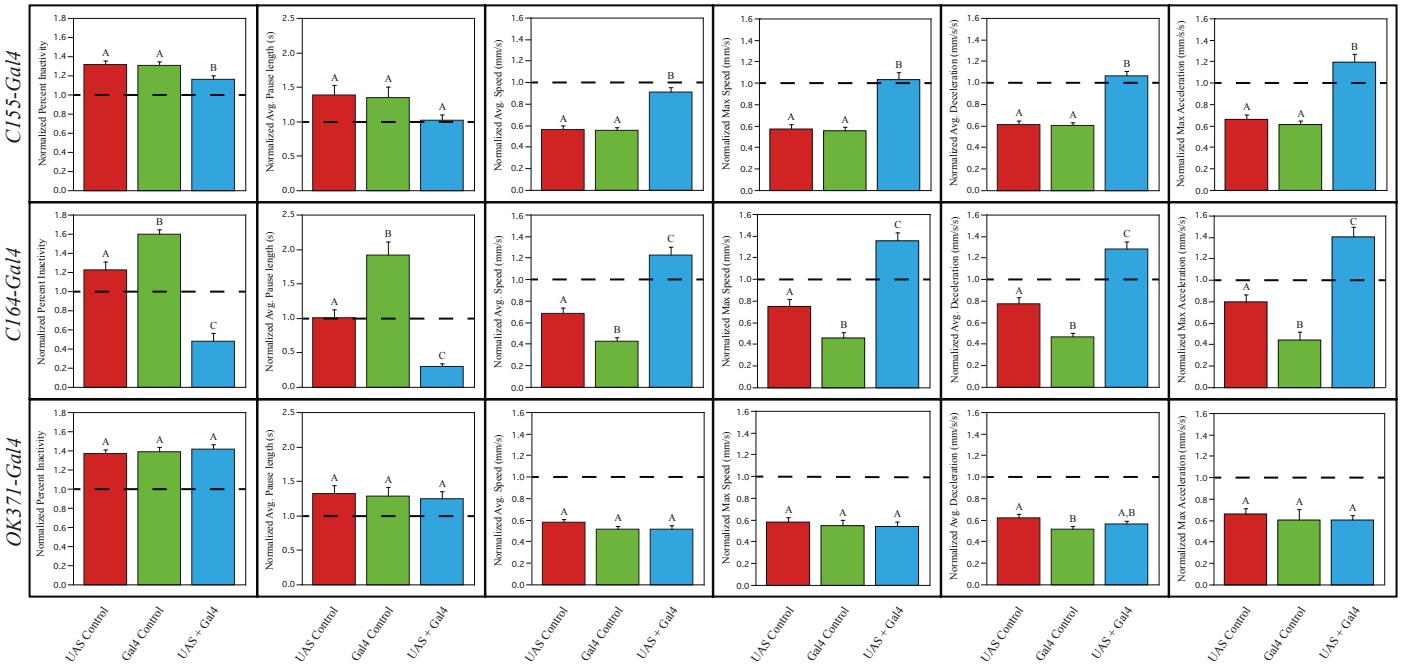

FIGURE S3.—Additional parameters from behavioral rescue experiments. Consistent with "representative" parameters in Figure 5, loss of *CASK*-β affects performance in all parameters tested for, and produces dose-dependent increases in motor initiation, decreases in motor maintenance, and decreases in speed and acceleration. As in Figure 5, the UAS and GAL4 controls each have one copy of the respective transgene (either UAS-CASK-β or a Gal4 driver) in a homozygous *CASK*-β null background. The UAS+Gal4 condition implies that flies contain one copy of both UAS-CASK-β and Gal4 driver, all in a homozygous *CASK*-β null background.

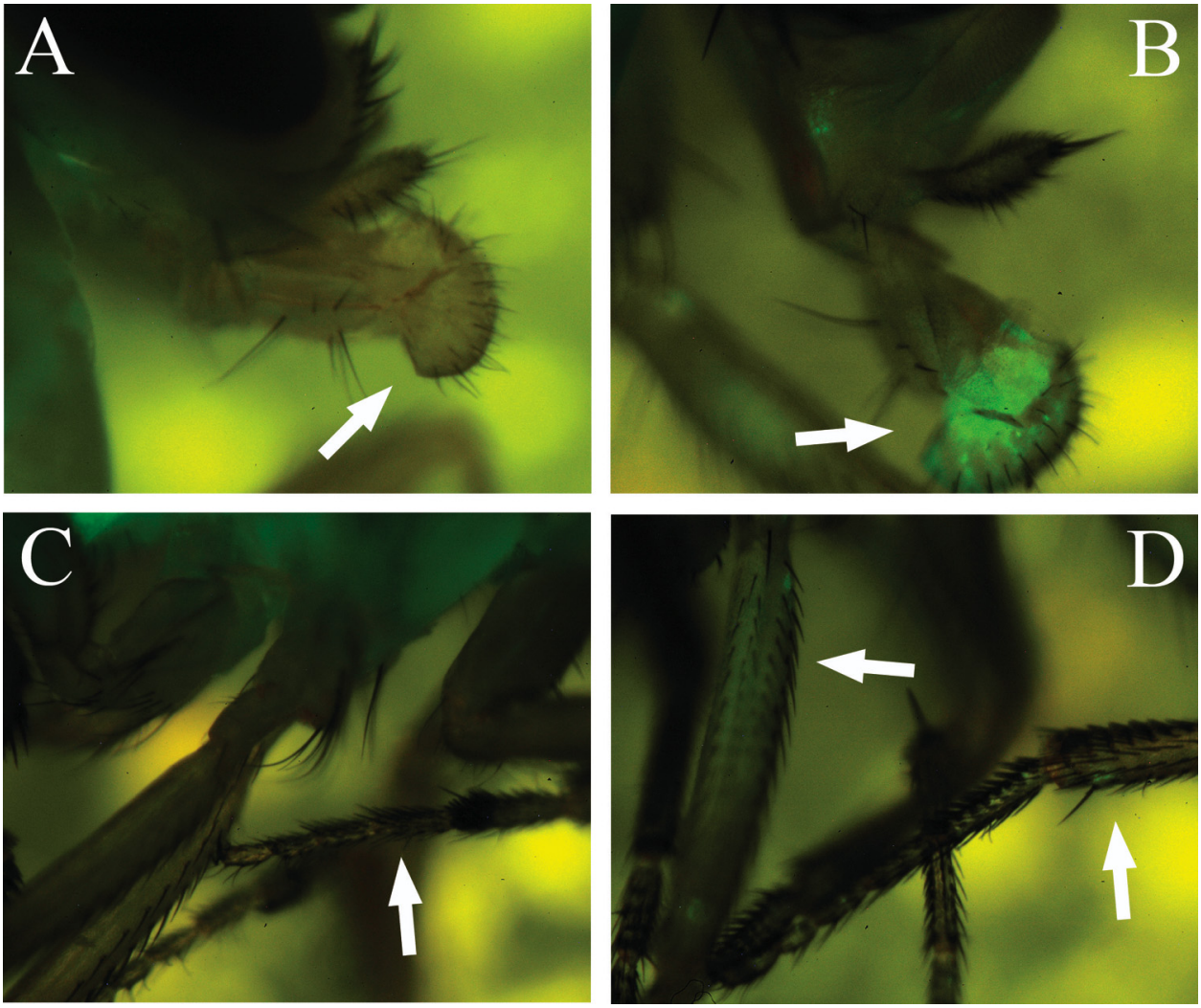

FIGURE S4.—*C164-Gal4* does not express in peripheral sensory neurons in the male. Pictures were taken using a fluorescent dissecting microscope of flies expressing *UAS-mCD8GFP* under control of the *C164-Gal4* driver. Male flies (A and C) do not appear to express GFP in the periphery, while female flies (B and D) do. Arrows denote GFP fluorescence in the proboscis and legs of females, and the lack of fluorescence in these structures in males. Since only male flies were run in behavioral experiments, it can be assumed that no CASK-β was present in the periphery of the assayed flies.

TABLE S1

Primer sets used for deletion mapping (top), quantitative real-time PCR (middle), and RT-PCR (bottom)

Primer Position	PCR Left Primer	PCR Right Primer
1	TGTTTTTGCTGCTCATCTCG	GGTACGCAAGCAAGTGCATA
2	TATGCACTTGCTTGCGTACC	AGCGAATCGCATTAGCACTC
3	AGATACCAACGGGTTTCTGG	ACGATGCACACACGAAATGT
4	ACATTTCGTGTGTGCATCGT	GGACCTGCAGAATGTGTCCT
5	TTTCCGTTGCAAAATTCACA	GATGCCTCGACTCAATTGCT
6	TCAAAATGACCGAAGACGAAAT	GCGATTCAAGGTCGTCATTT
7	CCAAATTCAGCTTGGTGACA	CGAACAAGGTCGTCTCATTG

cDNA Primed	QRT-PCR Left Primer	QRT-PCR Right Primer
CASK-α/β	GACGGTCGACAAGACCAA	ACTGGCACCTTGACAACCT
rp49	ATCCGCCCAGCATACAG	TCCGACCAGGTTACAAGAA

cDNA Primed	Isoform-specific Left Primer	Isoform-specific Right Primer
CASK-α	CTCTTCAATCCGTGCAGCTC	ACTGGCACCTTGACAACCT
CASK-β	TCAAAATGACCGAAGACGAAAT	ACTGGCACCTTGACAACCT

Deletion mapping primers correspond to the schematic in Figure 2A.

TABLE S2

F Values for all one-way ANOVA's

Figure	Condition	ANOVA F Value
3A	Courtship Initiation	$F_{(2, 72)}$=48.5697, *P*<0.0001
3B	Courtship Latency	$F_{(2, 72)}$=38.2590, *P*<0.0001
3C (left)	Courtship Habituation (immature male)	$F_{(1, 43)}$=0.4022, *P*=0.5293
3C (right)	Courtship Habituation (pheromone)	$F_{(2, 44)}$=6.4311, *P*=0.0035
4B	Percent Inactivity	$F_{(4, 445)}$=28.8689, *P*<0.0001
5	Initial Pause Length	$F_{(4, 449)}$=30.4813, *P*<0.0001
5	Avg. Bout Length	$F_{(4,332)}$=8.0918, *P*<0.0001
5	Avg. Pause Length	$F_{(4,436)}$=26.6235, *P*<0.0001
5	Avg. Speed	$F_{(4,332)}$=30.3987, *P*<0.0001
5	Avg. Peak Speed	$F_{(4,332)}$=26.4964, *P*<0.0001
5	Max Speed	$F_{(4,332)}$=34.1318, *P*<0.0001
5	Avg. Acceleration	$F_{(4,332)}$=44.0489, *P*<0.0001
5	Avg. Deceleration	$F_{(4,332)}$=45.5042, *P*<0.0001
5	Max Acceleration	$F_{(4,332)}$=32.5515, *P*<0.0001
6	N Initial Pause Length (*C155-Gal4*)	$F_{(2,244)}$=7.7353, *P*=0.0006
6	N Avg. Bout Lengh (*C155-Gal4*)	$F_{(2,236)}$=2.6264, *P*=0.0745
6	N Avg. Peak Speed (*C155-Gal4*)	$F_{(2,236)}$=35.4524, *P*<0.0001
6	N Avg. Acceleration (*C155-Gal4*)	$F_{(2,236)}$=51.6936, *P*<0.0001
6	N Initial Pause Length (*C164-Gal4*)	$F_{(2,186)}$=8.4727, *P*=0.0003
6	N Avg. Bout Length (*C164-Gal4*)	$F_{(2,160)}$=29.6185, *P*<0.0001
6	N Avg. Peak Speed (*C164-Gal4*)	$F_{(2,160)}$=53.6895, *P*<0.0001
6	N Avg. Acceleration (*C164-Gal4*)	$F_{(2,160)}$=53.1004, *P*<0.0001
6	N Initial Pause Length (*OK371-Gal4*)	$F_{(2,215)}$=0.7630, *P*=0.4675
6	N Avg. Bout Length (*OK371-Gal4*)	$F_{(2,203)}$=2.4359, *P*=0.0901
6	N Avg. Peak Speed (*OK371-Gal4*)	$F_{(2,203)}$=2.3716, *P*=0.0959
6	N Avg. Acceleration (*OK371-Gal4*)	$F_{(2,203)}$=3.5537, *P*=0.0304
S2	Percent Inactivity	$F_{(4,318)}$=18.0303, *P*<0.0001
S3	N Percent Inactivity (*C155-Gal4*)	$F_{(2,237)}$=5.7384, *P*=0.0037
S3	N Avg. Pause Length (*C155-Gal4*)	$F_{(2,237)}$=3.0330, *P*=0.0500
S3	N Avg. Speed (*C155-Gal4*)	$F_{(2,236)}$=33.6290, *P*<0.0001
S3	N Max Speed (*C155-Gal4*)	$F_{(2,236)}$=35.9531, *P*<0.0001
S3	N Avg. Deceleration (*C155-Gal4*)	$F_{(2,236)}$=51.8441, *P*<0.0001
S3	N Max Acceleration (*C155-Gal4*)	$F_{(2,236)}$=34.3021, *P*<0.0001
S3	N Percent Inactivity (*C164-Gal4*)	$F_{(2,163)}$=58.1883, *P*<0.0001
S3	N Avg. Pause Length (*C164-Gal4*)	$F_{(2,160)}$=26.4092, *P*<0.0001
S3	N Avg. Speed (*C164-Gal4*)	$F_{(2,160)}$=57.7496, *P*<0.0001
S3	N Max Speed (*C164-Gal4*)	$F_{(2,160)}$=51.4950, *P*<0.0001
S3	N Avg. Deceleration (*C164-Gal4*)	$F_{(2,160)}$=59.8870, *P*<0.0001
S3	N Max Acceleration (*C164-Gal4*)	$F_{(2,160)}$=36.8120, *P*<0.0001
S3	N Percent Inactivity (*OK371-Gal4*)	$F_{(2,204)}$=0.3917, *P*=0.6764
S3	N Avg. Pause Length (*OK371-Gal4*)	$F_{(2,204)}$=0.0846, *P*=0.9189
S3	N Avg. Speed (*OK371-Gal4*)	$F_{(2,203)}$=1.6047, *P*=0.2035
S3	N Max Speed (*OK371-Gal4*)	$F_{(2,203)}$=0.2105, *P*=0.8104
S3	N Avg. Deceleration (*OK371-Gal4*)	$F_{(2,203)}$=3.2489, *P*=0.0408
S3	N Max Acceleration (*OK371-Gal4*)	$F_{(2,203)}$=0.2602, *P*=0.7711