2mit, an Intronic Gene of *Drosophila melanogaster* *tim2*, Is Involved in Behavioral Plasticity

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Abstract

**Background:** Intronic genes represent ~6% of the total gene complement in *Drosophila melanogaster* and ~85% of them encode for proteins. We recently characterized the *D. melanogaster* *tim2* (*tim2*) gene, showing its active involvement in chromosomal stability and light synchronization of the adult circadian clock. The protein coding gene named *2mit* maps on the 11th *tim2* intron in the opposite transcriptional orientation.

**Methodology/Principal Findings:** Here we report the molecular and functional characterization of *2mit*. The *2mit* gene is expressed throughout *Drosophila* development, localizing mainly in the nervous system during embryogenesis and mostly in the mushroom bodies and ellipsoid body of the central complex in the adult brain. *In silico* analyses revealed that *2mit* encodes a putative leucine-Rich Repeat transmembrane receptor with intrinsically disordered regions, harboring several fully conserved functional interaction motifs in the cytosolic side. Using insertionional mutations, tissue-specific over-expression, and down-regulation approaches, it was found that *2mit* is implicated in adult short-term memory, assessed by a courtship conditioning assay. In *D. melanogaster, tim2* and *2mit* do not seem to be functionally related. Bioinformatic analyses identified *2MIT* orthologs in 21 *Drosophila* species, 4 *Lepidoptera* and in *Apis mellifera*. In addition, the *tim2-2mit* host-nested gene organization was shown to be present in *A. mellifera* and maintained among *Drosophila* species. Within the *Drosophilidae 2mit-hosting tim2* intron, *in silico* analyses detected a neuronal specific transcriptional binding site which might have contributed to preserve the specific host-nested gene association across *Drosophila* species.

**Conclusions/Significance:** Taken together, these results indicate that *2mit*, a gene mainly expressed in the nervous system, has a role in the behavioral plasticity of the adult *Drosophila*. The presence of a putative *2mit* regulatory enhancer within the *2mit-hosting tim2* intron could be considered an evolutionary constraint potentially involved in maintaining the *tim2-2mit* host-nested chromosomal architecture during the evolution of *Drosophila* species.

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Introduction

Any gene whose entire coding sequence lies within the bounds of a larger external gene is defined as a nested (or embedded) gene. The most common eukaryotic nested genes are completely embedded within large introns of their hosts, frequently oriented in the opposite transcriptional direction [1,2]. Recent data suggest that nested genes are widespread among Metazoa, representing ~6% of the total gene complement in *Drosophila melanogaster*, while ~2.1 and 0.5% in *Caenorhabditis elegans* and *Homo sapiens* genomes, respectively [2,3]. In *D. melanogaster*, nearly 85% of nested genes are predicted to encode for proteins, while the remaining generate non-coding RNAs [3,4].

Several investigations have been performed in order to evaluate the potential biological or evolutionary meaning of the host-nested gene organization in eukaryotic genomes. It has been hypothesized that nesting is favored by the presence of functional and/or transcriptional regulatory interactions between nested and host members [5]. However, a recent comparative analysis of expression profiles for 109 human and 752 *D. melanogaster* host-nested pairs did not show any significant
Results

introns, which harbors four nested transcribed sequences, of Hydropathy (GRAVY) value of -0.396. A transmembrane process in which long intronic sequences provide a niche for reported that only 20-34% of embedded gene relationships has been implicated in light synchronization of the circadian clock in the theoretical pI of 6.53 and a slightly hydrophilic Grand Average genomic architecture across the species in those cases.

In 2010, we characterized the D. melanogaster timeless2 (tim2 or timeout) locus [7], the paralog of the circadian clock component timeless1 (tim1) [8]). tim2 is widely expressed during development, and in the adult brain is localized mainly in the T1 basket neurons of the optic lobes and in the central complex. tim2 is an essential gene involved in maintaining chromosome integrity during development. Moreover, it has been implicated in light synchronization of the circadian clock in the adult fly [7].

tim2 is a 75 kb complex locus composed of 18 exons and 17 introns, which harbors four nested transcribed sequences, CG34308, BK002510, 2mit, and AY118619. Among these, only 2mit, located on the 11th intron of tim2, represents an embedded protein-encoding gene [7].

Here, we report the molecular and functional characterization of the D. melanogaster 2mit nested gene. We showed that 2mit is actively transcribed during embryogenesis, localizing in the developing nervous system. In the adult brain its expression is localized mainly in the mushroom bodies (MBs) and ellipsoid body (EB) of the central complex. In silico analyses indicated that 2MIT is a Leucine-Rich Repeat (LRR) transmembrane protein. Using insertional mutations, tissue-specific over-expression and RNA interference-mediated down-regulation, we demonstrated that 2mit is involved in adult behavioral plasticity, evaluated as short-term memory by a courtship conditioning assay. When nested 2mit and host tim2 gene functions were compared, no evident functional relationship became apparent. However, bioinformatic analyses identified 2MIT orthologs among 21 genome-sequenced species belonging to the Drosophila genus, in 4 Lepidoptera and in the hymenopteran Apis mellifera. The chromosomal tim2 locus organization, with 2mit embedded within a tim2 intron, was found in A. mellifera and all the examined Drosophilidae. The possible presence of evolutionary constraints contributing to preserve the tim2-2mit host-nested gene association across Drosophila species will be discussed.

2mit gene structure and protein sequence analysis

D. melanogaster 2mit (FBgn0260793) is organized in 2 exons and 1 intron and maps in an opposite transcriptional orientation within the 11th intron of the tim2 locus (Figure 1A [7]). 2mit conceptual translation originates a 1141 aa protein (NP_650258) with a predicted molecular weight of ~125 kDa, a theoretical pI of 6.53 and a slightly hydrophilic Grand Average of Hydropathicity (GRAVY) value of -0.396. A transmembrane domain spanning 931-953 residues was recognized by different software tools with the N- and C-terminal regions localized in the extracellular space and cytoplasmic compartment, respectively. The N-terminal region was found to contain a signal peptide with a cleavage site at position 29. These analyses strongly suggest that the mature form of the 2MIT protein is ~122 kDa in size, 1113 aa long, and a type I single-pass transmembrane protein. The 902 aa N-terminus is suggested to be exposed to the extracellular (or luminal) space, while the 187 aa C-terminus is cytoplasmic (Figure 1B).

The extracellular region is suggested to be composed of a structured domain and a long disordered region. The latter is characterized by low complexity sequences (residues 522-755 and 795-911) with a Thr-rich domain spanning 530-705 residues and a partially overlapping Ala-rich domain between 552-617 residues. The cytosolic domain is predicted to be prevalently disordered and contains a Ser-rich domain. In this region, the ELM server recognized several functional motifs, such as binding sites for 14-3-3 protein/s, Forkhead-associated (FHA) 1 and 2 factors, and a phosphorylation site for protein kinase A (PKA; Figure 1B).

FlyBase reported a second 2MIT protein isoform predicted to be 13 residues longer, possibly originating from translational stop codon read-through [9]. The C-terminal region of this longer 2MIT isoform (1155 aa) contains a TRG_endocytic motif, which is implicated in vesicular trafficking of different molecules (Figure 1B).

A scan of the N-terminal sequence against domain and protein signature databases, such as Pfam and PROSITE, revealed the presence of some LRRs which correspond to structural units (with a LxxLxLxxN/CxL conserved pattern) consisting of a β-strand and an α-helix. Since LRR domains are organized in series, they can form non-globular, crescent-shaped structures, which create a solvent-exposed, elongated, and concave surface of parallel β-strands, acting as a scaffold for interactions with other proteins [10]. The LRR domain was modeled using a MANIFOLD approach [11], which combines the prediction of secondary structures and results obtained by different repeat prediction methods. The first step was to identify the correct number of repeated units. RADAR, TRUST, and Repetita tools revealed different numbers of repeats. A structural alignment of all predicted repeated units was built in order to calculate a consensus pattern and to identify other missing repeats in the region spanning between the 80 and 530 amino acid positions. It was thus possible to recognize 16 repeats matching the consensus xxxLxxLxxNxLxxLpxxoFxx sequence that is typical for the LRR domain (Figure 2A). Each repeat contained hydrophobic, conserved positions (mostly Leu residues), predicted to be buried internally and to have a structural role. Other polar/charged residues are likely exposed to the solvent and are probably involved in protein-protein interactions.

Most LRR proteins contain flanking regions that are an integral part of the LRR domain. In the D. melanogaster 2MIT protein two terminal variable regions flank the LRR-NT and LRR-CT repeats. These regions usually have a capping role which protects the first and last repeats, but may also have a functional role. The LRR-NT is predicted to be disordered,
contains low complexity regions, and includes a cluster of charged residues between the 64 and 91 positions. Secondary structure analysis of the LRR-NT revealed that there are three hydrophilic α-helices which may form a N-terminal cap. The LRR-CT is estimated to be about 70 residues long and contains four Cys that may form disulfide bonds (Figure 2A).

The template search identified the structure of the Toll-like receptor 3 (PDB code: 2A0Z, chain A) as the most structurally similar to the 2MIT LRR domain (Figure 2B).

**2mit expression during development**

2mit is transcribed throughout *D. melanogaster* development in two mRNA isoforms of ~5.4 and 3.8 kb, carrying a unique 5′ UTR and two length-differing 3′ UTRs (353 and 1892 nt excluding the polyA segment [7]). *In situ* hybridization experiments on whole-mount embryos detected a diffuse 2mit expression pattern at the blastoderm stage (stage 5; Figure 3A). During segmentation (stage 15), 2mit mRNA localized in the developing Central and Peripheral Nervous Systems (CNS, PNS; Figure 3C, D).

In adult heads of wild-type flies that underwent 12 h: 12 h light:dark cycles (12:12 LD conditions, with ZTs 0 and 12 corresponding to lights-on and -off, respectively), Northern blot analyses revealed the presence of a single transcript corresponding to the ~5.4 kb 2mit mRNA isoform, suggesting that at least in that anatomical structure the longer mRNA variant is the most represented 2mit transcript (Figure 3F). To evaluate potential circadian variations in 2mit expression levels, quantitative PCR (QPCR) experiments were performed on adult heads, sampled every 4 h during the day, in both 12:12 LD and constant darkness (DD) regimes. Under 12:12 LD conditions, 2mit transcript levels showed an oscillating profile with a slight but significant variation over the 24 h cycle.
In particular, we revealed an increase in $2mit$ mRNA levels at approximately the end of the night/beginning of the day, between ZT 20 and ZT 0, and a reduction at ZT 8 (Newman-Keuls post-hoc test: ZT 8 vs ZT 0 or ZT 20 $p < 0.05$). After two days of DD, $2mit$ expression became constitutive ($F_{5,12} = 0.56$ $p = 0.76$, not significant; Figure 3G).

$2mit$ mRNA localization pattern was evaluated by in situ hybridization experiments on whole-mount adult fly brains sampled at ZT 0, when $2mit$ expression was known to be high. Specific hybridization signals were observed mainly in the MBs and EB of the central complex (Figure 3H-L), structures primarily involved in learning, memory, and locomotor activity control [12,13]. In the MBs, $2mit$ mRNA staining was observed in the neuronal somata (Kenyon cells) and at the level of both the vertical ($\alpha/\alpha'$) and medial ($\beta$, $\beta'$, $\gamma$) lobes, representing compact axonal structures (Figure 3H, I). Additional $2mit$ expression was visualized in the sub-esophageal ganglion (SOG; Figure 3H). Diffuse and weak signals were noted in both optic and antennal lobes (OLs, ALs; Figure 3H), probably as a result of non-specific hybridization staining. In fact, similar signals were observed when the $2mit$ sense probe was used in the same regions of the negative controls (Figure 3N).

Our $2mit$ mRNA analyses are consistent with high-throughput expression data from FlyBase indicating that the highest $2mit$ expression levels occur during embryogenesis, between 14 and 20 h after fertilization, and that transcription is restricted to nervous system structures at both larval and adult stages. Moreover, a recent study listed $2mit$ (CG 17319) among those genes preferentially expressed in the MBs [14].

$2mit$ is involved in courtship conditioning memory but not in learning

We previously demonstrated that the c03963 transposon insertion line (Exelixis Gene Disruption Project), carrying a PiggyBac (PB) element ~20 kb upstream of the $2mit$ ATG start codon, was characterized by a ~50% $2mit$ mRNA decrement and normal $tim2$ mRNA levels estimated at the third larval stage (L3 [7]; Figure 1A). After out-crossing with $w^{118}$ flies for eight generations, a ~50% $2mit$ mRNA depletion and unaffected $tim2$ mRNA levels compared to $w^{118}$ controls were confirmed in c03963 homozygous L3 ($2mit^{c03963}$) by QPCR (Table 1). We also analyzed mRNA levels of the other internally transcribed sequences in the $tim2$ locus, showing that the PB insertion in $2mit^{c03963}$ homozygous L3 did not cause any significant modifications in their expression compared to
Figure 3. 2mit mRNA expression in w118 flies. (A-D) Embryos at different developmental stages hybridized with the antisense 2mit probe. (A) Stage 5. (B) Stage 9. (C-D) lateral and frontal views of stage 15. (E) Negative control showing a stage 15 embryo hybridized with the 2mit sense probe. Bar in (E) represents 25 µm for (A)-(E). (F) Northern blot from wild-type adult heads sampled every 3 h in 12:12 LD conditions. 2mit indicates the single 2mit transcript revealed in adult heads. rp49 represents the rp49 housekeeping mRNA. M: RNA Ladder molecular marker. (G) 2mit mRNA levels [mean ± standard error of the mean (SEM)] sampled every 4 h in 12:12 LD (white bars) and DD (black bars) conditions. For each condition, 3 replicates were performed. In 12:12 LD, significant variations in 2mit mRNA levels were detected ($F_{5,10}$=7.89, $p <0.01$). In DD, no significant modifications in 2mit mRNA levels were detected ($F_{5,12}$= 0.56, $p= 0.76$). (H-L) 2mit mRNA localization in whole-mount adult brains sampled at ZT 0. (H-L) Wild-type adult brains hybridized with the 2mit antisense probe. (H) 2mit mRNA signals are visible at the level of the Kenyon cells (arrow) and in the different lobes of the MBs (image shows a ~6 µm Z-projection along the antero-posterior axis). (I) and (L) ~5 µm Z-projections obtained from the same brain showing 2mit mRNA signals in the Kenyon cells (arrows; I) and in the EB (L). (M) 2mitΔc03963 adult brain hybridized with the 2mit antisense probe. Weak signals are detected in the MB lobes and EB. (N) Wild-type adult brain hybridized with the 2mit sense probe (negative control). (M) and (N) are ~10 µm Z-projections. The following abbreviations are used: ol: optic lobe; al: antennal lobe; mb: mushroom bodies; mbl: mushroom bodies lobes; eb: ellipsoid body; sg: subesophageal ganglion; cg: central ganglion. α/α': vertical mushroom bodies lobes; β, β', γ: medial mushroom bodies lobes. Bar in (N) represents 50 µm for (H), (M) and (N), and 25 µm for (I), (L), and the 2X magnification inset in (M).

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w1118 controls (mRNA levels in 2mit<sup>03963</sup> and w1118 individuals: CG34308: 1.13 ± 0.33 vs 1 ± 0.0; F<sub>1,8</sub> = 0.15 p = 0.71; BK002510: 1.27 ± 0.57 vs 1 ± 0.0; F<sub>1,8</sub> = 0.22 p = 0.64; AY118619: 0.94 ± 0.17 vs 1 ± 0.0; F<sub>1,8</sub> = 0.93 p = 0.37; all p values are not significant). We next determined 2mit mRNA levels in 2mit<sup>03963</sup> homozygous adult heads, showing ~20% 2mit mRNA transcription levels compared to those of w1118 controls (Table 1). In addition, a weak 2mit mRNA signal was detected in 2mit<sup>03963</sup> homozygous adult brains by in situ hybridization experiments. In particular, we observed faint 2mit staining in the neuronal fibers of the EB and MB lobes (Figure 3M). No evident 2mit mRNA signals were detected in the brain region where MB cell bodies are located (Figure 3M), as probably they are under the detection limit of the in situ hybridization technique performed in this study.

2mit<sup>03963</sup> homozygous flies were viable and fertile. No lethal phenotypes were observed during post-embryonic development (Table 2). Adult 2mit<sup>03963</sup> homozygous flies did not display any gross morphological abnormalities and their overall brain organization was similar to that of wild-type flies. In addition, they did not show any impairment in light perception, evaluated as phototactic behavior (Table S1), as well as in locomotor activity (Figure S1).

2mit mRNA levels were also analyzed in five other strains (f00075, f06803, MB03271, MB08962, and MB08132, Exelixis Gene Disruption Project; Figure 1A) carrying PB or Minos (MB) transposons in proximity or within the 2mit gene. None of these strains showed significant modifications in 2mit mRNA levels (Table 1); thus they were excluded from subsequent analyses.

Given the specific brain 2mit mRNA hybridization pattern, we decided to assess whether 2mit plays a role in the behavioral plasticity associated with learning and memory. Therefore, 2mit<sup>03963</sup> homozygous adult males were analyzed using the courtship conditioning assay [15,16]. This test is based on natural sexual behavior and measures the reduction in courtship levels of male flies which have previously courted non-receptive, mated females [17]. For each genotype, we...
measured the Courtship Index (CI), defined as the time spent by a male courting an anesthetized virgin female during a 10 min observation period, in conditioned and sham males [15,16]. In OR-R control flies, the CIs of conditioned males were significantly reduced compared to those of sham individuals, indicating short-term memory (STM) formation (Mann-Whitney U test: OR-R: p<0.01; Figure 4A). As expected, w1118 sham males showed lower CI levels compared to OR-R sham controls under light conditions since white-eyed, vision-defective males have difficulty tracking females during courtship [18]. However, w1118 males showed STM formation, as the CIs of conditioned males were significantly lower with respect to those of sham controls (Mann-Whitney U test: w1118: p<0.05; Figure 4A). Similar results were also described by another study on flies carrying the w1118 allele in a Cantonized strain (w CantonS [19]). Therefore, we considered the w1118 flies a suitable control to study memory phenotypes using the courtship conditioning paradigm in 2mit03963 homozygous flies, which essentially have a w1118 genetic background, except for the PB transposon insertion detectable by a mini-w' marker gene.

The CIs of 2mit03963 sham males resulted similar to those of OR-R sham controls (Mann-Whitney U test: p=0.36, not significant; Figure 4A), indicating that 2mit03963 homozygous males possess normal virgin female perception. However, in 2mit03963 flies, the CIs of conditioned males were not significantly different from those of sham controls (Mann-Whitney U test: p=0.38, not significant; Figure 4A). Analogous results were obtained after comparing the CIs of sham and conditioned males in the classic memory mutant dunce (dnc1), used as negative control (Mann-Whitney U test: p=0.32, not significant; Figure 4A). Since during the test we did not observe evident impairments in courtship behavior and any abnormality in locomotor activity of 2mit03963 flies (Figure S1), these data suggest the presence of memory defects in 2mit03963 mutant males.

In addition, when tim2/+ heterozygous males for two different alleles (tim2o06979 and tim2o03027) have been analyzed, the CIs of conditioned males resulted significantly lower compared to those of sham controls (Mann-Whitney U test: p<0.05 for both tim2o06979/+ and tim2o03027/+ individuals; Figure 4A), suggesting that the tim2 depletion in tim2/+ heterozygous flies does not cause memory impairment.

To understand whether 2mit03963 memory defects were associated with training (learning) deficiencies, we determined the training index (TI), defined as the ratio between CIs during the final (CI) and initial 10 min (CI) of the training period [15,16], in conditioned 2mit03963 mutant and w1118 control males. TI values ≤ 0.5 are generally typical of wild-type flies, while TIs > 0.5 are characteristic of conditioning defective individuals [16]. Both 2mit03963 and w1118 flies exhibited TIs < 0.5 and the comparison between 2mit03963 and w1118 did not show any significant difference (Mann-Whitney U test: p=0.6, not significant; Figure 4B), indicating that 2mit03963 males reduced their courtship behavior in the presence of a mated female, thus displaying learning activity.

In order to determine whether memory defects in 2mit03963 flies were related to 2mit depletion, we generated three independent transgenic lines (2mitO53, 2mitO44, 2mitO77) for a UAS-2mitHA cDNA chimeric construct designed for 2mit over-expression studies. The presence of a 2MIT-HA chimeric protein was visualized by Western blot from flies in which 2MIT-HA over-expression was ubiquitously activated using an Actin5C-Gal4 (ActGal4) driver in a wild-type 2mit genetic background (Figure 5A). We identified a ~150 kDa band in the three ActGal4> 2mitO lines that was absent in the negative control (ActGal4>CyO) and likely represents 2MIT-HA. The discrepancy in molecular weight between the visualized band

![Figure 4. Memory and learning in 2mit03963 homozygous mutant flies.](image-url)
and the one expected from the estimation of 2MIT-HA theoretic weight (~122 kDa) may have resulted from anomalous detergent binding and denaturation in SDS-PAGE migration, which for transmembrane proteins may explain till ~45% molecular weights variations [20]. The three ActGal4>2mitO lines overexpressed different degrees of the 2MIT-HA protein, with higher levels in ActGal4>2mitO\(^{6+}\) flies and lower ones in ActGal4>2mitO\(^{4+}\) individuals.

Both 2mit mRNA and 2MIT-HA chimeric protein productions were then evaluated in adult brains of flies over-expressing the UAS-2mitHA construct at the level of the MBs (using the OK107 Gal4 driver) in a wild-type 2mit\(^{6+}\) genetic background. In OK107 Gal4>2mitO brains, 2mit mRNA and 2MIT-HA protein signals co-localized in the Kenyon cells and axonal lobes of the MBs (Figure S2).

Using genetic crossing and the pan-neuronal elavGal4 driver, we generated three elavGal4>2mitO lines, over-expressing 2MIT at the CNS level in a mutant 2mit\(^{6+}\) background (elavGal4>2mitO\(^{6}\), 2mit\(^{3+}\); elavGal4>2mitO\(^{6+}\); 2mit\(^{3+}\) and elavGal4>2mitO\(^{4+}\); 2mit\(^{3+}\)). 2mit mRNA levels were then checked in dissected adult brains by QPCR, which revealed 2mit mRNA over-expression in all three elavGal4>2mitO, 2mit\(^{3+}\) transgenic lines compared to w\(^{1118}\) controls, with higher values in elavGal4>2mitO\(^{6}\), 2mit\(^{3+}\) flies (~9-fold higher) and lower in elavGal4>2mitO\(^{4+}\); 2mit\(^{3+}\) individuals (~2-fold higher; Figure 5B).

In order to evaluate 2MIT’s ability to rescue the 2mit\(^{6+}\) mutant memory phenotype, we determined the CIs of conditioned and sham male flies for the three elavGal4>2mitO, 2mit\(^{3+}\) lines (elavGal4>2mitO\(^{6}\), 2mit\(^{3+}\); elavGal4>2mitO\(^{6+}\); 2mit\(^{3+}\) and elavGal4>2mitO\(^{4+}\); 2mit\(^{3+}\)) and relative controls (elavGal4>+/+; 2mit\(^{3+}\); Æ>2mitO\(^{6}\); 2mit\(^{3+}\); Æ>2mitO\(^{4+}\); 2mit\(^{3+}\); 2mit\(^{3+}\); Figure 5C). In all three elavGal4>2mitO, 2mit\(^{3+}\) lines, the CIs of conditioned males were significantly reduced with respect to those of sham individuals, indicating a substantial STM rescue (Mann-Whitney U test: elavGal4>2mitO\(^{6}\), 2mit\(^{3+}\); p< 0.005; elavGal4>2mitO\(^{4+}\), 2mit\(^{3+}\); p< 0.0001; elavGal4>2mitO\(^{6+}\); 2mit\(^{3+}\); p< 0.005). Memory defects were maintained in negative control flies carrying the elavGal4 driver or the UAS-2mitHA construct alone in a mutant 2mit\(^{3+}\) background, with the CIs of conditioned males similar to those of sham flies in all control lines (Mann-Whitney U test: elavGal4>+/+; 2mit\(^{3+}\); p = 0.24; Æ>2mitO\(^{6}\), 2mit\(^{3+}\); p = 0.81; Æ>2mitO\(^{4+}\), 2mit\(^{3+}\); p = 0.33; Æ>2mitO\(^{4+}\); 2mit\(^{3+}\); p = 0.43; all p values are not significant). Taken together, these data suggest that 2mit is required for Drosophila memory formation.

**2mit downregulation alters memory phenotype**

To confirm the involvement of 2mit in STM formation, we produced three independent transgenic lines (2mit KD\(^{6+}\); 2mit KD\(^{6+}\); 2mit KD\(^{3+}\); 2mit KD\(^{3+}\)) carrying a UAS-construct for 2mit knockdown (KD) analyses. No lethal phenotypes were observed when 2mit KD was generally induced in both neuroblasts and neurons (with the i(3)31Gal4 driver) or in mature neurons (with the elavGal4 driver) during embryonic development (Table 2). Pan-neuronal elavGal4>2mit KD produced different levels of 2mit mRNA depletion in dissected adult brains of the three...
transgenic lines. In fact, 2mit expression levels ranged from 0.17 ± 0.01 in 2mit KD61.1 to 0.34 ± 0.01 in 2mit KD6.1 compared to 1.00 ± 0.19 of w1118 controls (Figure 6A).

The STM phenotype in the elavGal4>2mit KD lines was then analyzed (Figure 6B). The pan-neuronal 2mit mRNA KD affected STM formation, since the CI values of conditioned flies resulted comparable to those of sham males in all of the elavGal4>2mit KD lines (Mann-Whitney U test: elavGal4>2mit KD6.1: p= 0.17; elavGal4>2mit KD61.1: p= 0.13; elavGal4>2mit KD6.1: p= 0.88; all p values are not significant). Moreover, significant differences were observed in the CIs of conditioned and sham flies in all negative controls (Mann-Whitney U test: elavGal4> +: p< 0.05; ++ 2mit KD6.1: p< 0.0001; ++ 2mit KD61.1 and ++ 2mit KD6.1: p< 0.005), suggesting once again that 2mit plays a specific role in the Drosophila memory phenotype.

We then evaluated the CIs of conditioned and sham males in transgenic flies, in which 2mit was silenced mainly in the whole MB structure, using the OK107- and c772Gal4 drivers, or in α, β, and γ MB lobes, with the MB247Gal4 driver [21]. Additional 2mit KD could be produced in other brain regions, since these drivers result weakly active also in the OLs and ALs (OK107Gal4), the OLs, ALs, EB and SOG (c772Gal4), and the OLs and glia cells (MB247Gal4) [21].

In two out of three OK107Gal4>2mit KD transgenic lines (2mit KD6.1 and 2mit KD6.1) and in all of the c772Gal4>2mit KD and MB247Gal4> 2mit KD lines (2mit KD6.1, 2mit KD61.1, and 2mit KD6.1), the CIs of conditioned and sham males were not statistically different, indicating altered memory formation (Mann-Whitney U test: with the OK107Gal4 driver: 2mit KD6.1: p= 0.18; 2mit KD61.1: p= 0.36; with the c772Gal4 driver: 2mit KD6.1: p= 0.67; 2mit KD61.1: p= 0.72; 2mit KD61.1: p= 0.42; with the MB247Gal4 driver: 2mit KD6.1: p= 0.88; 2mit KD61.1: p= 0.12; 2mit KD61.1: p= 0.27; all p values are not significant; Figure 6C). On the contrary, normal STM was observed in all the appropriate negative controls (++; 2mit KD lines: Figure 6B; Mann-Whitney U test: p> 0.05; c772Gal4> + and MB247Gal4> +: p< 0.05; Figure 6C). The third OK107Gal4>2mit KD61.1 transgenic line did not show any impairment in the STM phenotype, since in that case the CIs of conditioned males were significantly reduced from those of sham controls (Mann-Whitney U test: p< 0.05; Figure 6C). The absence of STM defects in that line could have been due to an inefficient 2mit down-regulation generated by the combination of the OK107Gal4 driver and the 2mit KD transgene, specifically in OK107Gal4> 2mit KD6.1 flies. In fact, when 2mit KD was pan-neuronally activated in 2mit KD6.1 flies by elavGal4, a 2mit mRNA down-regulation in dissected brains and parallel defects in STM were detected. In addition, the use of both c772- and MB247Gal4 drivers produced STM deficiencies in 2mit KD61.1 flies.

Finally, no STM deficiencies were detected when 2mit silencing was induced in different neurons of the central complex, using the c232- and 52YGal4 drivers. In fact, the CIs of conditioned males were significantly reduced compared to those of sham controls for all these 2mit KD-driver combinations (Mann-Whitney U test: c232Gal4> + and c232Gal4> 2mit KD6.1: p< 0.005; c232Gal4> 2mit KD61.1: p< 0.0001; c232Gal4> 2mit KD6.1: p< 0.05; 52YGal4>+ and c232Gal4> 2mit KD61.1: p< 0.05).

Figure 6. 2mit knockdown in adult flies. (A) 2mit mRNA relative levels (mean ± SEM) in the dissected brains of three independent elavGal4>2mitKD (6.1; 16.2; 61.1) and w1118 controls. Plot of 6 replicates. F3,20 = 1571, p< 0.0001; Newman-Keuls post-hoc test: each elavGal4>2mitKD line vs w1118 controls: p< 0.001. (B) Courtship Indices in sham (white bars) and conditioned (black bars) males for elavGal4>2mitKD (6.1; 16.2; 61.1) and appropriate controls [elavGal4>++; ++ 2mit KD (6.1; 16.2; 61.1)]. Mann-Whitney U test showed no significant differences between the CIs of sham and conditioned males in all the elavGal4>2mit KD (elavGal4>2mit KD6.1: p= 0.17; elavGal4>2mit KD61.1: p= 0.13; elavGal4>2mit KD6.1: p= 0.88). Significant differences between the CIs of conditioned and sham males were found in the control lines elavGal4> +: ++ 2mit KD6.1: ; ++ 2mit KD61.1: ; ++ 2mit KD6.1: ; ++ 2mit KD61.1: (C) Courtship Indices in sham (white bars) and conditioned (black bars) males for OK107Gal4>2mitKD (6.1; 16.2; 61.1), c772Gal4>2mitKD (6.1; 16.2; 61.1), MB247Gal4>2mitKD (6.1; 16.2; 61.1) and the control lines OK107Gal4++, c772Gal4++, MB247Gal4++. Mann-Whitney U test showed no significant differences between the CIs of sham and conditioned males in two out of three OK107Gal4>2mit KD lines (OK107Gal4>2mit KD6.2: p= 0.18; OK107Gal4>2mit KD61.1: p= 0.36), in all the c772Gal4>2mit KD flies (c772Gal4>2mit KD6.1: p= 0.67; c772Gal4>2mit KD61.1: p= 0.72; c772Gal4>2mit KD6.1: p= 0.42) and in all the three MB247Gal4>2mit KD lines (M247Gal4>2mit KD6.1: p= 0.88; MB247Gal4>2mit KD61.1: p= 0.12; MB247Gal4>2mit KD6.1: p= 0.27). Significant differences between the CIs of conditioned and sham males were found in the control lines OK107Gal4++, c772Gal4++, MB247Gal4++ and in the OK107Gal4>2mit KD61.1 transgenic line. In (B) and (C) data are expressed as mean ± SEM, with the number of tested flies indicated above each bar. The number of asterisks indicates the significance level: *: p< 0.05; **: p< 0.005; ***: p< 0.0001; ns: not significant.

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initially determined the both PR C profile of when pulsed at the beginning (ZTs: 13, 15, and 17) or at the end (ZTs: 21 and 23) of the night, respectively (Figure 7). The response of the circadian clock [7], we investigated whether 2MIT orthologs showed an incomplete coverage in 8 Drosophilidae species (D. simulans, D. sechellia, D. yakuba, D. erecta, D. ananassae, D. pseudoobscura, D. persimilis, D. willistoni, D. mojavensis, D. viridis, and D. grimshawi; Drosophila 12 Genomes Consortium 2007, http://rana.lbl.gov/drosophila/), in 4 Lepidoptera (Manudra sexta and Bombyx mori moths and Danaus plexippus and Heliconius melpomene butterflies) and in the following insect species: Culex quinquefasciatus, Apis mellifera, Aedes aegypti, Pediculus humanus, and Acyrthosiphon pisum. Due to the presence of gaps in the assembly of contigs, the multi-alignment process of 2MIT orthologs showed a significant degree of conservation (Table 3 and File S1). In all 2MIT proteins, the N-terminal signal peptide, LRR domains, the ELM server [26], and transmembrane helix were identified (File S1). In 2MIT ortholog multi-alignment for the 21 Drosophilidae species, the 2MIT putative LRR domains of orthologs. Moreover, the 2MIT putative LRR domains of Lepidoptera and A. mellifera were characterized by the presence of two additional repeated units. Maximum Likelihood analysis produced a 2MIT phylogenetic tree which mirrored the species tree. Among Drosophilidae, 2MIT phylogenesis followed both subgenus and subgroup classification of the Drosophila genus (Figure 8A). Within the 2MIT cytoplasmic region, the ELM server [26] identified different binding motifs common to almost all species (Figure 8B). Descriptions for these linear motifs can be found.

Figure 7. Phase response curve of 2mit<sup>03963</sup> and w<sup>1118</sup> flies. Analysis of variance genotype X time interactions (ZT): F<sub>5,434</sub>=1.76 p=0.12, not significant. Advance and delay phase shift responses are represented respectively as positive and negative values. 2mit: 2mit<sup>03963</sup> homozygous mutant flies. Data are expressed as mean ± SEM; (Ns) and [Ns] indicate the number of 2mit<sup>03963</sup> and w<sup>1118</sup> flies analyzed, respectively.

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Drosophila melanogaster 2MIT orthologs and phylogeny

Searching for 2MIT orthologs with tBlastN (using D. mel 2MIT as a query) against non-redundant sequences identified 2MIT ortholog in 12 Drosophilidae genomic sequences released in 2007 (D. melanogaster, D. simulans, D. sechellia, D. yakuba, D. erecta, D. ananassae, D. pseudoobscura, D. persimilis, D. willistoni, D. mojavensis, D. viridis, and D. grimshawi; Drosophila 12 Genomes Consortium 2007, http://rana.lbl.gov/drosophila/), in 4 Lepidoptera (Manudra sexta and Bombyx mori moths and Danaus plexippus and Heliconius melpomene butterflies) and in the following insect species: Culex quinquefasciatus, Apis mellifera, Aedes aegypti, Pediculus humanus, and Acyrthosiphon pisum. due to the presence of gaps in the assembly of contigs, the multi-alignment process of 2MIT orthologs showed an incomplete coverage in 8 Drosophilidae species (D. simulans, D. sechellia, D. yakuba, D. erecta, D. ananassae, D. pseudoobscura, D. persimilis, and D. willistoni) and in all the non-Drosophilidae species, except for A. mellifera. It was possible to extend the 2MIT coding region for Drosophilidae and Lepidoptera, using the Augustus gene prediction tool [25] on available whole-genome-shotgun sequences, and a full-length 2mit coding sequence was obtained (Table 3).

The nested 2mit gene does not show a functional relationship with its timeless2 host gene

A functional relationship could be shared by nested and host genes [5]. In Drosophila, homozygous tim2/tim2 flies die very early during pupal development. As heterozygous tim2/+ adult flies are characterized by a modified light synchronization response of the circadian clock [7], we investigated whether 2mit shares similar functions in the adult brain. Since the natural Is's polymorphism in the circadian clock gene tim1 significantly influences circadian light responses [23,24], we initially determined the Is-tim1/s-tim1 genotype, showing that both 2mit<sup>03963</sup> and w<sup>1118</sup> flies were homozygous for the Is-tim1 variant. Subsequently, we analyzed 2mit<sup>03963</sup> and w<sup>1118</sup> adult male responses to 20 min light pulses given at different times during the night (ZTs: 13, 15, 17, 19, 21, and 23), generating a phase response curve (PRC). As expected, w<sup>1118</sup> control flies showed delayed or advanced phase shifts in locomotor activity when pulsed at the beginning (ZTs: 13, 15, and 17) or at the end (ZTs: 21 and 23) of the night, respectively (Figure 7). The PRC profile of 2mit<sup>03963</sup> flies was similar to that of w<sup>1118</sup> controls (genotype X time (ZT) interactions: F<sub>5,434</sub>=1.76, p=0.12, not significant; Figure 7), suggesting that 2mit is not involved in light synchronization of the adult fly circadian clock.
Table 3. Protein information on 2MIT identified orthologs.

| Gene ID     | Species                          | Protein Predicted protein | %I | %S |
|------------|----------------------------------|---------------------------|----|----|
| *FBgn0208793 | D. melanogaster                  | 1141 1141                 | 100| 100|
| *FBgn0126657 | D. grimshawi                     | 1177 1177                 | 62 | 77 |
| *FBgn0080830 | D. pseudoobscura                 | 822 1158                  | 66 | 74 |
| *FBgn0198065 | D. virilis                       | 1160 1160                 | 62 | 74 |
| *FBgn0146946 | D. mojavensis                    | 1117 1117                 | 56 | 65 |
| *FBgn0241583 | D. yakuba                        | 824 1153                  | 95 | 98 |
| *FBgn0221118 | D. willistoni                    | 823 1162                  | 66 | 74 |
| *FBgn1099306 | D. erecta                        | 811 1100                  | 87 | 90 |
| *FBgn0191991 | D. simulans                      | 798 1001                  | 93 | 94 |
| *FBgn0096918 | D. ananassae                     | 812 1138                  | 71 | 78 |
| *FBgn0160729 | D. persimilis                    | 817 1161                  | 75 | 63 |
| *FBgn0180817 | D. sechellia                     | 411 1084                  | 71 | 75 |
| *FBgn00100265 | D. eugracilis                   | NA 1157                  | 85 | 89 |
| *FBgn01023804 | D. rhopaloa                    | NA 1147                  | 91 | 96 |
| *FBgg00104651 | D. biarmipes                     | NA 999                  | 90 | 94 |
| *FBgg00108324 | D. bipunctata                    | NA 1141                 | 74 | 80 |
| *FBgg00104256 | D. elegans                      | NA 1154                 | 83 | 88 |
| *GL987664 | D. ficusphila                    | NA 1188                 | 81 | 85 |
| *JH1111146 | D. kikkawai                      | NA 1144                 | 77 | 82 |
| *JH112787 | D. takahashii                    | NA 1143                 | 80 | 85 |
| *AJA002003167 | D. miranda                      | NA 1155                 | 65 | 73 |
| EGB10366  | A. mellifera                     | 999 999                 | 36 | 53 |
| EHML002549 | H. melpomene                     | NA 825                 | 36 | 53 |
| Ensoa0348 | B. mori                          | NA 802                 | 39 | 57 |
| EJH387114 | D. plexippus                     | NA 710                 | 39 | 53 |
| *AJAI00100612 | M. sexta                       | NA 792                 | 38 | 57 |

Annotated and predicted protein products are listed (numbers indicate aa length). I %: % Identity; S %: % Similarity; *: FlyBase ID; £: Ensembl; § GenBank ID; NA: not available.
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on the ELM web-site. In particular, in all 2MIT orthologs we detected recognition sites for N-arginine dibasic convertase (CLV_NDR_NDR1) and subtilisin-like pro-protein convertases (CLV_PCSK_KEX2_1), known to be involved in post-transduction maturation of several target proteins, as well as ligand sites for 14-3-3 protein/s (LIG_14-3-3_3) and a PKA phosphorylation site (MOD_PKA_2), implicated in fundamental cellular processes such as signal transduction and cell-cycle control. Moreover, at least one ligand site for FHA factors (LIG_FHA_1 and/or LIG_FHA_2), a protein domain involved in many signaling processes, and one intracellular sorting signal motif (TRG_ENDOCYTIC_2 and/or TRG_ER_diArg_1) were found in all the 2MIT ortholog C-terminal regions analyzed.

Analyses of tim2-2mit chromosomal organization in Drosophilidae, Lepidoptera and Apis mellifera

Comparative analyses of the D. melanogaster 2mit-harboring chromosomal region with those of the 12 Drosophila species sequenced in 2007 made it possible to identify conserved synteny using a 200kb-gene sliding window. Specifically, in all 12 species, 2mit was found to localize in an opposite transcriptional orientation within the tim2 11th intron. Moreover, 2mit was always identified in an opposite transcriptional direction in the same contig of the tim2 gene in the 9 newly sequenced Drosophila species released in 2013, which presented only wgs data. Analyses of the A. mellifera genome database (release 4.5) showed that the 2mit honey bee (Am 2mit) ortholog maps internally to the Am tim2 intron 14 on opposite strands. However, the tim2-2mit host-nested gene structure was not maintained in the 4 lepidopterous species (EnsemblMetaZooa, release 19).

Finally, we analyzed the D. melanogaster genomic region around the c03963 transposon, using a sliding window of approximately 400 nucleotides both upstream and downstream of the insertion site, searching for potential transcription factors (TFs). In particular, we identified a 6 bp TF binding site specific for the zinc-finger neuronal SNAIL repressor within the 11th intron of the D. melanogaster tim2 gene (5’CAGGTG3’, mapping ~20 kb upstream of the 2mit coding region). Comparative analyses performed on the 21 Drosophilidae identified SNAIL binding site conservation in the tim2 11th intron in all species with the exception of D. virilis, D. ananassae, D. grimshawi, and D. rhopaloa, which are characterized by incomplete assembly coverage in the region upstream of 2mit.

Discussion

2mit was originally identified as an intronic protein-coding gene located in an opposite transcriptional orientation within the 11th intron of the Drosophila melanogaster tim2 locus. Its transcription at all developmental stages gives rise to two 2mit mRNA variants with different 3’ UTR lengths (~5.4 and ~3.8 kb [7]). A recent FlyBase release (FB2013_03) suggested that AY118619 is part of the 2mit gene, representing a portion of the 1.3 kb 2mit 5’ UTR. This conceptually inferred analysis predicts that 2mit is composed of 4 exons and 3 introns and transcribed in a ~8.8 kb mRNA, ~3 kb longer than the unique ~5.4 kb 2mit mRNA isoform detected by Northern blot in adult fly heads. Furthermore, 2mit[10368] homozygous flies carrying the PB transposon ~20 kb upstream of the 2mit coding region and in the second intron of the AY118619 sequence have shown marked 2mit mRNA depletion and unaltered AY118619 mRNA levels. These data suggest that 2mit and AY118619 are two independent embedded genes in the tim2 locus and confirm our previous results indicating that 2mit is organized in 2 exons and 1 intron [7].

In silico analyses have indicated that D. melanogaster 2mit encodes a transmembrane protein carrying a LRR domain in the extracellular portion and a Ser-rich region in the cytoplasmic portion, which might represent a putative binding site for different molecular factors. LRR domains are widespread and highly conserved structural motifs with a primary function in protein-protein interactions [10]. LRR-bearing proteins in Eukaryotes have been shown to be key components in several biological processes, such as embryonic development, cell adhesion and signaling, and extracellular matrix assembly [27]. Among Metazoans, LRR proteins are fundamental in neuronal circuit development, including axon/dendrite guidance and synapse formation [28]. In particular, both transmembrane and secreted LRR proteins...
Seem to play a key role in the alignment of pre- and postsynaptic membranes, ensuring efficient neuronal communication [29-31]. Furthermore, LRR proteins have been found to be involved in the regulation of adult nervous system structural plasticity in mammals [28]. In Drosophila, transmembrane LRR proteins such as CAPRICIOUS (CAPS) and TARTAN (TRN) are involved in regulating axon and dendrite targeting during the development of neuromuscular, olfactory and visual systems [28]. Both \textit{caps} and \textit{trn} amorphic alleles cause lethal phenotypes during embryonic or postembryonic development [32,33].

Since \textit{2mit} resulted generally expressed in the CNS and PNS during embryonic segmentation, it can be hypothesized that 2MIT plays a role similar to that of other LRR transmembrane proteins involved in neuronal development. However, no lethal phenotypes were observed in \textit{2mit} \textit{c03963} homozygous flies and analogous results were obtained when \textit{2mit} silencing was induced in neuroblasts and neurons during embryogenesis. In addition, \textit{2mit} \textit{c03963} homozygous adult flies have not shown any evident morphological abnormalities. The \textit{2mit} \textit{c03963} allele is a hypomorphic variant of the \textit{2mit} gene, since \textit{2mit} mRNA decrements in \textit{2mit} \textit{c03963} homozygous larvae and adults, respectively. The hypothesis that \textit{2mit} plays an essential role during \textit{Drosophila} development cannot be excluded, since \textit{2mit} residual expression in \textit{2mit} \textit{c03963} homozygous flies might allow normal neurogenesis and/or vitality.

In wild-type flies, \textit{2mit} expression was detected in adult brains, indicating that this gene plays a role during adulthood. In particular, \textit{2mit} mRNA was identified in both the neuronal somata and axonal lobes of the MBs, and at the level of the EB neuronal fibers. The presence of \textit{2mit} mRNA in the MBs is

**Figure 8. 2MIT Phylogenetic analysis.** (A) Unrooted phylogenetic tree of 2MIT protein in 21 \textit{Drosophilidae}, 4 \textit{Lepidoptera} and \textit{Apis mellifera}. Statistical support for nodes on the trees was evaluated by the bootstrapping values (×500) shown in each branch point obtained by the Maximum-Likelihood method included in the MEGA 5.0 Software. (B) Colored bars at the right side of the phylogenetic tree represent binding motifs recognized by different factors in 2MIT ortholog cytoplasmic regions. CLV_NDR_NDR1: N-arginine dibasic convertase site; CLV_PCSK_KEX2_1: subtilisin-like proprotein convertases cleavage site; LIG_14-3-3_3: 14-3-3 ligand site; MOD_PKA_2: PKA phosphorylation site; LIG_FHA_1 and LIG_FHA_2: FHA phosphopeptide ligands; MOD_N-GLC_1: N-glycosylation site; TRG_ER_diArg_1: di Arginine retention/retrieving signal; TRG_ENDOCYTIC_2: sorting signal motif; LIG_EVH1_1: EVH1 ligands.

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consistent with data reported by Kobayashi and colleagues, who mentioned 2mit among those genes preferentially expressed in Drosophila MBs [14]. In addition, 2mit mRNA signals in both the MB axonal structures and EB neuronal fibers of wild-type brains seemed to be specific since they were less visible in 2mit hypomorphic mutants and absent in negative controls. Given the nature of the 2MIT predicted protein, these data might suggest that 2mit mRNA is subject to neuronal transport and translational controls. Local control of mRNA translation has been demonstrated within dendrites in several organisms, including Drosophila, and it is known to mediate long-lasting synaptic plasticity in the mature nervous system [34]. Different studies have recently provided evidence that regulated mRNA transport and translation occur in both developing and mature axons (for a review, see 35).

In the courtship conditioning test, 2mit hypomorphous homozygous adult flies have shown normal courtship behavior and courtship suppression during training. These data indicate that 2mit hypomorphous homozygous males are able to perceive females by integrating visual, olfactory and sensory stimuli, fundamental for courtship behavior [36]. However, 2mit conditioned males were not able to maintain courtship suppression, evaluated within 5-10 min after training, in the presence of a virgin female, suggesting defects in STM retention. STM impairments were related to 2mit mRNA deletion since the pan-neuronal 2MIT-HA chimeric protein over-expression in a 2mit background was able to rescue this mutant phenotype. Moreover, STM defects similar to those observed in 2mit mutant flies have been noted when 2mit was silenced in the whole CNS with the elavGal4 driver or using the more brain-restricted lines OK107-, c772-, and MB247Gal4, which strongly expressed GAL4 in the MBs. Additional weaker GAL4-positive regions common to OK107-, c772-, and MB247Gal4 drivers are located in the OLs [21]. Taken together these data restrict the brain regions potentially involved in the 2mit-mediated STM phenotype to the MBs and OLs.

In 1999, McBride and colleagues demonstrated that early memory phases in courtship conditioning assays are determined by neuronal circuits outside MBs (such as visual structures and antennal lobes), since chemically MB-ablated flies showed normal memory when tested immediately after training under light conditions [37]. Memory impairments appeared later (with memory decrements found at 30 min and no memory at 60 min after training), indicating that STM consolidation of courtship conditioning required the MB activity. In addition, the same study showed that the ALs play a role in STM retention up to 30 min after training [37]. These data are consistent with the general idea that the memory formation process is a multi-step phenomenon, involving different anatomical structures, including the ALs and OLs, with the MBs representing the brain region for the ultimate storage of memory [38,39]. Defects in the immediate recall of memory in courtship conditioning assays have been demonstrated for dunce and ruta baga mutants (e.g. [40]). Both of these genes are involved in the cAMP signaling pathway, which is fundamental in olfactory learning and memory processes occurring in the MBs [41]. Even if both genes are mainly expressed in the MBs, they are also transcribed at low levels in several other brain regions, including OLs and/or ALs [42,43].

Under our experimental conditions, 2mit was localized mainly at the level of the MBs in wild-type adult brains, and hybridization signals not clearly distinguishable from the background were detected in both ALs and OLs, at least at ZT 0. Although further studies are required in order to better understand the 2mit’s role in Drosophila STM, it is interesting to note that in silico analyses have identified in the 2MIT cytosolic portion two specific motifs for PKA and 14-3-3 protein/s, known to be involved in the control of Drosophila memory. In particular, PKA is part of the cAMP signaling pathway [44,45] and two LEONARDO (LEO) 14-3-3 protein ζ isoforms have been shown to modulate memory, acting via an additional signaling pathway, which includes SLOWPOKE Ca2+-dependent K+-channel and SLOB [46,47]. Expressed in the CNS during embryogenesis and mainly in the MBs and EB in the adult brain, leo shows spatial and temporal expression profiles similar to those of the 2mit gene [47]. Amorphic leo alleles cause embryonic lethality, while hypomorphic variants determined STM defects [47]. It is therefore tempting to speculate that 2MIT functions as a transmembrane LRR neuronal receptor, which in the adult fly influences the memory phenotype as part of a signal transduction pathway.

Our investigations concerning the tim2 and 2mit relationship in D. melanogaster suggest that there is no functional correlation. In fact, tim2 is an essential gene required for chromosome stability, which in different organisms has been demonstrated to encode a replisome component [48,49]. In the adult fly, tim2 has been implicated in circadian light entrainment, probably exerting a function different from that required during development [7]. 2mit might be involved in nervous system development, and we showed that it plays a role in adult STM. In addition, analyses of tim2 and 2mit hypomorphic alleles for STM and circadian light synchronization suggest that these two genes do not have overlapping functions in the adult.

Comparative genomic analyses have shown that the organization of tim2-2mit host-nested genes is present in A. melit Helena and Drosophilidae species, suggesting that a 2mit ancestral gene was located within the tim2 locus before the Hymenoptera-Diptera divergence, which occurred ~300 million years ago [50]. The embedded gene relationship was not maintained in M. sexta and B. mori moths or in D. plexippus and H. melpomene butterflies, indicating that some mobilization event/s involving the 2mit gene region occurred subsequently within the Lepidoptera lineage. It is however interesting to note that the tim2-2mit host-nested genomic architecture is preserved in all 21 Drosophila species. Among Drosophilidae, it has been estimated that only 20-34% of the embedded gene relationships is conserved [1,6], and for those cases the presence of some evolutionary constraints might be hypothesized. It has recently been proposed that nested genes have been conserved throughout evolution by cis-acting transcriptional regulatory sequences located within host introns [51]. Enhancer sequence conservation among Vertebrates was demonstrated in an intron of the LPS-responsive beige-like anchor (Lbra) gene, hosting the nested
Mab21l2 gene [52]. Lbra and Mab21l2 are not functionally related, but their host-nested gene organization has been maintained throughout Metazoan genomes, with the exception of some insect species [52]. In the D. melanogaster tim2 intron 11, a 6 bp binding site recognized by the SNAIL transcription factor [53] was identified in proximity of c03963 PB transposon insertion. SNAIL is known to restrict neuroectoderm and neural fate in invaginating mesoderm and to act as a regulator of neurogenesis in both the CNS and PNS during late embryogenesis. It has been hypothesized that SNAIL may act to repress non neural fates [54]. Comparative genomic analysis detected the presence of a SNAIL binding site in the 11th intron of the Drosophilidae tim2 locus. These data suggest that this neuronal regulatory sequence could represent a constraint that has maintained the tim2-2mit host-nested gene association during the evolution of Drosophila species.

Materials and Methods

Drosophila stocks and maintenance

Flies were raised on a standard agar-yeast-sucrose medium at 23°C in 12:12 LD. w1118, sine oculis1, duncan1, I(3)-31Gal4/TM6B, Actin5C-Gal4/CyO, elavGal4/CyO, OK107Gal4, and c232Gal4 strains were obtained from the Bloomington Drosophila Stock Center (http://flystocks.bio.indiana.edu). c772- and MB247Gal4 lines were received from C. Helfrich-Forster, University of Wuerzburg (Wuerzburg, Germany) and the 52YGal4 driver from J.D. Armstrong, University of Edinburgh (Edinburgh, Scotland, UK). Insertional strains c03963, f00075, f06803 (carrying a PB transposon) and MB03271, MB08132, MB08962, MB08132 (carrying a MB element) were obtained from the Exelixis Drosophila Stock Collection (drosophila.med.harvard.edu/). In these strains, PB or MB transposon insertions map in the tim2 intron 11. In particular, in MB08132 and f06803, insertions localize in the 2mit 3‘ UTR; in MB08962, the insertion maps in the unique 2mit intron; in c03963, MB03271 and f00075 strains upstream of the 2mit gene. PBtim2G697 (tim2G697) and PBtim2G0295 (tim2G0295) flies, carrying the PB transposon in the tim2 5‘ UTR and intron 8, respectively, were originally obtained from the Exelixis Drosophila Stock Collection and were characterized in [7].

2mitO and 2mit KD construct production and transgenic line generation

A 3453 bp 2mitHA chimeric construct (2mitO), characterized by 3423 bp 2mit full-length cDNA followed by an in-frame 27 bp HA (haemagglutinin) tag sequence and a stop codon, was generated for 2mit over-expression studies. Using specific primers (Table S2), 2mitO cDNA was initially amplified in four 5‘–3‘ serial fragments of 792, 1056, 1046 and 739 bp in length, which were cloned in a PCR –II-TOPO® vector (Invitrogen) and checked for errors by sequencing. The four fragments were then digested with appropriate restriction enzymes (Table S2), obtaining 5‘–3‘ 768 bp NotI-BamHI, 980 bp BamHI-SalI, and 964 bp SalI-Ndel consecutive 2mit cDNA fragments and a 736 bp Ndel-XhoI segment, coding the 3' 2mit region followed by 27 bp HA sequence. These segments were sequentially subcloned in a pBluescript® II S/K (+/-) vector (Invitrogen), obtaining the 3453 bp NotI-XhoI 2mitO cDNA, which was then transferred into a pUAST vector.

For KD studies, the UAS-2mit RNAi construct (2mitKD) was generated as in [55], using a 1234 bp fragment of 2mit cDNA (positions 2756-3989 in NM_142001.2) without off-target effects as predicted by a bioinformatic program of the Vienna Drosophila RNAi Center (VDRC, http://stockcenter.vdrc.at/). The 1234 bp cDNA fragment was amplified with the primers listed in Table S2.

Both 2mitO and 2mit KD transgenic lines were obtained by P-element-mediated transformation following standard procedures [56]. Three independent 2mitO transgenic lines were generated by the Drosophila Embryo Injection Service (Transflie, University of Ferrara, Ferrara, Italy). 2mitO[P] (insert on 3rd chromosome [Chr]), 2mitO [insert on 2nd Chr], 2mitO [insert on 2nd Chr]. Three independent 2mit KD transgenic lines were obtained in our laboratory: 2mit KD[P] (insert on 3rd Chr), 2mit KD[P] (insert on 2nd Chr) and 2mit KD[P] (insert on 2nd Chr). Insert localization along polytene chromosomes was determined by in situ hybridization [55].

Generation of Drosophila lines for molecular and behavioral analyses

Molecular and behavioral analyses were performed on the 2mitO strain obtained by out-crossing c03963 flies into a w1118 background for at least eight generations and on w1118 controls.

To evaluate the effects of 2mit over-expression in the 2mitO homozygous mutant background, using CyO/Sco; MKRS/TM6B balancing stock, we initially generated the elavGal4/CyO; 2mitO strain, and 2mitO M4/CyO; 2mitO strain and 2mitO M14/CyO; 2mitO lines, carrying elavGal4 or 2mitO constructs over a CyO balancer and the homozygous PB c03963 transposon insertion on the 3rd Chr. The 2mitO strain, 2mitO strain, characterized by the presence of both 2mitO and PB c03963 inserts on the 3rd Chr was generated via genetic recombination in F1 females, obtained by mating homozygous 2mitO and 2mitO parental lines and crossed to MKRS/TM6B balancing stock. Red-eyed 2mitO, 2mitO / TM6B or 2mitO; 2mitO / TM6B F2 flies were singly mated with w, MKRS/TM6B flies and checked for recombination via PCR, using specific primers to identify the PB c03963 insertion (Table S2). One F3 recombinant 2mitO, 2mitO / TM6B line was then selected for subsequent studies. The effects of 2mit over-expression in a 2mitO homozygous mutant background were evaluated on elavGal4; 2mitO; 2mitO flies and compared to elavGal4++. 2mitO and +/+; 2mitO flies and controls, obtained by mating 2mitO homozygous flies with those bearing the elavGal4 driver or 2mitO construct alone in the 2mitO background.

2 mit KD effects were analyzed in Gal4-driven 2mit KD flies compared to Gal4+++ and +/+ 2mit KD flies obtained by mating w1118 flies with individuals carrying either a Gal4 driver or the 2mit KD construct alone.

Evaluation of tim2 depletion effects was performed on heterozygous tim2/+ for two different tim2 alleles (tim2
c06978 and tim2(TM6B) obtained by mating tim2/TM6B flies to w118 flies.

RNA isolation, QPCR, and Northern blotting

Total RNA was obtained from L3, 3-5 day-old adult heads and dissected brains. 3-5 day-old flies raised in 12:12 LD conditions or after 3 days in DD were sampled at 3 or 4 h intervals. Adult heads were separated from bodies according to [57]. Brains were dissected in PBS, fixed in 4% paraformaldehyde in PBS for 30 min at 4°C and washed three times for 10 min in PBS. Total RNA was extracted from samples using Trizol™ Reagent (Invitrogen) following the manufacturer’s protocol. The cDNA was synthesized from 1 µg of total RNA using SSII Reverse Transcriptase (Invitrogen) and an Oligo(dT)20 primer. QPCR reactions were performed in a 10 µl reaction volume, containing 200 nM of specific primers (Table S2), 5 µl GoTaq® qPCR Master Mix (Promega) and ~30 ng of cDNA per sample. QPCR was performed in triplicate and repeated three times on an ABI7500 system (Applied Biosys), with the following amplification profile: 95°C for 2 min, 40 cycles of two-step amplification (95°C for 25 sec and 60°C for 60 sec), and melting curve (60–90°C with a heating rate of 0.5 °C/10 sec). To evaluate differences in gene expression we chose a relative quantification based on the standard curve method [58]. Levels of expression were compared with those of an endogenous control transcript (rp49) that did not appear to be differentially expressed under our experimental conditions.

Northern blotting was carried out as in [59], using a ~1.7 kb 3’ 2mit probe (3040-4722 positions in NM_142001.2) and rp49 full-length cDNA (U92431) as standard.

In situ mRNA hybridization and immunohistochemistry

mRNA in situ hybridization on embryos was carried out as in [60] with Fluorescein-labelled 980 nt antisense and sense 2mit RNA probes (2060-3039 positions in NM_142001.2). The 2mit sense probe was used as negative control. Hybridization signals were detected using an alkaline phosphatase-conjugated anti-Fluorescein antibody (1:2000, Roche) and NBT/BCIP substrates (Roche).

Biotin labeled RNA antisense and sense ~1.7 kb 2mit RNA probes (3040-4722 in NM_142001.2) were made for mRNA hybridization on whole-mount adult brains using the Biotin RNA Labelling Mix (Roche). The 2mit sense probe was used as negative control. In situ hybridization experiments were performed on 3-5 day-old adult brains collected at ZT 0, in 12:12 LD conditions. Sample collection, tissue fixation, and mRNA in situ hybridization procedures were performed as described in [7]. Samples were hybridized at 65°C overnight with 100 ng probe. Probe detection was performed using TSA™ Signal Amplification kit (PerkinElmer) following the manufacturer’s instructions, incubating samples at 4°C overnight in Streptavidin (1:100) and 3 h in tyramide solution (TSA™ Cyanine 3 System). To visualize the presence of both 2mit mRNA and 2MIT-HA chimeric protein in OK107Gal4-2mitO adult brains, in situ hybridization protocols did not include treatments with Proteinase K [7]. After in situ procedures, brains have been incubated at 4°C for 3 days with a rabbit anti-HA antibody (1:600; Sigma) and at 4°C overnight with a goat anti-rabbit IgG-Alexa 488 (1:250; Invitrogen). Samples were mounted in Vectashield H-1000 (Vector Laboratories) and microscopic analyses were performed using a Leica TCS SP5 II confocal microscope (Leica Microsystems). At least 10 brains for each genotype were analyzed. For each brain, optical sections (Z-series) were taken at 0.5 µm intervals. Post-acquisition analysis and Z-stack construction were performed with Fiji, an open source image processing package based on ImageJ (http://fiji.sc/wiki/index.php).

Western Blotting

Adult fly whole-bodies were homogenized in extraction buffer as in [61]. After 2 min of sonication, β-dodecyl maltopyranoside detergent was added (1% final concentration). Samples were placed on a rotating wheel for 1 h at 4°C to allow membrane solubilization. According to [62], 0.2 volumes of 5% sodium deoxycholate were added and samples were incubated 10 min on ice. Lysates were centrifuged twice (2800g, 4°C); supernatants were diluted in LDS loading buffer (Invitrogen) and DTT 1 M (0.73 X final concentration; Sigma) and placed 10 min at 70°C. SDS-PAGE was performed using 3-8% NuPAGE® Tris-Acetate pre-cast gel (Invitrogen). After blotting, nitro-cellulose membranes (Trans-Blot Transfer Medium; Bio-Rad) were incubated with a rabbit anti-HA antibody (1:1000; Sigma) and a goat anti-rabbit IgG-HP (1:100; Santa Cruz Biotechnology Inc.). Positive immunoreactivity was visualized using the ECL detection system.

Evaluation of ls/s-tim1 haplotypes

The single 294 Guanosine insertion/deletion polymorphism existing in the D. melanogaster tim1 gene was determined as in [24] using the Amplification of Refractory Mutations System (ARMS) PCR method on single flies. Primers are listed in Table S2.

Egg-to-adult viability

For each genotype, vitality test experiments were performed collecting ~100-300 embryos and counting developing L3, pupae, and adult flies.

Phototactic behavior

Phototactic behavior was tested in a maze consisting of a series of Y and T tubes (4 mm external diameter) interconnected by transparent 1.5 cm long plastic tubes similar to those proposed to test geotaxis behavior by [63]. Each maze had a single entrance at one side and eight terminal ends at the other. The eight terminal ends were closed with funnel traps, as described in [63]. Mazes were placed horizontally in a box, with black internal walls and a white LED light (130 lux) placed in a corner, corresponding to one of the maze terminal edges. Before testing, flies were maintained in dark conditions. Analyses were performed at the ZT 0-2 time interval on 3-5 day-old males. During each trial, 10 flies were placed at the entrance of the maze and after 1 h each fly received a score from 0 to 7, reflecting the number of positive choices towards the light source.
Locomotor activity analyses

3–5 day-old flies were individually transferred into transparent tubes (1.5 cm diameter and 4.5 cm length). Tubes were placed inside a black box under white light (141 lux) and locomotor activity was recorded during a 10 min period for each fly. Data were processed by AnyMaze software (Stoelting, Wood Dale, IL, USA). The following parameters were evaluated: total distance moved (m; sum of the distance between each point in the track); average speed (mm/sec); total number of immobility episodes (number of transitions from mobility to an immobile state exceeding 2 sec); total immobility time (sec; sum of duration of each immobility episode). Recordings were performed from ZT 1 to ZT 7.

Circadian locomotor analyses

Locomotor activity was recorded using the Drosophila Activity Monitoring System® (DAMSystem, Trikinetics Inc., Waltham, MA, USA). PRCs were obtained subjecting flies to 20 min light pulses (400 lux) delivered at ZTs 13, 15, 17, 19, 21, and 23. Phase changes were calculated as described in [64]. Positive and negative values represent advanced and delayed phase shift responses, respectively.

Memory analyses

Courtship conditioning assays were performed as described in [15,16]. Conditioned male flies and sham controls for each genotype were analyzed. Briefly, 4 day-old virgin males, previously kept alone as soon as they eclosed, were individually placed with a 4-5 day-old wild-type OR-R mated female for a 1 h training period (conditioned males) or were kept alone (sham controls) in a mating chamber (8 mm diameter and 3 mm high). Males were individually transferred to a new mating chamber and, within 5-10 min, were tested for 10 min with a CO2-anesthetized wild-type OR-R virgin female, collected that day. Male courtship activities towards virgin or mated females were videotaped under uniform white light (141 lux). The first and last 10 min of the 1 h training period and the 10 min test were inspected to record male courtship behavior. Males which copulated during the training period or courted less than 1 min during the first 10 min of training period were excluded from analyses. If males copulated during the test period or an anesthetized virgin awakened from anesthesia, the observation period was concluded. A courtship index (CI), defined as the amount of time a male spent courting during the 10 min test period, was calculated for each conditioned and sham male. The training index (TI), defined as the ratio between CIs in the final 10 min (CIf) and initial 10 min (CIi) of the training period, was calculated [16].

Statistical analyses

All molecular and behavioral data, except for those regarding memory analyses, were analyzed by parametric one- or two-way analysis of variance (ANOVA) and Neuman-Keuls post hoc test. CI data did not approximate normal distributions, evaluated with Liliefors (Kolmogorov-Smirnov) and Shapiro-Wilk tests, even after arcsine, arcsine squared, or arcsine square root transformation. Therefore, they were non-parametrically analyzed using the Mann-Whitney U test for pair-wise comparisons. Analyses were performed using the Statistica 5.0 package (Statsoft Inc.).

Bioinformatic tools

Sequence accession and annotation were performed through the FlyBase web platform (release FB2013_03; http://flybase.org; 12 Genomes Consortium 2007, http://rana.lbl.gov/drosophila/; modENCODE project, https://www.hgsc.bcm.edu/content/drosophila-modencode-project) or by the EnsemblMetazoa database (http://metazoa.ensembl.org/index.html). D. melanogaster 2mit nucleotide and amino acid sequences were compared with the non-redundant sequences available at the NCBI using BLAST and tBLASTN algorithms. The identified contigs were then analyzed with the Augustus gene prediction tool [25].

A multiple protein sequence alignment was obtained by using the MAFFT program (http://mafft.cbrc.jp/alignment/server/) and subjected to phylogenetic analysis by Maximum Likelihood method, via MEGA 5 software [65]. Genetic distances were calculated using the Jones–Taylor–Thornton algorithm [66] and statistical support for nodes on the tree was evaluated using bootstrapping (500 iteration cycles) [67]. Searching for transcription factor binding sites around 2MIT was scanned for the presence of repeated units using a multiple protein sequence alignment obtained from the Annie server (http://annie.bii.a-star.edu.sg), which integrates the prediction from several computational tools, such as CAST and SEG for low complexity region detection and SAPS for the analysis of amino acid composition. The transmembrane topology prediction was derived from the consensus of tools provided by Annie: HMMTOP, PHOBIUS, and TMHMM. We employed an integrative bioinformatic approach combining sequence and domain database searches with the consensus from predictions of protein structural features. The 2MIT sequence was used as a query to scan the domain databases InterPro and Pfam; the secondary structure was predicted using a consensus approach [69]. Prediction of intrinsic disorder and the presence of signal peptides was assessed using ESPRITZ [70] and SignaLP [71], respectively. The N-terminal sequence of 2MIT was scanned for the presence of repeated units using a combination of different classes of repeat prediction methods: RADAR [72], TRUST [73], and Repetita [74]. The predicted repeats were manually aligned in order to calculate the consensus pattern which defines the repeats in the 2MIT protein and to identify further mis-predictions [75]. The C-terminus was scanned for functional linear motifs using the ELM server [26].

Sequence feature analysis

2MIT protein sequence (SwissProt accession ID: Q9VFY9) annotation was obtained from the Annie server (http://annie.bii.a-star.edu.sg), which integrates the prediction from several computational tools, such as CAST and SEG for low complexity region detection and SAPS for the analysis of amino acid composition. The transmembrane topology prediction was derived from the consensus of tools provided by Annie: HMMTOP, PHOBIUS, and TMHMM. We employed an integrative bioinformatic approach combining sequence and domain database searches with the consensus from predictions of protein structural features. The 2MIT sequence was used as a query to scan the domain databases InterPro and Pfam; the secondary structure was predicted using a consensus approach [69]. Prediction of intrinsic disorder and the presence of signal peptides was assessed using ESPRITZ [70] and SignaLP [71], respectively. The N-terminal sequence of 2MIT was scanned for the presence of repeated units using a combination of different classes of repeat prediction methods: RADAR [72], TRUST [73], and Repetita [74]. The predicted repeats were manually aligned in order to calculate the consensus pattern which defines the repeats in the 2MIT protein and to identify further mis-predictions [75]. The C-terminus was scanned for functional linear motifs using the ELM server [26].

Alignment construction

The structural template for the 2MIT LRR domain was found using MANIFOLD based on sequence and secondary structure. Initial alignments were generated through systematic parameter variation from an ensemble of similar alternatives.
Given the problematic nature of repeated sequences, the best initial alignment was used as a starting point only. Manual refinement was performed using knowledge obtained from the structural alignment of the different predicted repeats. Knowledge of key residues and secondary structure was used to anchor the aligned repeats.

Molecular modeling

The model for the 2MIT LRR domain was constructed using the HOMER server (URL: http://protein.cribi.unipd.it/), which uses the conserved parts of the structure to generate a raw model, which is then completed by modeling the divergent regions with a fast divide and conquer method [76]. Side chains were placed with SCWRL3 [77] and the energy was evaluated regions with a fast divide and conquer method [76]. Side chains were placed with SCWRL3 [77] and the energy was evaluated with FRST [78]. The final models were subjected to a short steepest descent energy minimization with GROMACS [79] to remove energy hotspots before calculating the electrostatic surface with APBS [80]. The structure is visualized using PyMOL (DeLano Scientific, URL: http://pymol.sourceforge.net/).

Supporting Information

Figure S1. Locomotor activity in 2mit-03963 and w1118 flies. (A) Total distance. F3,106=0.21 p=0.88; (B) Average speed. F3,106=0.21 p=0.88; (C) Number of immobility episodes. F3,106=0.04 p=0.98; (D) Total immobility time. F3,106=0.11 p=0.95. 2mit : 2mit-03963 homozygous mutant flies. Data are expressed as mean ± SEM with the number of tested flies indicated above each bar.

(TIF)

Figure S2. 2mit mRNA and 2MIT-HA chimeric protein in the MBs of OK107Gal4>2mitO flies. Localization of 2mit mRNA (red, A) and 2MIT-HA chimeric protein (green, B) in the MBs of an OK107Gal4>2mitO Fly brain; signals are merged in (C). 2mit mRNA and 2MIT-HA chimeric protein co-localize in the Kenyon cells (arrow) and axonal lobes. * 2mit mRNA and 2MIT-HA signals in non-MB cells activated by the OK107Gal4 driver. Images are ~12 µm Z-projections. The following abbreviations are used: α/ α': vertical mushroom bodies lobes; β, β', γ: medial mushroom bodies lobes. Bar in (C) represents 15 µm for (A)-(C).

(Figure S3. Memory formation in c232Gal4>2mit KD and 52YGal4>2mit KD flies. Courtship Indices in sham (white bars) and conditioned (black bars) males for c232Gal4>2mit KD and 52YGal4>2mit KD flies (6.1; 16.2; 61.1) lines and relative controls [c232Gal4> +, 52YGal4>+ and -> 2mit KD (6.1; 16.2; 61.1)]. Data are expressed as mean ± SEM with the number of tested flies indicated above each bar. The CIs of the sham flies were significantly different from those of the conditioned males in all c232Gal4- and 52YGal4->2mit KD lines and relative controls. The number of asterisks indicates the significance level: *: p < 0.05; **: p < 0.005; ***: p < 0.0001.

(Figure S3)

File S1. Multiple alignment of 21 Drosophilidae, 4 Lepidoptera and Apis mellifera 2MIT sequences performed by MAFFT software and manually refined.

(Fasta)

Table S1. Phototaxis behavior in 2mit-03963 flies.

(Docx)

Table S2. Primers used in amplification and cloning experiments.

(Xls)

Author Contributions

Conceived and designed the experiments: FS RC. Performed the experiments: FB AB CB OR MC EL SCET. Analyzed the data: FS AB EL SCET RC. Wrote the manuscript: FS AB EL SCET.

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