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.
INTRODUCTION

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 (PETERSON et al. 2010; PISANI et al. 2005). 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.

Membrane-associated guanylate kinase (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 further 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 towards characterizing the small isoforms, which we designate 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 interaction with the CaMK-like domain (HODGE et al. 2006; LU et al. 2003).

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 it is still unclear as to the molecular cause of the locomotor deficit following the disruption of CASK expression, 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 which 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 transheterozygote 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).

In order 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 which 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
transheterozygote 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°C under a 12 hr:12 hr light:dark 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) (Bellen et al. 2004; Spradling et al. 1999). 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 a transheterozygote 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 (Mahir 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 for UAS-CASK 10.20 (the cDNA used corresponds to the RB mRNA in FlyBase), which was provided by Peter Bryant (UC Irvine, Irvine, CA), and C164-Gal4, which was provided by Vivian Budnik (UMass Medical School, Worcester, MA).
**P-Element Mutagenesis:** The P element excision screen was performed using line EY07081, which harbors a P element inserted 1751 base pairs 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 \( w; +/+; MKRS\Delta2-3Sb/TM2Ubx\Delta2-3 \) flies. The resulting F1 female progeny were crossed with third chromosome double balancer flies (\( w; +/+; TM3Sb/TM6B \)), and the F2 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 Dr. Gisela Wilson (University of Wisconsin, Madison, WI). This antibody was raised in guinea pigs immunized with GST-CMG\( _{152-897} \) as previously described (MARBLE et al. 2005). A monoclonal antibody was used for actin normalization (Millipore). Both primary antibodies were used at a concentration of 1:1000. HRP-conjugated anti-mouse (GE Healthcare) and guinea pig (Jackson Laboratories) secondary antibodies were both 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 that either 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 1X 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 roughly 1 kb immediately upstream and downstream of the P element insertion site (see Supplemental Table 1 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, NJ).
RNA Quantification And Identification: RNA was extracted from equal numbers of anesthetized whole flies using Tri Reagent (Molecular Research Center, Inc.). RNA samples were then denatured at 65°C for 5 min, and chilled on ice. 5 µl of RNA 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 (Supplemental Table 1). 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 (Supplemental Table 1).

Courtship Habituation: Male flies were collected within 6 h of eclosion under anesthesia and sorted individually into test tubes containing yeast-free media. At five days old, a male was placed with a decapitated immature male in a mating chamber (8 mm in diameter, 3 mm deep) and tested for courtship response. The courtship index (CI) is the percentage of time 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 didn’t start courtship during the 10 min observation period, a score of 600 was given. For the habituation assay, a male was paired with either 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 CI’s. When the habituation index = 1, this indicates that there has been no courtship habituation, since the courtship level of trained males is equivalent to that of sham trained males. ≥ 20 males were tested for each condition. All courtship experiments were performed under dim red lights (> 700 nm) in a controlled environment room (25°C, 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 (Supplemental Figure 1).
**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 ten under anesthesia. Following a two day recovery period at 25°C, 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 5 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 (percent inactivity, initial pause length, average bout length, average pause length, average speed, average max speed, average peak speed, average acceleration, max 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, 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 three or more 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 less than 18 sec long were excluded for all parameters, with the exception of the initial pause length parameter. For this parameter, all trials with at least five seconds of uninterrupted tracking from time point zero 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°C with 70% humidity.

**Circadian Rhythm Analysis:** Male flies aged 1-2 days were individually sorted into 65 x 5-mm glass tubes, each containing 5% agarose with 2% sucrose at one end. Following a 3 day entrainment period in a 12hr:12hr light/dark cycle (LD), 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°C. 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 (PBS), fixed for 15 min in 4% paraformaldehyde, and mounted using Vectashield. Images of the brains were taken on a Leica TCS SP5 confocal microscope at 20x 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 (DFA) 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 ANOVA’s are listed in Supplemental Table 2.

**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 which contains a P{EPgy2}
element within the first intron at the 5’ end of the gene (BELLEN et al. 2004; SPRADLING et al. 1999). The P element was mobilized and 259 candidate excision lines were identified by their lack of eye color (see Methods and Materials). 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 row), and actin (bottom row) 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 are comparable to those of Canton S (CS) wild type, and it is indicated as “Con” in Figure 1A. 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 mRNA, or the unique region presumed to lie in the N-terminal region of CASK-α (see Supplemental Table 1 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. Lines P29 and T119 both 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 of the coding region. Sequencing also revealed that these lines both harbor a small duplication of the deleted region elsewhere in the genome (denoted by the * in Figure 2C), but these duplications do not appear to rescue CASK expression.

Both the P18 and P46 lines are missing bands #3-6 (Figure 2B), which correspond to a large portion of the 5’ UTR and 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 promoter region and remaining coding exons (denoted by ** 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; SUN et al. 2009; ZORDAN et al. 2005) 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; GAILEY et al. 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 CASKP18 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 can not 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 CASKP18 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 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 (Fig. 3C, left side). 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 (Fig. 3C, right side), 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 besides CASK (DIMITRATOS 1999; MARTIN and OLLO 1996), 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 which suggest that 307/313 flies have a severe motor defect, (MARTIN and OLLO 1996; SUN et al. 2009; ZORDAN et al. 2005) and might have trouble initiating any kind of behavioral response involving movement. The courtship index of the 307/313 males to immature males were indeed extremely low (Figure 3A) and many of these males never initiated courtship during 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 percent 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 side). This suggests that CASK-β plays a dose-dependent role in locomotion.

$307/313$ flies demonstrate a more severe increase in percent inactivity, but $CASK^{P18}/Df(3)x313$ flies show activity levels similar to $CASK^{P18}$ (Figure 4B, right side, $P<0.05$). Similar trends can also be seen with $CASK^{P18}$ in trans with other deficiencies, such as $Df(3)x307$ and $Df(3)excel6187$ (Supplemental Figure 2). 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, multivariate analysis of variance (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 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 which 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-\( \alpha \) 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-\( \beta \) 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-\( \beta \) decreases, the length of each bout of activity also decreases, indicating an inability to maintain locomotion once initiated (motor maintenance). Average speed, average max speed, and average peak speed (Figure 5, middle row) all followed the same trend, where loss of CASK-\( \beta \) expression caused a dose-dependent decrease in the resulting speed. Average acceleration, average deceleration, and max acceleration (Figure 5, bottom row) also behaved similarly; loss of CASK-\( \beta \) 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 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\textsuperscript{P18} flies appear to demonstrate a locomotor phenotype which 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\textsuperscript{P18} animals ($P<0.05$), with all other parameters trending towards 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\textsuperscript{P18} likely result from loss of CASK-\(\alpha\), although we cannot rule out a genetic interaction between the additional mutations on these two deficiency chromosomes. It should be noted that although CASK\textsuperscript{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-\(\beta\) expression in the nervous system:** To confirm that these motor phenotypes stem from loss of CASK-\(\beta\), we assayed the ability of a CASK-\(\beta\) cDNA transgene (used previously in Hodge et al. 2006) to rescue locomotor performance using the UAS/Gal4 binary expression system (Brand and Perrimon 1993; Fischer et al. 1988; Phelps and Brand 1998). We found that expression of CASK-\(\beta\) with a weak pan-neuronal driver (C155-Gal4) partially rescued all parameters which were previously deficient in CASK\textsuperscript{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 Supplemental Figure 3. Because expression of C155-Gal4 tends to be weak in adulthood, a partial rescue phenotype was not surprising. Reconstitution of CASK-\(\beta\) 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-\(\beta\).

**CASK-\(\beta\) 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 7a 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 peripheral sensory neurons (Supplemental Figure 4). Furthermore, the ellipsoid body, which is known to be a major locomotor control center of the insect brain (Martin et al. 1999; Strauss 2002; Strauss and Heisenberg 1993), 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 (Maehr 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 (DAM) System indicated that CASKP18, 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 h 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 are 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, as 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, so as 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.

**The CASK locus encodes two distinct MAGUKs:** CASK proteins have been defined as a subfamily of MAGUK protein 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 which 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 (Figures 4B, 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. Along with this,
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 can not 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, subesophageal ganglion (SOG), pars
intercerebralis, and parts of the central complex (fan shaped body), while the periphery is
completely devoid of expression (Figure 7, Supplemental Figure 4). Interestingly, the ellipsoid body, which is known primarily for its role in locomotion (MARTIN et al. 1999; STRAUSS 2002; STRAUSS and HEISENBERG 1993), 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 has suggested that alterations in the regulation of neurotransmitter release at the NMJ in 307/313 larvae and adults (SUN et al. 2009; ZORDAN et al. 2005) 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 CASKP18 flies. Instead, the site of action is within a pre-motor 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 (MATSUI et al. 2009; SERWAY et al. 2009; STRAUSS 2002). These cells could also include populations of antennal lobe neurons involved in sensory processing, or smaller groups of neurons which are denoted in the schematic (Figure 7), but are difficult to identify based on 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. Along with this, 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 3A,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 transheterozygous for two overlapping deficiencies. These deficiencies eliminate CASK-α as well as CASK-β, and also contain mutations in genes besides $CASK$ (DIMITRATOS 1999; MARTIN and OLLO 1996), 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 3A,B). Along with this, 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 4B-C, 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 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 both of these movement disorders, as well as many others, have been developed and characterized, and show deficits similar to those of CASK<sup>P18</sup> 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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FIGURE LEGENDS

Figure 1- Characterization of CASK mutants. (A) Immunoblot of candidate P element excision lines show that lines P18, P29, P46, and T119 appear to be null for CASK-β protein, while 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 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.

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 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 both are missing band #3, which corresponds to the region immediately surrounding the original insertion site. Lines P18 and P46 are both 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 both 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 *), while P18 has an additional insertion at the deletion site (denoted by **).
Figure 3- CASK<sup>P18</sup> mutants display reduced levels of courtship, but not a reduction of pheromone sensitivity. (A) Loss of CASK-β lowers 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<sup>P18</sup> and 307/313 flies. The behavioral changes in the 307/313 flies are more severe than CASK<sup>P18</sup> flies in both cases. Letters signify significant differences between groups. (C) Courtship habituation assays performed with either immature male exposure (left side) or with direct exposure to isolated immature male pheromone (right side) produced no plasticity defect in CASK<sup>P18</sup> 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 immature male condition, but these flies did demonstrate a significantly larger and more variable habituation index than either control or CASK<sup>P18</sup> 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, as ANOVA and pairwise comparisons were performed separately for these two conditions.

Figure 4- CASK<sup>P18</sup> 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<sup>P18</sup>), P18 (CASK<sup>P18</sup>), P18/313 (CASK<sup>P18</sup>/Df(3R)x313), and 307/313 (Df(3R)x307/Df(3R)x313) flies. (B) Percent inactivity (total percent 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<sup>P18</sup> 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.

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 ($P<0.05$).

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. Dotted 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 ($P<0.05$).

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), subesophageal ganglion (SOG). Interestingly, the ellipsoid body (EB) is completely devoid of expression. There are also several
other small unidentified clusters of cells which 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). Scale bar = 50 μm.
Figure 1 (Drosophila CASK Regulates Locomotion)
Figure 2  (Drosophila CASK Regulates Locomotion)
Figure 3 (Drosophila CASK Regulates Locomotion)
Figure 4 (Drosophila CASK Regulates Locomotion)
Figure 5 (Drosophila CASK Regulates Locomotion)
Figure 6 (Drosophila CASK Regulates Locomotion)
Figure 7 (Drosophila CASK Regulates Locomotion)