

An Unusual Cation Channel Mediates Photic Control of Locomotion in *Drosophila*

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Summary

A unique family of putative ion channels that are related to voltage-gated sodium and calcium channels has been identified in genomic and cDNA studies of metazoans [1, 2]. Aside from evidence for expression of family members in the nervous system [2, 3], little is known about the operation of the channel or its functional significance. In the present study, this conserved family's sole *Drosophila* member, a gene known both as CG1517 [4, 5] and as *Dm α 1U* [1], is shown to correspond to the *narrow abdomen* (*na*) gene and is the locus of a set of mutations that affect sensitivity to anesthetics [6–8]. Immunohistochemistry of adult heads reveals that the channel is expressed in the neuropil of the central complex and optic lobe; expression is severely depressed in the mutants. In addition to previously described defects, the mutant phenotype is demonstrated here to include dysfunction in the coupling between light and locomotor behavior. Most dramatically, mutant flies have an inversion of relative locomotor activity in light versus dark. The involvement of the channel in daily rhythms of the fruit fly is especially provocative because the human ortholog lies in a candidate region linked to bipolar disorder [9], a disease frequently associated with altered diurnal behavior [10].

Results and Discussion

Identification of the Locus

The mutations of interest, which have effects on abdominal morphology and locomotor behavior as well as anesthetic sensitivity [6–8], had previously been mapped to a portion of the 12E region of the X chromosome of *Drosophila* [8, 11]. A P element insert that inactivates the gene has been reported [8], but all existing stocks of this line lack a physical aberration in the 12E region (Z. Guan and R.L.S., unpublished data). In the absence of a physical landmark, we used SSCP analysis [12, 13] of amplified segments of DNA from the region [4], as well as subsequent sequencing, to assign the mutations to a gene. As shown in Figure 1, the mutations map to CG1517, a protein predicted by hydropathy analysis to be a member of the four-repeat ion channel superfamily. Searching protein databases with the entire CG1517

ORF indeed shows significant hits on many voltage-gated calcium and sodium channels. However, although most of these have E values of about 10^{-40} , a few have much better scores (E values $< 10^{-300}$). These impressive hits, whose strength derives largely from the high conservation of the putative intracellular domains of the protein, define the novel ion channel family. The family seems to be limited to metazoans, with no obvious members found amongst microbial genomes; vertebrate members include a rat ortholog (NP_705894, described in reference [2]) and a human ortholog (NP_443099). In a previous survey of ion channels [1], this gene family has been called α 1U to indicate both its relatedness to α subunits of voltage-gated calcium and sodium channels and its uniqueness within that superfamily. Two of the alleles of *Dm α 1U* (*har38* and *har85*, named for altered halothane resistance) are identical and alter a predicted splice junction. Compared to their Oregon-R parent (R.L.S., unpublished data) and the iso-1 wild-type strain [4], they change the indicated 5'-splice junction from GTttGT to GTtTA (lowercase letters indicate bases that differ from consensus [14]); this favors the use of an out-of-frame cryptic splice site (see below). The parent of the *na* mutant (named for its original phenotype, a narrow abdomen) has been lost, making SSCP uninformative. However, relative to the iso-1 strain, the only remarkable sequence alteration of CG1517 that is found in the mutant strain (GenBank accession number AY160083) is a deletion of 9 nt that changes the predicted peptide sequence in a putative membrane-spanning segment from SDAVTLTLLFTAE to SDTVLFTAE.

Of the three traits previously associated with these mutations, abdominal morphology is hard to score quantitatively and is very sensitive to genetic background; it will not be discussed further. A genetic experiment confirmed that sequence alteration of *Dm α 1U* is responsible for the other two phenotypes. In a set of lines selected to have undergone recombination in the region (for a diagram of the cross, see Supplementary Figure S1 available with this article online), there was perfect agreement between the inheritance of the altered splice site of *har85* and both sensitivity to halothane, as judged by the distribution test [11], and “hesitant” walking [6], as judged by the time for unanesthetized flies to climb the side walls of a vial. To further probe for linkage between these traits and the sequence change, we backcrossed a recombinant line that had inherited the *har85* splice site and the nearby visible marker. Two rare lines that maintained the marker but had lost the hesitant-walking phenotype were isolated; both had normal sensitivity to halothane, and both had undergone a crossover that had replaced the *har85* splice site with the wild-type allele. The complete correspondence between the sequence change and the traits leads us to conclude that alteration of CG1517 is responsible for the anesthetic and locomotor phenotype.

Expression of the Putative Channel

The abundance and distribution of the product of the *Dm α 1U* gene (also referred to as the *na* gene in honor

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Genomic DNA

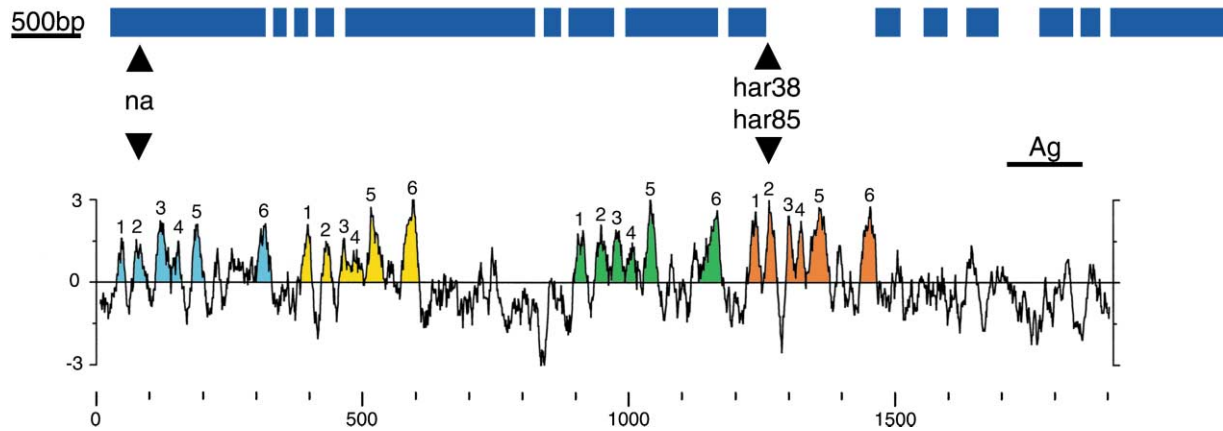


Figure 1. Molecular Analysis of the *Dmα1U* Gene

Below the solid line representing genomic DNA from the 12E region [4] are the positions of predicted exons of CG1517 (taken from GenBank: AE003495.2, gi:1072841). Below these are indicated the positions of three noncomplementing mutations. Below the exon diagram is a hydrophathy plot [35], with a 14 amino acid window, of the deduced ORF of CG1517. The positions of four groups of predicted transmembrane segments are highlighted, as are the location of the genetic alterations and the region of the ORF (Ag) used to raise a polyclonal antibody. As a consequence of our preliminary studies of the 5' end of the transcript of the gene (R.L.S., unpublished data), the amino terminus is indicated to start 320 amino acids downstream from that originally proposed. As for other ion channels [36], the hydrophilic nature of the predicted leader sequence implies that the first transmembrane segment functions as the Type II signal anchor [37] that initiates membrane integration.

of the first-reported allele [15]) was evaluated with a polyclonal antibody raised to a bacterially expressed portion of the gene (Figure 1). A survey of material taken at various developmental stages showed that adult heads are the richest source of channel protein, suggesting that the gene is more involved in physiology than development. Within the head the channel is concentrated in synaptic regions (neuropil) but not in areas of the brain occupied primarily by cell bodies or axon tracts (Figure 2A). Many areas of the brain express the channel, but staining is not uniform. Most notable is the high channel density in the lateral triangles of the ellipsoid body (Figures 2A and 2B), a structure largely devoted to the generation and coordination of locomotor patterning in flies [16, 17]. Also of interest is the pattern of expression in the optic lobe, where prominent staining is seen in several distinct layers of the medulla and in the lobula complex, structures important for higher order processing of visual information [18]. The channel is also seen in a single row in the distal lamina but is not apparent in the retina. Comparison of sections from male and female heads reveals no obvious differences in distribution or intensity (R.L.S., unpublished data). Overall, the pattern of staining of *Dmα1U* shows distinct similarities (strong expression in ellipsoid body, proximal medulla, and lobula complex) to that reported for markers of GABA-ergic transmission [19, 20]. There is a similarly provocative but imperfect match to the pattern (strong expression in lateral triangles, a single layer of the distal lamina, and multiple layers of the medulla and lobula complex) reported for cholinergic markers [21, 22]. Although these patterns are broad and therefore might be fortuitous, the overlap in distribution raises the possibility that the *Dmα1U* channel is involved

in the release of one or more of these neurotransmitters or in modulation of their postsynaptic effects.

All existing alleles of the *na* gene dramatically reduce antibody staining in the brains of mutant flies (Figure 2C). This depression is confirmed by Western blots, in which specific bands are readily detected in extracts of wild-type heads (see below) but are undetectable in the mutants (R.L.S. and H.A.N., unpublished data). Thus, although the mutations do not completely destroy the gene (Figure 1) and our assays cannot exclude the presence of small amounts of gene product, we can conclude that the existing alleles of *Dmα1U* substantially depress gene function. This is consistent with earlier reports that the mutant phenotype is not significantly enhanced by replacement of any of these alleles with a chromosome deficient for the region [6, 8, 11]. It should be pointed out that our evaluation of gene expression relies on an antibody to a single region of the predicted ORF (Figure 1). It is thus possible that our analysis is blind to some splice isoforms. However, mutations in the first and last of the four repeats similarly disrupt antibody staining (Figure 2C); an isoform that skipped both of these repeats would yield a much shortened form of the channel. In conventional members of the superfamily, isoforms that eliminate two of the four repeats have in fact been observed [23–25]. But, to our knowledge, these have never been shown to form functional channels and are often postulated to serve as negative regulators. Thus, we think it unlikely that our analysis has missed an important source of functional gene product.

As to the basis for the lower levels of channel protein, for the *na* mutation we presume that, as a result of an altered trans-membrane span, the protein is misfolded

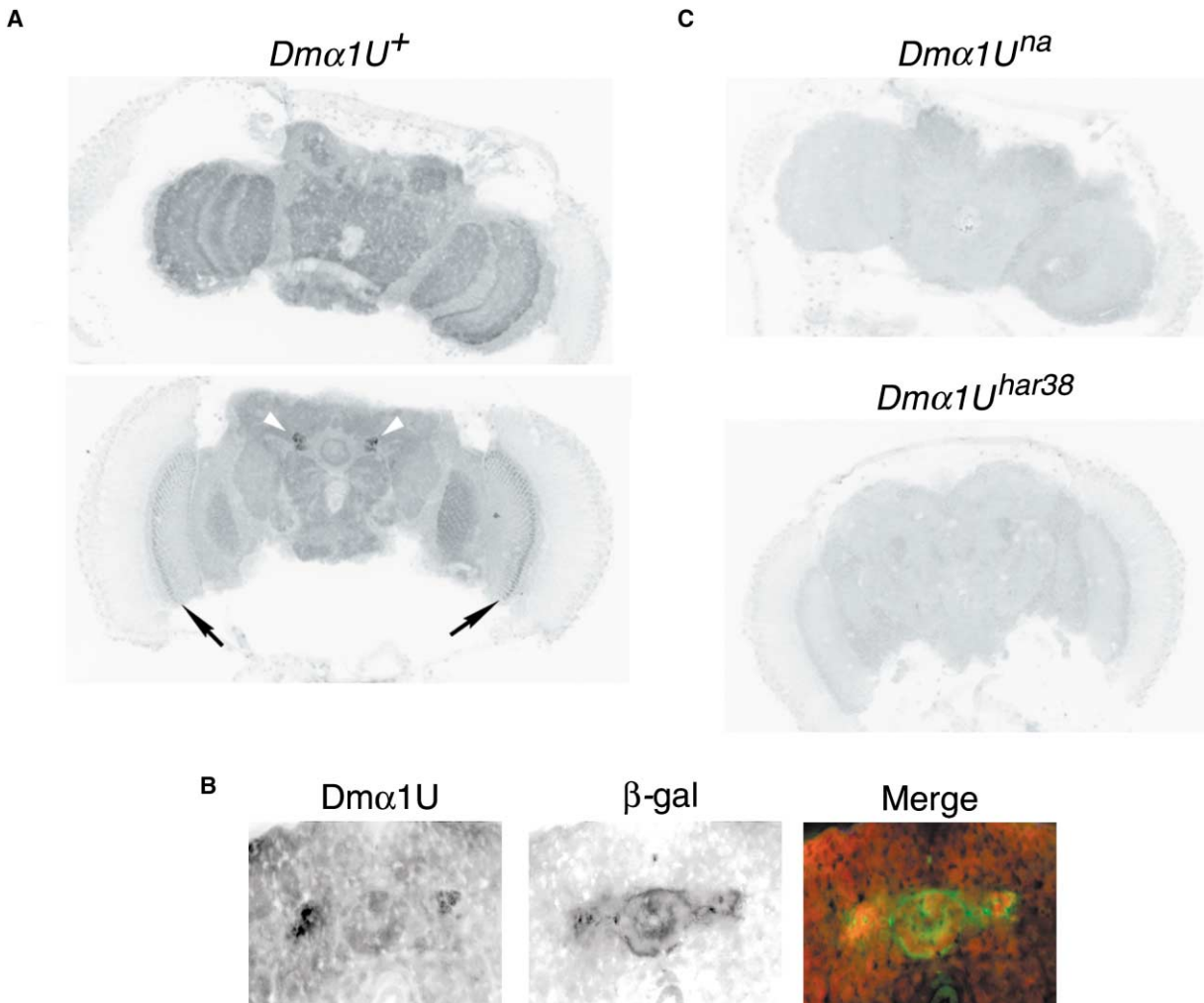


Figure 2. Anatomical Distribution of Channel Protein

(A) *Dmα1U⁺* flies. Cryosections (10 μm thick) of adult heads were fixed with 2% paraformaldehyde and stained with a 1:100 dilution of antiserum. The antiserum was raised in rabbits (Research Genetics, Huntsville, AL) to a segment of the ORF (shown in Figure 1A) that had been cloned into an expression vector (pET28c; Novagen, Madison, WI). After affinity purification (QUICKPure System; Sterogene Bioseparations, Carlsbad, CA), the antiserum was preadsorbed with a non-cognate antigen. After staining for 20 min and two brief washes, antibody was detected with Alexa Fluor 546 goat anti-rabbit IgG (Molecular Probes, Eugene, OR). Thin arrows point to a row of stained cells at the distal edge of the lamina, and arrowheads point to dense staining in a pair of structures near the ellipsoid body. To avoid a background fluorescent signal from pigment that escapes from the eyes, we used white-eyed flies for these sections and for those of panel (C).

(B) Identification of the heavily stained structure in the central brain. Red-eyed flies in which a *UAS-tau-lacZ* construct [38] was driven by GAL4 expressed from a *c232* insert [17] were sectioned, fixed, and stained as above with anti-CG1517 (left panel). The same sections were also stained with anti-β-galactosidase (Promega), which was detected with Alexa Fluor 488 goat anti-mouse IgG (middle panel). The merged image (right panel) shows costaining in the lateral triangles.

(C) *Dmα1U* mutant flies. When the same procedure as that in panel A is used, no specific staining is seen above a general background, one that persists equally in wild-type and mutant heads, when antibody is preadsorbed with the cognate antigen (R.L.S., unpublished data).

and subsequently degraded [26]. For *har38*, RT-PCR of RNA from adult heads shows that almost all transcripts from the region affected by the mutation are a little larger than wild-type. Sequencing of the principal RT-PCR product identified the change as the use of a cryptic splice site, located 41 nt downstream of the wild-type site (R.L.S. and H.A.N., unpublished data). The shifted reading frame results in premature termination of translation prior to the antigenic determinant (Figure 1); whether the truncated protein is degraded is unknown.

Mutant Phenotype

Because the existing alleles of the *na* gene attenuate expression of the protein, the phenotype of these strong hypomorphs should reveal the contribution of the putative channel to fruit fly biology. To make a proper comparison, we used lines in which the mutations had been repeatedly backcrossed to make them congenic with the wild-type Canton-S strain [11]. The mutant flies are noticeably smaller than controls but are not obviously sick or deformed. Furthermore, the mutants have a brain

Table 1. Activity Levels and Rhythmicity of Wild-Type and *Dmα1U* Flies

	LD				DD	Rhythmicity					
	L Activity		D Activity			Activity counts	Period (hr)	Power	R	B	A
	counts	(%)	counts	(%)	(%)						
Female											
+	50 ± 7	(68.3)	21 ± 3	(31.7)	32 ± 4	23.9 ± 0.1	50 ± 12	90	10	0	10
<i>Dmα1U^{na}/+</i>	48 ± 5	(67.5)	25 ± 4	(32.5)	32 ± 5	24.0 ± 0.1	37 ± 6	68	14	18	28
<i>Dmα1U^{har*/+}</i>	52 ± 3	(78.0)	15 ± 1	(22.0)	31 ± 2	23.9 ± 0.1	47 ± 5	72	20	8	75
<i>Dmα1U^{na}</i>	9 ± 2	(23.4)	32 ± 5	(76.6)	28 ± 3	24.5 ± 0.5	5 ± 4	22	11	67	9
<i>Dmα1U^{har*}</i>	17 ± 2	(33.6)	38 ± 4	(66.4)	32 ± 3	24.5 ± 0.4	9 ± 2	18	27	54	55
<i>Dmα1U^{na}/Dmα1U^{har*}</i>	19 ± 2	(32.7)	36 ± 3	(67.3)	30 ± 2	24.8 ± 0.4	8 ± 2	17	15	67	81
Male											
+	21 ± 1	(62.9)	13 ± 1	(37.1)	17 ± 1	24.0 ± 0.1	48 ± 5	83	7	10	72
<i>Dmα1U^{na}</i>	7 ± 1	(25.5)	21 ± 2	(74.5)	18 ± 2	25.3 ± 0.8	3 ± 1	5	18	77	44
<i>Dmα1U^{har*}</i>	10 ± 1	(30.0)	22 ± 1	(70.0)	18 ± 1	26.0 ± 0.9	2 ± 1	6	19	75	100

Activity was measured as counts per 30 min. Activity percentage (%) refers to the average percentage of individual flies' activity during the lights-on (L) or lights-off (D) period of LD. Rhythmicity, period analysis, and power (periodogram peak-significance line) were determined with Clock Lab analysis software [41]. Flies were categorized as rhythmic (R; power ≥ 15), borderline rhythmic (B; 0 < power < 15), or arrhythmic (A; power = 0). The designation *har** is used to indicate that data from stocks bearing the *har38* and *har85* alleles were pooled.

anatomy that is grossly normal and, despite the “hesitant” walking phenotype, do not show defects such as bang sensitivity or temperature-sensitive paralysis in assays for gross alterations of excitability (our unpublished data). This suggests that, despite its widespread distribution (Figure 2A), the *Dmα1U* channel is not essential for the basic operation of the nervous system. Instead, it raises the possibility that the channel is particularly important for a restricted set of functions. Here we show that regulation of the daily rhythm in locomotor activity is one such function.

When mutant flies that had been entrained to diurnal lighting (LD = 12 hr light:12 hr dark) were subsequently monitored under constant darkness (DD), all the mutant lines exhibited a similar phenotype. As summarized in Table 1, the majority of flies were arrhythmic, and only a small fraction were strongly rhythmic. A heteroallele analysis, with flies obtained by intercrossing mutant and wild-type lines, showed that the mutations are recessive and noncomplementing for this phenotype (Table 1). Although the defect in DD is significant, a more striking and uncommon aberration was seen during the LD entrainment. Wild-type flies are diurnal, with a greater proportion of their activity occurring during the daytime [27], but the mutants exhibit most of their activity at night (Figures 3A–3C). Under these lighting conditions, just as described above for flies in constant darkness, the locomotor pattern of trans-heterozygotes is indistinguishable from that of homozygotes (Table 1), and this finding argues strongly that both the LD and DD phenotypes reflect alterations in the *Dmα1U* gene and not some adventitious variation.

Although establishing the mechanism(s) by which the *Dmα1U* channel influences the operation of daily locomotor behavior will require much future effort, we have made an initial examination of a few key parameters. First, we tested for daily variation in channel protein. When Western blots of adult heads were taken at various times during LD, they revealed no significant change

in the amount or apparent size of the bands that are specifically recognized by our antibody (a representative blot can be seen as Figure S2 of the Supplementary Materials available with this article online). This indicates that expression of the channel is not under circadian regulation, a conclusion consistent with the absence of CG1517 from lists of genes identified in microarray studies as having daily variation in transcript levels (reference [28] and references cited therein). Second, again judged by Western blots of adult heads during LD (Figure 4), mutant flies exhibit a significant oscillation in the abundance and electrophoretic mobility of a key clock component, Per protein [29]. Although this experiment cannot rule out changes in a few pacemaker neurons or subtle changes in rhythm amplitude, the phase of Per cycling in the mutants argues against a gross mistiming of clock function as a molecular basis for the inverted day-night behavior. Consistent with this interpretation is the fact that those mutant flies that display strong rhythms in DD have normal period and phase. Note that, for such flies (e.g., Figure 3F), the phase in DD is opposite to that seen during LD; this finding argues that the circadian clock had not undergone a large shift or complete inversion of phase. If it had, activity in DD and LD would be expected to have the same (aberrant) phase. Instead, the behavior of the strongly rhythmic mutant flies suggests that, in the absence of channel function, locomotor activity is altered by light despite normal clock function. Here, it should be noted that *Dmα1U* mutants show two other effects of light on motion. Light suppresses a rhythmic (approximately 0.3 Hz) movement of the proboscis and antennae that is prominent in the mutants (S. Rajaram and H.A.N., unpublished observations), and (in white-eyed derivatives of the mutants) light enhances the climbing deficit that is induced by a general anesthetic [30]. Thus, an aberrant motor response to photic input is a general feature of the mutants.

Non-circadian effects of light on locomotor activity have been termed masking [31]. Accordingly, *Dmα1U* is

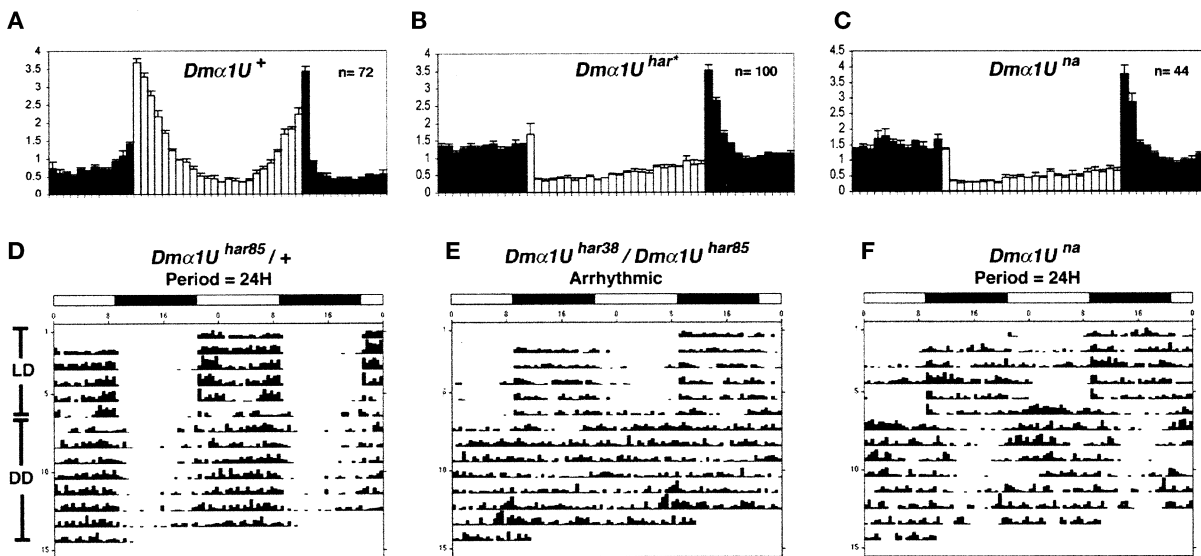


Figure 3. Diurnal Activity Patterns in Wild-Type and *Dmα1U* Mutant Flies

(A–C) Average activity. The activity of individual flies was monitored as described [39] in 30 min time blocks over 4 days of LD. The histograms represent average activity levels (\pm sem) over a 24 hr period, light and dark periods of each day being denoted by white and black bars, respectively. The activity of each fly was normalized, and individual days were then averaged into a single day, presented as arbitrary units and not absolute values. (A) Wild-type flies display a crepuscular activity pattern during LD conditions; activity peaks occur near the time of the lights-on and lights-off transitions. Wild-type flies anticipate both the lights-on and lights-off transitions by increasing their activity prior to those transitions. Overall activity is higher during the lights-on period than the lights-off period. (B and C) *Dmα1U* mutant flies show suppressed activity levels during the lights-on period. Additionally, these flies fail to anticipate the lights-off transition. Most mutant flies do not have a lights-on activity peak, although this peak does occur in some flies. In panel (B), the designation *har** is used as in Table 1.

(D–F) Representative actograms of individual wild-type and *Dmα1U* mutant flies. (D) During diurnal conditions, the activity of a heterozygote is like that of wild-type: highest at the beginning and end of the lights-on period (shown as a white bars above the actogram). When flies are shifted into constant darkness (DD; day 6), the activity pattern remains similar to that of LD, except that the activity peak corresponding to the lights-on transition is not maintained. (E) In this heterozygous mutant fly, activity during LD is concentrated in the lights-off period (black bars above actogram). In DD, the mutant displays an arrhythmic activity pattern. (F) An uncommon mutant fly that displays a rhythmic activity pattern in DD. In such flies, the phase of activity during DD is similar to that of wild-type flies and is anti-phase to the activity pattern observed in mutant flies during LD.

implicated either in enhancing positive masking, i.e., stimulation of locomotor activity by light, or in suppressing negative masking effects of light. Interestingly, similar LD activity patterns have been observed in *glass cryptochrome* (*gl^{60j} cry^p*) double mutants in which light input to the circadian clock is eliminated [32, 33]. It would be parsimonious to infer that *Dmα1U* is involved in transmission of photic information to the clock, but the possibility that the channel is an element that mediates circadian locomotor output must also be entertained. In addition, we do not know whether the frequent loss of rhythmicity of mutant flies in DD (Table 1) reflects the presumptive input/output defect or implies an additional role for *Dmα1U* in the robust operation of the central clock itself. Nonetheless, the results presented in this

paper, especially the inversion of light-dark behavior, argue for a prominent role of this novel ion channel in regulating the locomotor response to light.

Conclusions

The evidence we have presented indicates that the fly ortholog of a unique family of ion channels is an important modulator of neural function. Although a complete null mutation might reveal additional defects, those shown by the strong hypomorphs we have studied indicate the functions that are most critically dependent on the channel. The altered coupling between visual signals and locomotion is most dramatic, but the mutants still display defects, such as altered sensitivity to general anesthetics [30, 42], even in the absence of visual input.

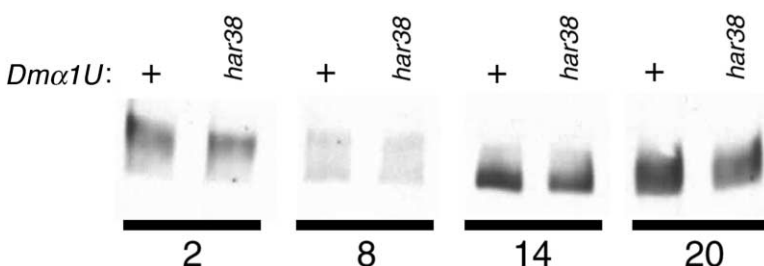


Figure 4. Western Blot Analysis of the Daily Variation in *per* Gene Product

Heads that were collected at the indicated Zeitgeber Times (where ZT0 is lights-on and ZT12 is lights-off) from adult flies of the indicated genotype. Extracts were made, electrophoresed, and probed as described [40].

It remains to be seen whether this novel channel is a direct anesthetic target, is important for the function of cells that contain such targets, or influences anesthesia more indirectly [34]. Finally, it must be noted that the human ortholog (represented by UniSTS-N40916) not only lies in the region implicated as containing a susceptibility locus for bipolar disorder but is virtually coincident with the peak of the LOD score [9]. Of course, other candidates from this region must also be considered, and it is dangerous to extrapolate too far from data on fruit fly behavior. However, our demonstration that hypomorphic mutations of this gene result in altered diurnal behavior, an endophenotype frequently associated with bipolar disorder [10], should serve to make this candidate a primary focus of attention.

Supplementary Material

Two supplementary figures are available with this manuscript online at <http://images.cellpress.com/supmat/supmatin.htm>.

Acknowledgments

We thank Yi Jun Shi and Tai Min for help with cryosectioning and Kevin Keegan for writing an Excel macro for analysis of light-dark behavior data. We also acknowledge Ben White and Tom Bonner for their comments on the manuscript and Kate Osborne for help with DNA sequencing.

Received: August 14, 2002

Revised: September 27, 2002

Accepted: October 9, 2002

Published: December 23, 2002

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