# Neurotoxic protein expression reveals connections between the circadian clock and mating behavior in *Drosophila*

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**To investigate the functions of circadian neurons, we added two strategies to the standard *Drosophila* behavioral genetics repertoire. The first was to express a polyglutamine-expanded neurotoxic protein (MJDtr78Q; MJD, Machado–Joseph disease) in the major *timeless* (*tim*)-expressing cells of the adult brain. These Tim-MJD flies were viable, in contrast to the use of cell-death gene expression for *tim* neuron inactivation. Moreover, they were more arrhythmic than flies expressing other neurotoxins and had low but detectable *tim* mRNA levels. The second extended standard microarray technology from fly heads to dissected fly brains. By combining the two approaches, we identified a population of Tim-MJD-affected mRNAs. Some had been previously identified as sex-specific and relevant to courtship, including mRNAs localized to brain-proximal fat-body tissue and brain courtship centers. Finally, we found a decrease in the number of neurons that expressed male-specific forms of the *fruitless* protein in the laterodorsal region of the brain. The decrease was not a consequence of toxic protein expression within these specialized cells but a likely effect of communication with neighboring TIM-expressing neurons. The data suggest a functional interaction between adjacent circadian and mating circuits within the fly brain, as well as an interaction between circadian circuits and brain-proximal fat body.**

Circadian rhythms are widespread in nature. As a consequence, many aspects of behavior, metabolism, and physiology show cyclical changes during each 24-h period. These include locomotor activity rhythms, which are intimately connected to the ≈75 pairs of known clock neurons within the adult *Drosophila* brain (e.g., ref. 1). These cells express cycling levels of clock mRNA and protein, including products of the clock genes *period* and *timeless* (*per* and *tim*; ref. 2). The circadian molecular program also runs within several peripheral (noncentral brain) tissues, including eyes, Malpighian tubules, leg sensilla, and antennae (3). Peripheral tissues contribute to other circadian outputs such as olfaction (4) and reproductive behavior (5–7).

Several tools exist to eliminate or inactivate *Drosophila* pacemaker neurons, including agents that cause cell death, e.g., the proapoptotic genes *hid* and *reaper* (8), or those that inactivate neuronal function, e.g., ectopically expressed modified $K^+$ channels (9, 10), tetanus-toxin light-chain (11), and abnormal synaptic proteins (12). This second group typically inhibits synaptic transmission and, as a consequence, interneuronal communication, but the neurons are still alive. Importantly, the combination of *tim* or *per* drivers with tetanus toxin or modified potassium channels results in flies that are still rhythmic in a light–dark cycle (ref. 13; M. Nitabach, personal communication). Expression of an abnormal synaptic protein with a *tim* driver also does not affect locomotor activity rhythms. In contrast, the combination of *tim* or *per* drivers with *hid* or *reaper* is lethal (Y. Peng, personal communication).

The aim of the present study was therefore to assess behavioral and molecular phenotypes in the complete absence of clock-neuron function. To find a tool intermediate in strength between tetanus toxin and *hid*, we introduced the use of neurotoxic proteins to the circadian system and took advantage of their well described properties: slow, cumulative, and specific for the neurons that express them (14–17). To this end, we used the GAL4–upstream activating sequence (UAS) system to express UAS-neurotoxic protein constructs under control of the *tim* promoter. The most effective proteins rendered flies completely arrhythmic and potently decreased *tim* mRNA levels. By monitoring mRNA profiles with RNA from brains as well as heads, we were able to identify not only affected brain mRNAs but also affected sex-specific fat-body mRNAs. In conjunction with courtship assays and staining of *fruitless* gene products, the data indicate that clock neurons probably affect reproductive behavior through local interactions within the fly brain and/or the fat body.

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## Results

**Neurotoxic Protein Expression and Inactivation of Specific Neuronal Subpopulations.** To address whether neurotoxic protein expression has an advantage over the use of cell death genes, we expressed 16 different UAS-neurotoxic protein constructs in adult clock neurons with a well characterized *tim-gal4* driver (*tim-gal4* line 27). Locomotor activity profiles were assayed under standard 12-h light/12-h dark [light–dark (LD)] as well as constant darkness (DD) conditions, and the relative toxicity of the different proteins on rhythms was largely congruent with previous studies on the effects of these proteins on general neuronal function (refs. 18–22; Table 4, which is published as supporting information on the PNAS web site).

*tim-gal4*;UAS-MJDtr78Q (strong; MJD, Machado–Joseph disease) transgenic flies (henceforth referred to as Tim-MJD) had locomotor activity patterns as disabled as the most severe clockless mutants (Fig. 1*A*; see refs. 23–27) and were used for further studies. UAS-MJDtr78Q consists of the C terminus of ataxin 3 containing an expanded polyglutamine tract [78 instead of 27 glutamine residues (19)]. These expansions are observed in individuals with MJD. In contrast to Tim-MJD, MJDtr78Q expression in dopaminergic and serotoninergic neurons caused no detectable circadian abnormality but did cause a specific defect in female mating latency, a dopamine-dependent behavior (Fig. 4 *A* and *B*, which is published as supporting information on the PNAS web site; refs. 28 and 29).

It is unlikely that the Tim-MJD behavioral defect is a consequence of a more general toxicity, because overall locomotor activity levels were not significantly different from those of wild type (data not shown). However, Tim-MJD expression had a

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Abbreviations: LD, light-dark; MJD, Machado–Joseph disease; ZT, Zeitgeber time; UAS, upstream activating sequence; LN, latero-neuron; $LN_v$, ventral LN; $sLN_v$, small $LN_v$.

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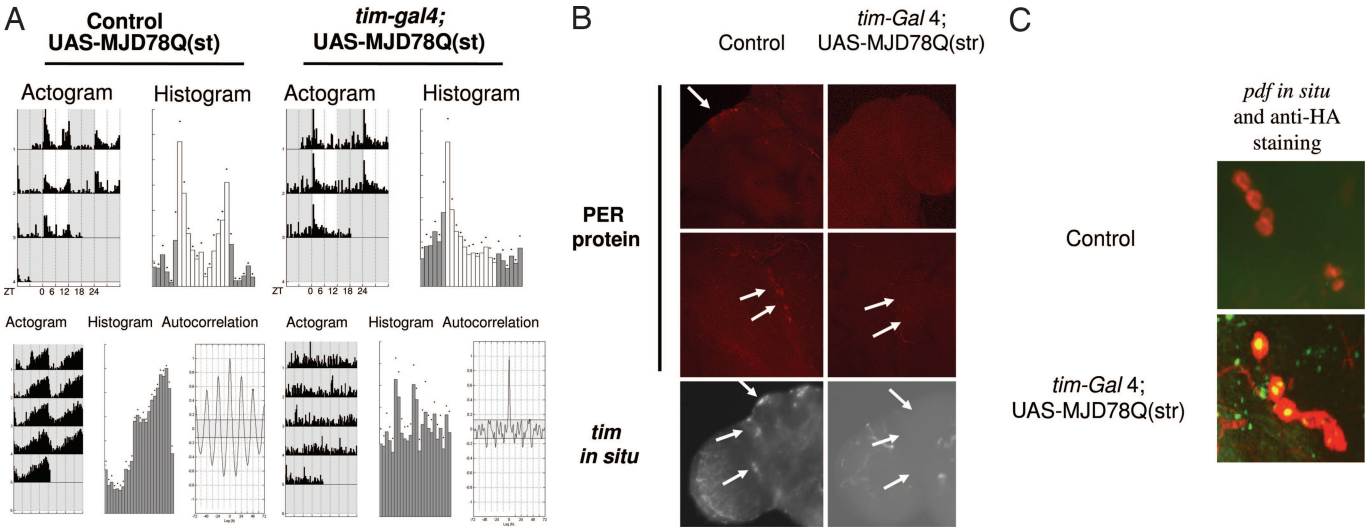


**Fig. 1.** MJDtr78Q can be used as a target for selective killing of neurons. (*A*) (*Upper*) LD activity (actograms, *Left*; histograms, *Right*) and (*Lower*) constant darkness (DD) activity. The lighting condition is indicated by the color of the background (actograms) or activity bin (histograms). Autocorrelation and rhythmic index were measured as described in ref. 52. $n = 16$ in all cases. (*B*) PER staining (*Top*), ZT20. Arrows point at the location of the pacemaker neurons. (*Bottom*) *tim in situ* hybridization (ZT14). Arrows indicate localization of all clock groups [for PER staining: dorsal neurons (DNs) in top graph and $LN_v$s in the bottom one; for *timeless in situ* DNs, $LN_v$s, and dorsal LNs]. None of these groups are present in Tim-MJD fly brains. (*C*) Anti-HA antibodies recognizing HA-tagged MJDtr78Q protein colocalize with *Pdf* detected by *in situ* hybridization.

prominent effect on lifespan (Fig. 4*C*). As a consequence, only young flies (1–3 days old) were used for subsequent behavioral and molecular studies.

**The Molecular Clock Is Affected in Tim-MJD Flies.** We monitored *tim* mRNA and *per* protein (PER) cycling in the brains of Tim-MJD flies by *in situ* hybridization and immunocytochemistry, respectively. Both *tim* mRNA and PER were undetectable at all time points in Tim-MJD brains (Fig. 1*B Right* and data not shown). This is in contrast to the well described robust cycling of both *tim* mRNA and PER in the clock cells of wild-type flies (Fig. 1*B Left* and data not shown). Although this suggests that pacemaker neurons are absent in Tim-MJD, *pigment-dispersing factor* (*Pdf*) mRNA, characteristic of large and small ventral latero-neurons ($LN_v$) neurons, was still present in the correct location in Tim-MJD fly brains (Fig. 1*C*). Indeed, there was no Tim-MJD effect on the number of large $LN_v$ cells, but we consistently observed a significant decrease in the number of *Pdf*-positive small $LN_v$s (Table 1).

**Clock Gene Expression Is Specifically Affected in Tim-MJD Flies.** To identify genes affected by inactivation of *tim*-expressing cells, we first compared the expression profile in LD conditions between wild-type and Tim-MJD fly-head RNA collected at one Zeitgeber time (ZT; ZT16): 552 genes were down- and 368 up-regulated (1.5-fold change, $P = 0.05$; Tables 5 and 6, which are published as supporting information on the PNAS web site). These included several clock mRNAs (Fig. 2 *A Upper*), suggesting an impact on the circadian program. *tim*, *vri*, *cry*, and *Pdp*1 were the most-affected mRNAs, although statistically significant differences were observed only for the first three (probably because of outlier data for *Pdp1* from one microarray replica; data not shown). Notably, *tim*, *vri*, and *Pdp1* had lower levels in Tim-MJD flies, whereas *cry* had higher levels. Of these four, only *cry* mRNA normally exhibits an expression trough rather than a peak at ZT16. The higher *cry* mRNA level therefore suggests a Tim-MJD effect on the clock cell transcriptional program rather than an ablation of most clock neurons.

**Table 1. MJD expression affects $sLN_v$**

| Genotype | No. of $sLN_v$s | No. of $lLN_v$s |
|---|---|---|
| Control ($n = 31$) | 3.50 ± 0.09 | 4.30 ± 0.16 |
| Tim-MJD ($n = 44$) | 2.20 ± 0.16 | 4.11 ± 0.23 |

The values represent the average cell number for each group ± SEM. There is a significant reduction in the number of small cells in Tim-MJD brains (*t* test = 1.78E-08) and no significant difference in the number of large cells (*t* test = 0.52) between Tim-MJD and *tim-gal4* flies. $lLN_v$s, large $LN_v$s.

Because head RNA contains eye and fat-body RNA as well as brain RNA, we compared the mRNA levels of several well described fat-body and eye genes between wild-type and Tim-MJD flies. There was no statistically significant difference in all known expressed eye-*rhodopsin* mRNAs and most known fat-body genes (data not shown). Tim-MJD flies also had no gross morphological abnormalities, including the eye. Finally, there was no difference in the levels of most known brain RNAs, exemplified by a few known neuropeptides likely to be brain-specific (data not shown and Fig. 2 *A Lower*). The exceptions were the neuropeptide mRNAs *corazonin* and *Pdf*; both genes are rhythm-related (30, 31).

However, we did observe effects on circadian mRNAs suspected of being outside the brain, i.e., putative fat-body circadian mRNAs (see below). Because clock-gene expression is known to take place in the eye as well as the fat body (30), and because both of these tissues are present in heads, the Tim-MJD effect on clock mRNAs (Fig. 2*A*) may not be restricted to the brain. We therefore decided to assay the Tim-MJD effect using brains instead of fly heads as a source of mRNA.

**Core Clock Genes and Cycling Transcripts Are Specifically Down-Regulated in Tim-MJD Brain RNA.** To this end, brain tissue was dissected from Tim-MJD and wild-type fly heads, collected at two opposite time points (ZT4 and ZT16), which were used to prepare total RNA and biotin-labeled probes; these were hybridized to high-density oligonucleotide Affymetrix microarrays (see *Materials and Methods*). The resulting signals were strong and reproducible, showing that dissected brains are a reliable source of high-quality RNA for microarray analysis (data not shown).

We then compared the expression profile of Tim-MJD brains at two opposite LD time points (ZT4 and ZT16) with similar data from wild-type brains. The levels of almost all core clock gene mRNAs are significantly decreased in Tim-MJD brains. However,

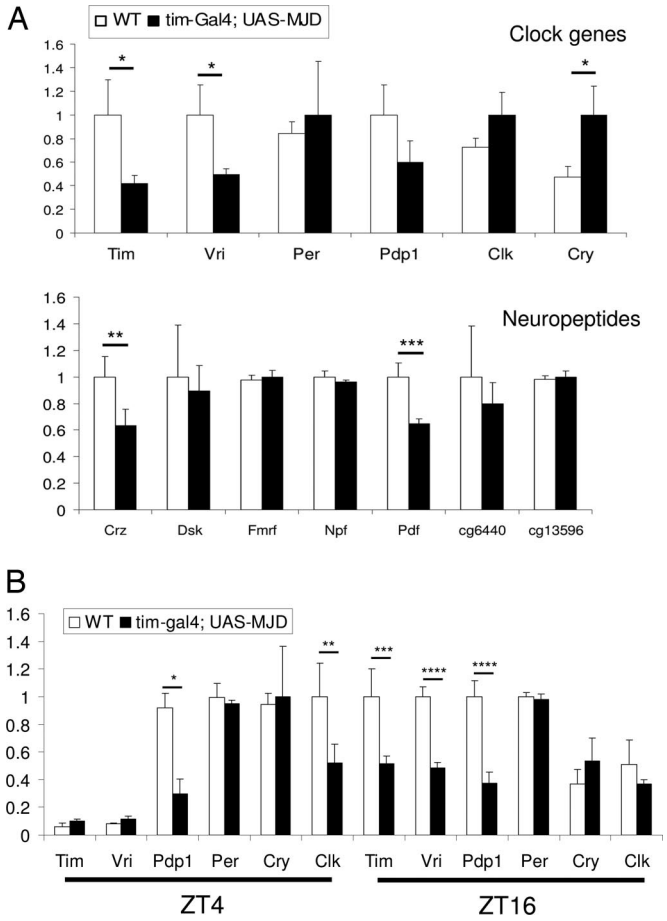


**Fig. 2.** MJDtr78Q expression in *tim*-expressing cells has a specific impact on clock genes. (*A*) Fly heads. We assigned a value of 1 to the maximum expression value for a given probe set between the two genotypes; the value in the other genotype was calculated as a fraction of this maximum value. Error bars indicate SEM. Asterisks show statistically significant differences in comparison with a control (*, $P < 0.03$; **, $P < 0.05$; ***, $P < 0.01$). For statistical inference, a *t* test was used. (*B*) Fly brains. Error bars indicate SEM. Asterisks show statistically significant differences in comparison with control (*, $P < 0.03$; **, $P < 0.05$; ***, $P < 0.02$; ****, $P < 0.01$). For each gene, the maximum value between the two genotypes and the two timepoints was chosen, and the expression values for this gene on the other genotype and timepoint were calculated relative to it.

circadian transcription is not completely inhibited, because residual oscillations of *tim*, *vri*, *cry*, and *Clk* were still detected (compare ZT16 with ZT4; Fig. 2*B*). The effect on clock mRNAs appears specific or preferential; they are more affected than noncircadian brain or nonbrain mRNAs (Fig. 5*A*, which is published as supporting information on the PNAS web site).

To address this specificity issue, we first used *t* test statistics combined with random permutation [significant analysis of microarrays (SAM) algorithm; ref. 32]. A *t* value for each probe set was computed at each time point. A value of zero implies there are no significant changes between the two fly strains; a positive value means down-regulation in Tim-MJD flies, whereas a negative value implies up-regulation of a particular probe set. The absolute value of the *t* score takes into account the deviation and fold difference; the bigger the absolute value, the more significant the change. We chose 2.5 as a threshold for the absolute *t* score and introduced an additional filter by choosing only genes that change >1.5 times between the two strains. With these values, most mRNAs do not change. However, 597 genes are down- and 739 up-regulated in at least one of the two time points in Tim-MJD brain RNA (data not shown; Tables 7 and 8, which are published as supporting information on the PNAS web site, respectively).

**Table 2. Multiple sex-specific genes are affected in Tim-MJD flies**

| Gene | Maximum *t* value |
|---|---|
| Sodh-1 | 7.39 |
| cg2736 | 4.94 |
| *cg1764* | 4.75 |
| *Yp2* | 4.6 |
| *Yp1* | 4 |
| cg15219 | 3.84 |
| cg5344 | 3.58 |
| cg5288 | 3.29 |
| cg3488 | 3.22 |
| cmp44E | 3.07 |
| Cyp4d21 | 2.72 |
| cg9645 | −7.82 |
| *cg7461* | −2.8 |
| | |
| cg16820 | 4.5 |
| cg5945 | 4.15 |
| cg17189 | −3.13 |
| takeout | −3.11 |
| cg7079 | −2.6 |
| cg10407 | −2.52 |
| | |
| Sxl | 3.5 |
| fruitless | 3.2 |

The top section corresponds to genes identified in ref. 38; the middle section corresponds to genes identified in ref. 39, and the bottom section corresponds to general sex-determination genes. The maximum *t* value correlates with the degree of increase (positive value) or decrease (negative value) of a given probe in Tim-MJD flies with respect to control (as explained in the text).

We then addressed the extent to which the 597 down-regulated mRNAs are biased toward the circadian system. First, a significant fraction overlaps with cycling mRNAs previously identified in two studies (33, 34) when compared with a random sample of 597 probes (Fig. 5*B*). Second, we calculated average *t* scores for these two groups of cycling mRNAs as well as a stress-related group. The cycling transcripts have an average *t* score significantly bigger than that calculated for the same number of random transcripts, whereas a negative average *t* score was obtained for the collection of 58 defense and heat-shock proteins (Fig. 5*C*). These mRNAs had been previously shown to be up-regulated by polyglutamine extended proteins in different systems (35–37).

**Courtship Behavior, Fat Body, and the Circadian Clock.** Brain dissections are designed to minimize contamination by peripheral tissues, but some perineuronal fat-body tissue remains attached to the brains. Indeed, many fat-body mRNAs (*yl*, *cg5665*, *Fbp1*, and *Fbp2*), as well as at least 500 mRNAs that are highly expressed in heads but not brain-enriched, were easily detectable in the brain RNA analysis; most are not significantly affected by Tim-MJD (data not shown). However, a small subset of fat-body mRNAs (e.g., *Yp1*, *Yp2*, *Yp3*, and *Adh*) is strongly down-regulated in Tim-MJD brains (Fig. 5*D*). Because expression of these mRNAs was much less affected by Tim-MJD in head RNA (data not shown), the effect appears preferential for the fat tissue immediately surrounding the fly brain (see below). Indeed, some previously identified cycling head mRNAs localize to brain-proximal fat tissue (38, 39) and are prominently featured in the list of Tim-MJD-affected transcripts. They include sex-specific fat-body mRNAs: Cyp4d21 (Table 2; also called *sxe1*; ref. 39) and mRNAs from the *takeout* gene family (Table 2; ref. 38). These two groups of transcripts are associated with sex-determination and sex-associated behaviors (38, 39). Two

**Table 3. Mating behavior during a 1-hr observation period**

| Genotype | % mated/total tested (1 hr) | % fertile flies (after 1-wk pairing) |
|---|---|---|
| Control males | 95 ($n$ = 20) | 100 ($n$ = 12) |
| *tim*-MJD males | 0 ($n$ = 18) | 92 ($n$ = 12) |
| Control females | 95 ($n$ = 20) | 100 ($n$ = 10) |
| *Tim*-MJD females | 24 ($n$ = 21) | 100 ($n$ = 12) |

These pairs were allowed to mate for a 1-wk period and then were scored for the presence of progeny after 10–12 days. Values in parentheses represent the number of vials scored.

additional genes implicated in sex-specific determination and behavior, *Sexlethal* and *fruitless* (*Sxl* and *fru*; ref. 40), are also significantly down-regulated in Tim-MJD flies (Table 2).

The effect of TIM-MJD on sex-specific mRNAs, coupled with previous links between circadian rhythms and mating (5, 6, 38), suggested these flies might exhibit a courtship phenotype. Indeed, a simple 1-hr mating assay (e.g., ref. 41) indicated that there was a significant decrease when Tim-MJD males or females were assayed with wild-type virgin females or males, respectively (Table 3). Fertility was not affected (data not shown).

**MJDtr78Q Expression in *timeless*-Expressing Neurons Affects *fru* Expression.** To address the possibility that the mating effect is a consequence of MJDtr78Q expression within brain courtship centers (reviewed in ref. 42), we assayed the relationship between the Tim-MJD pattern and expression of the *fruitless* courtship gene. To this end, we stained *tim-gal4*;UAS-GFP brains with an antibody that recognizes the male form of the *fruitless* protein $FRU^M$ (43) and found no overlap (data not shown and Fig. 3*A*). We noticed, however, that some laterally located $FRU^M$ cells were close to some clock neurons, probably dorsal LNs and/or dorsal neurons (Fig. 3*A Right*). This proximity might facilitate communication between the two systems and explain the Tim-MJD mating phenotype, at least in Tim-MJD males. We therefore analyzed $FRU^M$ expression by counting FRU-positive cells in three brain regions, defined as top, central, or lateral (Fig. 3*B*). For both the top and center regions, wild-type and TIM-MJD brains were indistinguishable. There was, however, a significant decrease ($P < 0.05$) in $FRU^M$-positive cells in the lateral region, which may be relevant to the Tim-MJD mating phenotype (see *Discussion*).

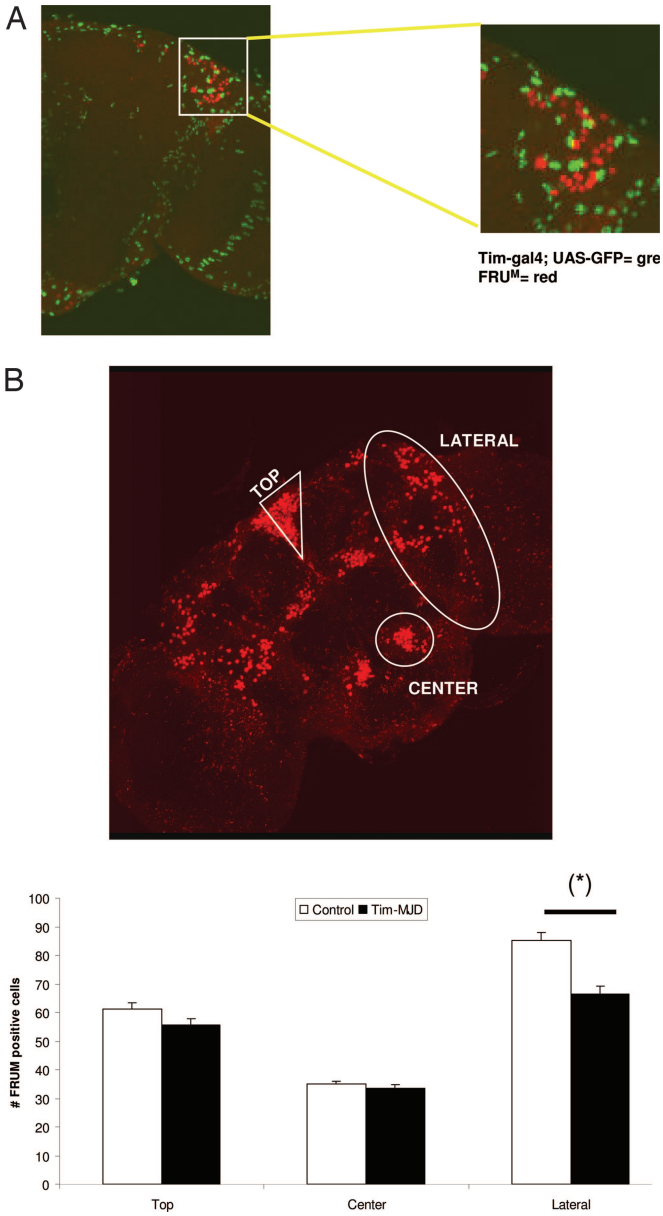


**Fig. 3.** Clock neuron control of sex-specific gene expression and mating behavior. (*A*) Some $FRU^M$-positive cells are located in close proximity to *tim*-gal4-positive cells. (*B*) The number of $FRU^M$-positive cells in the lateral part of TIM-MJD brains is significantly diminished ($P < 0.05$) compared with control flies (*tim*-gal4). After categorizing the $FRU^M$-positive cells inside each of the arbitrary groups shown (*Upper*, top, center, or lateral), their numbers were counted and the numbers were averaged. This arbitrary group corresponds to the following $FRU^M$ clusters previously described (43): top, cluster 2; center, cluster 7; and lateral, clusters 3, 4, 5, 6, and 14.

## Discussion

In this study, we combined two strategies, neurotoxic protein expression and brain microarrays, to investigate circadian clock gene expression and behavior. Some Tim-MJD-affected mRNAs had been previously identified as cycling, whereas others are sex-specific and relevant to courtship. Because Tim-MJD flies also exhibit a mating defect, we suggest that this phenotype reflects circadian neuron inactivation as well as an important courtship-relevant connection between these neurons and cells affecting reproductive behavior.

Our goal was to examine the behavioral and molecular phenotype in the absence of circadian neuron function. This had not yet been achieved, because the proapoptotic UAS genes *hid* and *reaper* (8) are embryonic lethal in combination with *per* or *tim* drivers. Importantly, *Ddc-gal4* is also lethal in combination with UAS-*hid* (data not shown). Other neuronal inactivation tools, e.g., UAS-tetanus toxin and UAS-modified $K^+$ channels, are rhythmic with *per* and *tim* drivers in LD cycles (10, 13). Although these UAS transgenes might cause arrhythmicity in combination with stronger circadian drivers, the fully arrhythmic phenotype of the same *tim* driver with UAS-MJDtr78Q indicates it is a stronger reagent.

Young flies expressing neurotoxic proteins under the control of the *tim-gal4* driver not only were viable but also had similar morphology, fertility, and locomotor activity levels compared with young control flies (data not shown). The only striking phenotypes were complete arrhythmicity under all conditions, mating defects, and a shortened lifespan (Figs. 1*A* and 4*C*; Table 3). Several lines of evidence suggest that the arrhythmic phenotypes are related to ablation/inactivation of the clock in *tim*-expressing fly brain neurons. It is possible that MJDtr78Q expression in *tim*-expressing glial and fat-body cells contributes to the shortened lifespan (2). Indeed, it was recently shown that MJDtr78Q expression in glial cells causes

a shortened lifespan (44). However, we did not find specific changes in gene expression of glial-specific transcripts in Tim-MJD flies (data not shown), suggesting that these cells are not strongly or universally affected by the *tim* driver.

Are Tim-MJD behavioral and molecular phenotypes due to clock-neuron death? Two independent observations indicate that MJDtr78Q expression predominantly leads to circadian transcriptional misregulation, at least in young flies. First, the gene expression changes in Tim-MJD resemble those observed in the clock transcription factor mutant $Clk^{Jrk}$. Of 552 genes down-regulated in heads of Tim-MJD, 104 (19%) were reported as down-regulated in $Clk^{Jrk}$, and only 27 (4.9%) were up-regulated in $Clk^{Jrk}$. A comparison of genes up-regulated in Tim-MJD heads (368 genes) with genes up-regulated in $Clk^{Jrk}$ has a similar proportion of identical transcripts (19%), making it unlikely that this down–down and up–up relationship between the two strains is fortuitous. Perhaps CLK is recruited to inclusions, and circadian transcription is inhibited because it is the only core pacemaker protein with a clear polyQ region.

Second, the Tim-MJD effect on the $LN_v$ cell population (Fig. 1 *C* and Table 1) is remarkably similar to that observed in another transcription factor mutant $cycle^{02}$ as well as in $Clk^{Jrk}$ (45). Because large $LN_v$s are born much later than small $LN_v$s ($sLN_v$s) (46), the apparent Tim-MJD selectivity for $sLN_v$s may reflect longer neurotoxic protein exposure. Nonetheless, $sLN_v$s are still present in young adult flies, suggesting that some circadian neurons survive MJDtr78Q expression from first larval instar to adulthood. Persistent adult expression may then explain the short lifespan.

Despite the presence of *Pdf* gene expression, disruption of *per* and *tim* transcription by Tim-MJD expression seems complete in the ≈75 pairs of pacemaker neurons (Fig. 1*B*). This contrasts with the more modest effect (50–70% decrease) on the levels of *timeless* mRNA and of other clock-related mRNAs from whole heads or brains (Fig. 2 *A* and *B*). Moreover, molecular oscillations still persist in Tim-MJD brains (Fig. 2*B* and data not shown), suggesting that cell-autonomous molecular oscillations continue outside of the pacemaker cells in Tim-MJD flies, at least under these LD conditions. If the remaining 30–50% of clock gene expression derives from a much larger number of extra-pacemaker clock cells, these must be low-expressing brain clock cells, which explains the failure to detect *tim* or *per* expression by immunostaining or *in situ* hybridization in Tim-MJD brains. Lower expression levels per cell would also explain the likely persistence of these neurons in Tim-MJD flies, i.e., expression levels would be below a toxicity threshold. Combined with relatively late *tim* expression during eye development, low expression levels might also contribute to the lack of a Tim-MJD rough eye phenotype.

Some cycling-head mRNAs come from the fat body as well as the eye and brain (38, 39), and some fat-body mRNAs are strongly affected in Tim-MJD flies. Strikingly, the MJD effect is dramatic in brain RNA but less strong or absent in head RNA for most of these fat-body transcripts. This is reminiscent of a previously reported difference in gene expression between brain-proximal and canonical fat-body cells (39); the former should constitute a more prominent source of fat-body signal in the dissected-brain samples than in the total head samples. That yolk-protein mRNAs are among the Tim-MJD-affected brain transcript population (Fig. 5*D*) suggests this fat-body subset is not restricted to behavioral function.

Although courtship and mating are mainly controlled by *fruitless*-expressing regions of the central nervous system (47), recent evidence suggests that the circadian clock, as well as the fat body, contributes to these behaviors (5, 7, 38, 39). Therefore, the mating phenotype could be a direct effect of disrupting the clock mechanism in this tissue. However, because brain $FRU^M$ expression in some neuronal groups is implicated in the early steps of male courtship (48), the decrease in $FRU^M$-expressing neurons in Tim-MJD (Fig. 3*B*) might also be relevant to the mating phenotype. This decrease might impact not only courtship-relevant targets within the brain but also brain-proximal fat-body gene expression. In this view, $FRU^M$ contributes to transmitting circadian information from brain clock centers to mating-relevant peripheral tissues such as the fat body. In any case, we suggest that circadian neurons affect courtship and mating behavior by communicating with brain courtship centers as well as peripheral tissues like the fat body.

## Materials and Methods

**Fly Strains.** The lines *tim-gal4* no. 27 (2), *Ddc-gal4* (28), and UAS-MJDtr78Q (weak, strong, and moderate; ref. 19) have been described.

***In Situ* mRNA Hybridization on Adult Brain Whole Mount.** *In situ* hybridizations to *tim* and *Pdf* mRNAs were as described (49, 50).

**Immunostaining and Assessment of Cell Numbers.** PER immunostaining was as described (51). For $FRU^M$ immunostaining, fly brains were treated as for PER immunostaining, but $FRU^M$ antibody and Cy5-conjugated anti-rat IgG were used. Neuronal marking was analyzed by using a Leica (Wetzlar, Germany) TCS SP2, scanning in the Cy5 channel for singly labeled brains (anti $FRU^M$) or scanning for Cy5 (red) and GFP (green) in colocalization assessments.

For cell counting, brains of Tim-MJD or control flies were stained and visualized as described above. $FRU^M$ expression from the whole brain was quantified by projecting a Z series of 1- to 2-$\mu$m sections. Cell-counting numbers were obtained by taking the total number of $FRU^M$-positive cells for each region. Five brains were analyzed in this way, and the number of cells for the different regions was counted twice. Average values for each brain were then used to perform ANOVA.

**Locomotor Behavior.** Male flies were monitored for 4 days in LD conditions, followed by 4–5 days in constant darkness, by using Trikinetics *Drosophila* Activity Monitors (Waltham, MA). Analyses were performed with a signal-processing toolbox (52). We used autocorrelation and spectral analysis to estimate behavioral cycle durations (periods) and the rhythm index (53) to assess rhythm strength. General locomotor activity of individual flies was measured as described (41).

**Assessment of Lifespan.** Ten groups (five of males and five of females), each containing 10 flies of a given genotype, were monitored for survivorship. The $MI_{50}$, defined as the time (in days) when half of the flies had died, was determined and averaged among five independent experiments.

**Courtship Behavior.** Male–female pairs were tested by placing them in the cylindrical chambers of a "mating wheel" (54) at 25°C. Wild-type males were collected soon after eclosion and grown individually in food vials for 3–4 days. Transgene-bearing females or males (3–5 days old) were introduced to wild-type males or females, respectively, and the time to copulation was determined for each pair during a 1-hr observation period. If the pair did not mate within this time frame, it was considered a "nonmating." Ten pairs were observed simultaneously, and a mean mating-initiation time ± SEM was tabulated for each genotype.

Mating latency comparisons were performed by using a one-way ANOVA (log-transformed data), with genotype as the main effect, as in ref. 41. For courtship behavioral analysis, statistics were performed by using JMP software (SAS Institute, Cary, NC).

**Microarrays.** ***Probe preparation.*** Total RNA was extracted from fly heads or brains by using the Mini RNA Isolation Kit (Zymo Research, Orange, CA), according to the manufacturer's protocol. All of the samples were collected at ZT4 and/or ZT16. For heads, cDNA synthesis was carried out as described in the Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA). The

cRNA reactions were carried out by using the BioArray High-Yield Transcript Labeling Kit (Enzo Biochem, New York, NY). In the case of *tim-gal4*;UAS-MJD and UAS-MJD brains, cDNA was synthesized by using the Ovation Biotin System according to manufacturer's protocol (Nugen, San Carlos, CA). Affymetrix high-density arrays for *Drosophila melanogaster* were probed, hybridized, stained, and washed according to the manufacturer's protocol.

***Data analysis.*** GeneChip.CEL files were analyzed by using R, the bioconductor package, and the gcrma algorithm (55, 56). Antilogarithm (base e) was applied to the data to obtain expression values.

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