The MAP Kinase p38 Is Part of Drosophila melanogaster’s Circadian Clock

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Abstract

All organisms have to adapt to acute as well as to regularly occurring changes in the environment. To deal with these major challenges organisms evolved two fundamental mechanisms: the p38 mitogen-activated protein kinase (MAPK) pathway, a major stress pathway for signaling stressful events, and circadian clocks to prepare for the daily environmental changes. Both systems respond sensitively to light. Recent studies in vertebrates and fungi indicate that p38 is involved in light-signaling to the circadian clock providing an interesting link between stress-induced and regularly rhythmic adaptations of animals to the environment, but the molecular and cellular mechanisms remained largely unknown. Here, we demonstrate by immunocytochemical means that p38 is expressed in Drosophila melanogaster’s clock neurons and that it is activated in a clock-dependent manner. Surprisingly, we found that p38 is most active under darkness and, besides its circadian activation, additionally gets inactivated by light. Moreover, locomotor activity recordings revealed that p38 is essential for a wild-type timing of evening activity and for maintaining ~24 h behavioral rhythms under constant darkness: flies with reduced p38 activity in clock neurons, delayed evening activity and lengthened the period of their free-running rhythms. Furthermore, nuclear translocation of the clock protein Period was significantly delayed on the expression of a dominant-negative form of p38b in Drosophila’s most important clock neurons. Western Blots revealed that p38 affects the phosphorylation degree of Period, what is likely the reason for its effects on nuclear entry of Period. In vitro kinase assays confirmed our Western Blot results and point to p38 as a potential “clock kinase” phosphorylating Period. Taken together, our findings indicate that the p38 MAP Kinase is an integral component of the core circadian clock of Drosophila in addition to playing a role in stress-input pathways.

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Introduction

Circadian clocks provide a key advantage to organism allowing them to prepare in advance for daily environmental changes. They control daily rhythms in physiology and behavior, as locomotor activity, sleep-wake cycles and hormonal secretion. A hallmark feature of these clocks is that they oscillate with free-running periods of ~24 h, even in absence of external time cues. Molecularly, circadian clocks depend on species-specific clock genes and proteins that interact in complex feedback loops to rhythmically control gene transcription (reviewed in [1–2]). However, research of the last few years demonstrated that not only rhythmic gene expression but also post-translational modifications, especially protein phosphorylation, play a crucial role in generating and maintaining circadian rhythms and most importantly in determining the speed of the oscillations [3–9].

Studies in Drosophila melanogaster have been instrumental in our understanding of clock mechanisms in general and mammalian ones in particular. In Drosophila’s main feedback loop, the core clock genes period (per) and timeless (tim) are rhythmically transcribed and translated into the proteins PER and TIM. Following phosphorylation by kinases (and/or dephosphorylation by phosphatases), both proteins accumulate in the cytoplasm and finally translocate back to the nucleus to inhibit their own transcription as well as that of clock-controlled genes (reviewed in [10]). Even if most of the clock proteins are phosphorylated within this molecular machinery, PER seems to be the clock component behaving as the primary “phospho-timer” [4,6]. Recent findings indicate that PER proteins in animals possess up to 25–30 phosphorylation sites [5,11] many of which undergo daily changes in phosphorylation. These temporal changes in PER phosphorylation are crucial for a functioning clock, since they modulate the stability of PER as well as the time of its nuclear entry, and in this way determine the pace of the clock [11–13]. While in the past it was thought that the amount of phosphate residues of clock proteins determines their degradation, studies nowadays show that it is rather site-directed phosphorylation that modulates clock protein function and stability [11–14]. So far, in Drosophila just a few kinases have been identified that interact with PER: DBT [15–17], SGG [12], CK2 [18–20] and proline-directed kinases as...

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The genome of the fruit fly encodes two functional p38 and even more elaborate as many cells express diverse isoforms of p38. The complexity of this p38 MAPK signaling network becomes even more elaborate as many cells express diverse isoforms of p38. The genome of the fruit fly encodes two functional p38 orthologues - p38a and p38b [25–26]. Phosphorylation of both is well described in respect of Drosophila development [27–28,37], stress and immune response [25–27], [38–40]. Interestingly, various studies in mammals [41–42] and fungi [43–44] addition-
(hereafter also referred as p-p38) also showed clear labeling in the protocerebrum (Fig. 1F), but p-p38 staining of clock neurons was restricted to much fewer cells. We found reliable staining only in the DN1a,s (Fig. 1G) and in one experiment also in the l-LN,s (not shown). This discrepancy might be due to the specificity of p-p38 antibody, which rather represents the current activation pattern than expression pattern of p38. Generally, tiny amounts of activated kinases are sufficient for effective signaling in transduction cascades. Thus, the amount of activated p38 might be well below the detection limit of the p-p38 antibody in the majority of clock neurons. In addition, p38 may be temporally phosphorylated as shown for the hamster SCN, where activated p38 was only high in the late day and early night [41]. Indeed, we noticed that p-p38 levels in the DN1a,s as well as staining in the entire cortex of the brain depended also on the time of day and were only high towards the end of the night (Fig. 1D,E2 compared to Fig. 1F,G2).

To finally exclude any unspecific antibody labeling, antibody staining on two p38 null mutants, w^{1118} + p38a^{A4} and yw;p38b^{A51} + (from now on referred to as p38a^{A4} and p38b^{A51}, respectively), was performed at ZT21 and p-p38 staining intensity was measured in DN1a,s. Both p38 mutants displayed a significant reduction in phosphorylated p38 to 50% of wildtype level (p< 0.05; Fig. S2). This clearly verifies the authenticity of the p-p38 antibody labeling, but also suggests the existence of both p38 isoforms in these cells.

Taken together, even if we did not get a complete overlap, p38b-driven GFP expression as well as p38 antibody staining indicates that both p38a and p38b are expressed in several clock neurons, most probably in the PDF-positive l-LN,s and s-LN,s as well as in the DN1a,s. This finding coincides with other studies: Microarray studies on LN,s detected enriched p38a mRNA levels in the s-LN,s as compared to other brain regions [50]. Furthermore, Mef2, a transcription factor well recognized as a downstream target of p38 MAPK signaling in Drosophila muscle [27] and mammalian myocytes, lymphocytes and neurons [29,51–53], was shown to localize in all subgroups of Drosophila clock neurons [54] indicating p38 MAPK signaling in these cells.

Darkness and clock dependent phosphorylation of p38 MAPK in DN1a,s

So far, Drosophila studies mainly focused on p38 MAPK expression over a longer period of time, especially with regard to development [26,37,55]. Since the observed changes in the amount of phosphorylated p38 in the DN1a,s at ZT9 and ZT21 (Fig. 1D–G) might also point to daily oscillating gene expression, we examined mRNA levels of p38a and p38b in the course of a day.

Quantitative real-time PCR (qPCR) from head extracts of Canton S wildtype flies revealed an allover higher expression level of p38b compared to p38a throughout the day (p<0.001; Fig. 2A). This is consistent with data published in a microarray-based atlas of gene expression in Drosophila (Flyatlas - http://www.flyatlas.org). Moreover, we did not discover any circadian oscillations of p38 isoforms on the transcriptional level, which is reminiscent on findings in fungi [44] and mammals [41,56]. Very similar to our study, the latter papers demonstrated rhythmic phosphorylation of p38 during the night and low levels during the day as we could show for the DN1a,s. This would argue for a night-time specific function of p38 within the clock of these neurons. Nevertheless, we have to admit that the DN1a,s are not the clock neurons that are most important for the control of behavioral rhythmicity. Future studies have to show, whether a cyclic activation of p38 does also occur in the s-LN,s.

p38b knockdown and overexpression in clock neurons induce period lengthening

Locomotor activity recordings are a well-suited technique for investigating circadian behavioral rhythms in Drosophila melanogaster. When entrained to LD cycles wildtype flies display a typical bimodal activity pattern with an anticipatory morning and evening activity peak around lights-on and lights-off. In constant darkness this rhythmic locomotor behavior proceeds with its internal individual period reflecting the pace of the endogenous clock. To examine the role for p38 MAPK within the circadian system, we used transgenic RNA interference (RNAi) to reduce p38b RNA levels and thus p38b activity in different subsets of clock neurons, and screened for altered behavioral rhythms in LD as well as in constant dark conditions (DD). For RNAi-mediated p38b knockdown a w;UAS-p38bRNAi;+ line was combined with different drivers as well as a UAS-dicer2;++;+ line (dicer2). We first used dicer2;tim(UAS)-Gal4;+, a driver line with a broad expression pattern that allows ubiquitous expression in all clock cells. Daily activity patterns of dicer2;UAS-p38bRNAi;tim(UAS)-Gal4;+ flies were similar to those of control flies showing normal wildtype LD behavior with activity peaks around lights-on and lights-off (Fig. 3A). To test the effectiveness of p38b transgenic RNAi, we performed qPCR on head extracts and found no significant reduction in p38b mRNA level in our experimental line. This may be due to a small number of p38b-positive clock neurons compared with the total number of p38b-expressing neurons within the brain (Fig. S1 compared to Fig. 1). Thus, w;UAS-p38bRNAi;+ was additionally combined with da-Gal4, a line that expresses Gal4 in most tissues throughout development [60]. Using the broader driver, we finally observed a significant decrease in p38b mRNA level in w;UAS-p38bRNAi;da-Gal4/+ compared to respective controls, confirming the effectiveness of our p38bRNAi line.

MAPK p38 and Drosophila’s Circadian Clock

For studying oscillations in active p38 in more detail, immunochemistry on Canton S wildtype brains was carried out in LD 12:12 at different times of day. Triple-labeling with anti-p-p38, anti-VRI and anti-PDF revealed daily oscillation in p38 phosphorylation in DN1a,s, with low levels during the light phase (ZT1-9) and significantly higher levels in the dark (ZT13-21) (p<0.05; Fig. 2B). Furthermore, the average number of p-p38-positive DN1a,s per hemisphere was significantly higher at night than during the day (p<0.05; Fig. S3). The diurnal oscillation in phosphorylated p38 in DN1a,s strongly points to a clock-mediated activation of p30 within the circadian system. To test whether these diurnal variations in active p38 are indeed clock-controlled or just represent a direct response to darkness, p38 staining intensity in the DN1a,s was measured under constant conditions at CT6 and CT18. Interestingly, similar to our observations in LD, the level of active p38 was significantly lower in the subjective day than subjective night confirming our hypothesis of a clock-controlled phosphorylation of p38 (p<0.001; Fig. 2C). Since previous studies in mice [57–59] and hamsters [41] also suggested a light dependent regulation of ERK and p38 activity in the SCN, we further exposed flies at CT6 and CT18 for 15 minutes to light and dissected brains before and after light pulse treatment. While levels of active p38 at CT 6 remained constant, light pulse at CT 18 led to a significant decrease in p-p38 signal (p<0.05; Fig. 2D).

These results indicate an additional light-induced regulation (depression) of p38 activity.

Taken together, our findings are in strong favor of a clock-controlled phosphorylation of p38. Both p38a and p38b are constantly expressed throughout the day and display no circadian regulation on transcriptional level. Activation of p38 MAPK, however, seems to be clock-regulated, showing high levels of active p38 during the night and low levels during the day as we could show for the DN1a,s. This would argue for a night-time specific function of p38 within the clock of these neurons. Nevertheless, we have to admit that the DN1a,s are not the clock neurons that are most important for the control of behavioral rhythmicity. Future studies have to show, whether a cyclic activation of p38 does also occur in the s-LN,s.
Figure 1. p38 MAPK expression pattern in adult male Canton S brains. p38 MAPK distribution within the circadian clock was investigated immunohistochemically with an antibody directed against Drosophila p38b (A–C) and against phosphorylated human p38 (D–G). A–C: Staining with anti-p38b (green) in Canton S wildtype brains was visible in many cell bodies close to the lateral clock neurons, but co-labeling with anti-VRI MAPK p38 and Drosophila’s Circadian Clock.
Since we found no behavioral phenotype in LD, we next focused on locomotor behavior of dicer2;UAS-p38bRNAi/tim(UAS)-Gal4;+ flies under constant conditions using $\chi^2$-periodogram analysis. Surprisingly, 93% of the flies were arrhythmic (Table 1) and only 7% showed rhythmic locomotor behavior with a prolonged free-running period of 25.3 h (p < 0.001; Fig. 3A; Table 1). Considering the fact that besides clock neurons dicer2;tim(UAS)-Gal4;+ additionally drives expression in glia cells, we wanted to rule out a glia-specific effect on rhythmicity and period length. Therefore, we restricted p38b knockdown solely to the PDF-expressing clock neurons, the s-LNvs and the l-LNvs, using the more specific clock driver dicer2;Pdf-Gal4;+. Dicer2;UAS-p38bRNAi/Pdf-Gal4;+ flies showed a later onset of evening activity and a higher activity after lights-off than control flies in LD (Fig. 3B) as well as a significantly prolonged free-running period of 24.8 h in DD (p < 0.05; Fig. 3B; Table 1). Only about half of the flies were arrhythmic as opposed to 93% of dicer2;UAS-p38bRNAi/tim(UAS)-Gal4;+ flies (Table 1). These findings suggest that p38 has indeed a functional role within the circadian system and that its specific knockdown in the clock neurons mainly delays evening activity and lengthens the free-running period.

To further confirm our hypothesis of p38 functioning in the clock three additional constructs were expressed to interfere with endogenous p38b: two UAS-p38b kinase-dead transgenes (UAS-p38bKD3 and UAS-p38bKD8) and a dominant-negative UAS-p38b transgene (UAS-p38bDN-S). Interestingly, simultaneous expression of UAS-p38bKD3 and UAS-p38bKD8 in either PDF- or TIM-expressing clock neurons by using the more specific clock driver dicer2;Pdf-Gal4;+ caused a later onset of evening activity and a significantly lower activity after lights-off than control flies in LD as well as in DD (p < 0.05; Fig. 3B; Table 1). Only about half of the flies were arrhythmic as opposed to 93% of dicer2;UAS-p38bRNAi/tim(UAS)-Gal4;+ flies (Table 1). These findings suggest that p38 has indeed a functional role within the circadian system and that its specific knockdown in the clock neurons mainly delays evening activity and lengthens the free-running period.

**Figure 2. Daily p38 mRNA (A) and protein expression (B–D) in Canton S wildtype.** A: Quantitative real-time PCR on head extracts revealed constant mRNA expression throughout the day with lower higher levels of p38b compared to p38a (p < 0.001). B: Antibody staining with anti-p-p38 on adult brains displayed rhythmic phosphorylation of p38 in DN1as in LD with significant higher p-p38 levels occurring during the night than in the day (p < 0.05). C: A highly significant reduction of active p38 in DN1as at CT6 compared to CT18 in DD indicates a clock-controlled activation of p38 (p < 0.001). D: Only a 15 minute light pulse (LP) during subjective night (CT18) and not during the subjective day (CT6) leads to a reduction in active p38 in DN1as, suggesting a clock-dependent photic reduction of active p38. The "C" in D indicates control brains without 15 minute light pulse (LP). Error bars show SEM. Significant differences (p < 0.05) are indicated by *, highly significant differences (p < 0.001) by **.

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expressing neurons resulted in a delayed onset of evening activity and a prolonged free-running period under DD compared to respective controls, but did not cause any arrhythmicity (p < 0.001; Fig. S5; Table 1). This phenotype became even more obvious when the dominant-negative p38b transgene was expressed in all clock cells (using w;tim(UAS)-Gal4;+ or only in the LNvs (using yw;Pdf-Gal4;+): evening activity was delayed in LD and, in DD, free-running period was lengthened for about 2 h in UAS-p38bDNS;tim(UAS)-Gal4/+ flies (p < 0.001; Fig. 4A; Table 1) and for about 2.5 h in UAS-p38bDNS;Pdf-Gal4/+ flies as compared to controls (p < 0.001; Fig. 4B; Table 1). Again, no higher fraction of arrhythmic flies was observed (Table 1).

The high number of arrhythmic flies after knockdown of p38b, which was not observed with any of the other constructs, points to putative off-target effects of the p38bRNAi construct. Off-target effects can never be excluded with the RNAi technology and are
Table 1. Rhythmicity and period length of all investigated genotypes in constant darkness (DD) according to χ²-periodogram analysis.

| Genotype | n  | arrhythmic (%) | rhythmic (%) | Period (hr ± SEM) | Power² (%V ± SEM) |
|----------|----|----------------|--------------|-------------------|--------------------|
| UAS-p38bRNAi/w+ | 32 | 3 | 97 | 23.4±0.04 | 35.4±1.49 |
| dicer2Pdf-Gal4/w+ | 31 | 0 | 100 | 24.3±0.05 | 38.5±1.84 |
| dicer2tim(UAS)-Gal4/w+ | 32 | 3 | 97 | 24.1±0.06 | 38.5±1.91 |
| dicer2UAS-p38bRNAi/Pdf-Gal4/w+ | 31 | 42 | 58 | 24.8±0.11 | 38.3±3.23 |
| dicer2UAS-p38bRNAi/tim(UAS)-Gal4/w+ | 28 | 93 | 7 | 25.3±0.35 | 42.4±8.00 |
| UAS-p38bRNAi/tim(UAS)-Gal4/w+ | 31 | 13 | 87 | 23.8±0.04 | 33.9±1.52 |
| Pdf-Gal4/w+ | 30 | 3 | 97 | 24.0±0.07 | 35.6±2.25 |
| w/lim(UAS)-Gal4/w+ | 32 | 0 | 100 | 24.1±0.04 | 40.1±2.05 |
| wUAS-p38bRNAi/Pdf-Gal4/UAS-p38bRNAi/w+ | 32 | 6 | 94 | 25.1±0.07 | 35.5±1.48 |
| wUAS-p38bRNAi/tim(UAS)-Gal4;UAS-p38bRNAi/w+ | 30 | 3 | 97 | 25.1±0.08 | 35.6±1.89 |
| UAS-p38bRNAi/w+ | 32 | 6 | 94 | 24.0±0.04 | 34.2±1.56 |
| UAS-p38bRNAi/Pdf-Gal4/w+ | 32 | 3 | 97 | 26.5±0.09 | 30.0±0.93 |
| UAS-p38bRNAi/tim(UAS)-Gal4/w+ | 30 | 3 | 97 | 25.9±0.06 | 34.1±1.93 |
| UAS-p38bRNAi/w+ | 32 | 22 | 78 | 23.2±0.06 | 22.2±1.07 |
| UAS-p38bRNAi/Pdf-Gal4/w+ | 31 | 0 | 100 | 25.3±0.04 | 35.5±1.85 |
| UAS-p38bRNAi/tim(UAS)-Gal4;UAS-p38bRNAi/w+ | 29 | 0 | 100 | 25.5±0.06 | 41.7±2.24 |
| w/+;UAS-p38bRNAi/w+ | 32 | 0 | 100 | 23.8±0.05 | 44.7±2.57 |
| dicer2Pdf-Gal4/w+;p38bRNAi/w+ | 32 | 0 | 100 | 24.6±0.06 | 47.9±2.62 |
| dicer2tim(UAS)-Gal4/w+;p38bRNAi/w+ | 32 | 6 | 94 | 24.5±0.07 | 39.1±1.81 |
| dicer2UAS-p38bRNAi/w+;p38bRNAi/w+ | 32 | 12 | 88 | 23.9±0.05 | 26.3±1.37 |
| dicer2UAS-p38bRNAi/tim(UAS)-Gal4;UAS-p38bRNAi/w+ | 27 | 41 | 59 | 24.9±0.08 | 26.3±1.99 |
| w¹¹18 | 32 | 10 | 90 | 23.3±0.07 | 24.5±1.05 |
| p38bAT1 | 29 | 47 | 53 | 23.2±0.10 | 23.7±1.35 |
| p38bRNAi | 29 | 14 | 86 | 23.6±0.08 | 32.4±1.30 |
| p38bets | 28 | 21 | 79 | 23.7±0.07 | 28.3±1.91 |

n indicates the number of tested flies per genotype that survived locomotor recordings. Power and period values were averaged over all rhythmic flies for each genotype.

1FLies with power values <0.2 were defined as arrhythmic.

2Power is a measure of rhythmicity and is given in % of variance.

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more likely to occur if the RNAi construct is expressed in many neurons as was the case with the tim-gal4 driver. When p38bRNAi was expressed with pdf-gal4, that drives in only 16 neurons in the brain, the number of arrhythmic flies was significantly lower (χ²-test: χ² = 17.02; p<0.001). Thus, it is most likely that the p38bRNAi construct has off-target effects causing arrhythmicity in addition to its specific effects on clock speed. To test, whether the molecular clock is still running in DD after knockdown of p38b, we immunostained brains of dicer2;UAS-p38bRNAi/Pdf-Gal4/+ flies with anti-PER and anti-TIM throughout the circadian cycle on day 4 in DD (Fig. S6). We found that the molecular cycling persisted, just the phase of the oscillation was delayed in comparison to controls, which is consistent with the long period of the flies. We conclude that p38 mainly affects the speed of the clock and has little if any effects on the stability and robustness of the molecular clock cycling.

Since p38bRNAi knockdown and expression of non-functional p38b cause period lengthening of locomotor free-running rhythms, we next wondered what would happen if we overexpress wildtype p38b (UAS-p38b+) in different subsets of clock neurons. Surprisingly, although p38b overexpression might have been expected to give the opposite effect of p38bRNAi (i.e., short locomotor free-running period), UAS-p38b+/Pdf-Gal4/+;+ and UAS-p38b+/tim(UAS)-Gal4/+;+ exhibited significantly later evening activity in LD and longer locomotor free-running rhythms in DD that were similar to those of p38bRNAi and p38b/dicer2;Pdf-Gal4/+ flies (p<0.001 and p<0.001 respectively; Fig. S7 and Table 1). This suggests that there is an optimal level of p38b for provoking locomotor activity rhythms with normal period.

Taken together our results indicate that wildtype levels of functional p38b are required for wildtype timing of evening activity and normal/wildtype free-running rhythms under constant conditions. Furthermore, already p38b knockdown or overexpression restricted to the LNvs (PDF-neurons) is sufficient to cause free-running rhythms with long period. This is well consistent with the dominant role of the s-LNvs, in which we found p38 expression, in controlling rhythms under constant darkness (reviewed in [61]). Since the oscillation speed was significantly affected by p38b manipulation, we rather assume a function of p30 MAPK in the core of the clock than in its input pathway.

p38a knockdown recapitulates the p38b knockdown phenotype

As shown before, the p38a isoform appeared to be co-expressed in the clock neurons (Fig. S2) raising the question whether p38a...
has also a possible function within the circadian system. To test this, we down-regulated p38a either in all clock cells (using dicer2;tim(UAS)-Gal4;+) or only in the LNvs (using dicer2;Pdf-Gal4;+), as done before for p38b. The effectiveness of p38a transgenic RNAi was successfully confirmed via qPCR on w;+;UAS-p38aRNAi/da-Gal4 fly heads (p < 0.001; Fig. S4).

Down-regulation of p38a had generally weaker effects than down-regulation of p38b: it did not significantly delay evening activity in LD and, in DD, period was not lengthened as dramatically as after manipulation of p38b protein level (Fig. 5). Nevertheless, \( \chi^2 \)-periodogram analysis revealed that both experimental lines (dicer2;tim(UAS)-Gal4;+;UAS-p38aRNAi/+ and dicer2;Pdf-Gal4;+;UAS-p38aRNAi/+) had significantly longer free-running periods than the respective controls (p < 0.001 and p < 0.05 respectively; Fig. 5A,B; Table 1). This result strongly argues for a clock-related role for p38a besides p38b.

Complete loss of either p38b or p38a does not disturb circadian rhythms

After we found that down-regulation of p38b or p38a significantly affected the flies' free-running rhythms, we aimed to test whether a complete loss of either isoform does affect rhythmicity in a similar way. To our surprise this was not the case. Although \( p38b^{D45} \), a \( p38b \) null mutant, displayed a slightly enhanced percentage of arrhythmic flies in DD, this was also the case for its precise excision line \( p38bpex41 \), indicating some background effect on rhythmicity (Table 1). Additionally, both

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Figure 4. Locomotor activity rhythms of flies expressing a dominant-negative form of p38b (p38b\(^{DN-S}\)) in Drosophila clock neurons and respective controls. Both, expression of a dominant negative form of p38b in either all clock neurons (UAS-p38b\(^{DN-S}\);tim(UAS)-Gal4;+) or just in a subset of clock cells, the PDF-positive LNvs (UAS-p38b\(^{DN-S}\);Pdf-Gal4;+), resulted in a diurnal activity profile with a significantly delayed evening activity onset in comparison with respective controls (upper panels in A and B). This delay in evening activity is accompanied by a significantly prolonged free-running period in UAS-p38b\(^{DN-S}\);tim(UAS)-Gal4;+ (lower panels in A) as well as in UAS-p38b\(^{DN-S}\);Pdf-Gal4;+ flies (lower panels in B), when released into constant darkness. For recording and processing of activity data as well as for figure labeling see Figure 3.

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lines showed similar activity profiles under LD conditions and did not differ in their free-running period (Fig. 6A; Table 1). Even if p38a null mutants, p38a<sub>D1</sub>, showed a later onset of evening activity and a higher fraction of arrhythmic flies than the w<sup>1118</sup> controls (Fig. 6B and Table 1), the free-running period of the rhythmic flies was not different from that of the controls. The higher percentage of arrhythmic flies might be associated with the prominent role of p38a in immune stress response [39–40], inflammation [26,62] and lifespan [27]. Therefore flies lacking this isoform might be less healthy and display disrupted behavioral rhythms.

Our results suggest that the two isoforms might replace each other under certain conditions. According to Han et al. [26] p38a and p38b appear to have partial functional redundancy, because both isoforms are similarly activated in response to stress-inducing or inflammatory stimuli in cell culture experiments. This compensatory mechanism may be vital for the flies, when one of the isoforms lacks completely. But the compensatory mechanism may be elusive when one isoform is only down-regulated in specific neurons that are not necessary for survival (e.g. the clock neurons): Lengthened free-running rhythms in DD just occurred, when either p38b or p38a levels in clock neurons were reduced, but not completely absent from the entire fly. We therefore suppose that either isoform overtakes the clock specific function of the other one only in its complete absence. As p38a mRNA levels were not increased in p38b<sup>D45</sup> flies and p38b mRNA levels were not elevated in p38a<sup>D1</sup> (Fig. S8), normal wildtype p38a or p38b levels seem to be sufficient to drive circadian rhythms in complete absence of the other isoform.

**Figure 5. Locomotor activity rhythms of p38a knockdown flies and respective controls.** Average activity profiles of dicer2;UAS-p38b<sup>RNAi</sup>/tim(UAS)-Gal4/+ (upper panel in A) and dicer2;UAS-p38b<sup>RNAi</sup>/Pdf-Gal4/+ (upper panel in B) displayed wildtype-like behavior in LD with activity bouts around lights-on and lights-off and did not differ from those of control flies. Evening activity onset was not delayed in the two mutant strains. However, when released into constant darkness both, p38a knockdown in TIM- (lower panels in A) and PDF-expressing neurons (lower panels in B) resulted in significant prolonged free-running rhythms in comparison to respective controls. For recording and processing of activity data as well as for figure labeling see Figure 3.

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If our hypothesis is true, \( p38b^{-45};p38a^{-1} \) double mutants should show long free-running periods. Unfortunately, the combination of both null alleles turned out to be lethal. Similarly, double mutants lacking the \( p38a \) gene and carrying a hypomorphic \( p38b \) allele \( (p38b^{-25};p38a^{-1}) \) were hardly viable. Furthermore, flies that hatched had a very short life-span dying 3–6 days after emergence of the pupa, making it hard to investigate their free-running rhythms. Nevertheless, we were able to record the locomotor activity of two \( p38b^{-25};p38a^{-1} \) mutants for 5–6 days, which were entrained to LD 12:12 during pupal stage and immediately transferred into DD after eclosion (Fig. 6C). These flies free-ran with a long period until they died. In addition, we

Figure 6. Locomotor activity rhythms of \( p38b \) and \( p38a \) null mutants and hypomorph double mutant flies. Both \( p38 \) null mutants, \( p38b^{-45} \) (upper panels in A) and \( p38a^{-1} \) (upper panels in B), displayed wildtype-like behavior with activity bouts around lights-on and lights-off when recorded in LD 12:12. Even if evening activity onset of \( p38a^{-1} \) seems to be delayed compared to \( w^{1118} \), this delay did not result in a longer free-running period under constant darkness (lower panels in B). Similarly, flies, lacking the \( p38b \) gene, also showed comparable free-running rhythms as their respective controls (lower panels in A). Activity data in C show two representative single actograms of a double mutant strain with a hypomorphic \( p38b \) allele \( (p38b^{-25};p38a^{-1}) \). Since these flies are hardly viable and die within 3–6 days after emergence of the pupa, flies were already entrained to LD12:12 during pupal stage and subsequently monitored in DD conditions after eclosion. Even if periodogram analysis was not possible due to the short recording period, \( p38b^{-25};p38a^{-1} \) flies clearly showed a long free-running period when kept in constant darkness (C). For recording and processing of activity data as well as for figure labeling see Figure 3.

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could simultaneously down-regulate p38a and p38b in TIM-positive neurons (dicer2; UAS-p38bRNAi/tim(UAS)-Gal4; UAS-p38bRNAi/+). Such flies exhibited also significant longer free-running periods in DD than the relevant controls (p < 0.001; Table 1). Together, our findings strongly indicate that both p38 isoforms are involved in the control of locomotor activity rhythms under constant conditions and that they can partly replace each other.

**Expression of the dominant negative form of p38b phase delays the molecular circadian clock**

Delayed evening activity and long free-running rhythms are often associated with a delayed nuclear entry of PER and TIM, an event in the molecular cycle which is mainly regulated via phosphorylation of PER by proline-directed kinases and SGG [12] as well as TIM by SGG [63]. To see whether the nuclear entry of PER is affected by p38 MAPK, we immunostained respective controls and flies, in which the dominant-negative form of p38b was expressed in the LNvs (UAS-p38bDN; Pdfl-Gal4/+; +) in 1-hour intervals in LD and quantified the amount of nuclear PER in the s-LNvs and l-LNvs (Fig. 7). We chose UAS-p38bDN; Pdfl-Gal4/+; + since the delay in evening activity under LD conditions was most prominent compared to other p38 mutant strains. Interestingly, we found a significant delay of nuclear entry of PER in both types of clock neurons that perfectly matched the delayed evening activity.

**p38b affects the degree of PER phosphorylation**

There are several ways, how p38 could influence the phosphorylation degree of PER and this way influence the efficiency and speed of nuclear translocation: p38 may directly phosphorylate PER or it may activate or inhibit already known key kinases of PER. Accordingly, p38 was shown to phosphorylate and activate CK2 [34–35] making it possible that p38 lengthens the period of the molecular oscillations via CK2 finally leading to a delayed nuclear translocation of the PER-TIM heterodimer. Alternatively or in addition, p38 may work on phosphatases that reduce phosphorylation. Previous studies revealed that both, p38 [64] and CK2 [65], stimulate the activity of the protein phosphatase 2A (PP2A) in mammalian fibroblasts. PP2A on the other hand was shown to dephosphorylate and stabilize PER, thereby promoting PER’s nuclear translocation in *Drosophila* clock cells [66]. Consequently, reduction of PP2A activity resulted in long free-running periods, the same phenotype we observed after manipulation of p38 levels.

To test whether p38b affects the degree of PER phosphorylation, we performed Western Blots on head extracts of flies, in which the dominant-negative p38b transgene (UAS-p38bDN; +) was driven in all clock cells including the photoreceptor cells (in LD 12:12). This time, we did not use Pdfl-Gal4, since Western Blots mainly reflect PER oscillations of the compound eyes (the oscillations of the 150 PER-expressing clock neurons can barely be seen behind the oscillations of the ~1600 PER-expressing photoreceptor cells). Indeed, PER seemed to be less phosphorylated in flies with impaired p38b signaling (Fig. 8A). For a better comparison we repeated the Westerns blotting control and experimental flies for each ZT side by side (Fig. 8B). We found that PER was clearly less phosphorylated in the flies with impaired p38b signaling at all time points. This was most evident during the night being well consistent with the postulated high activity of p38 MAPK during darkness. We conclude that p38 promotes phosphorylation during the night. The lack of this phosphorylation may delay nuclear entry of PER during constant darkness and in this way lengthen the free-running period of the clock significantly.
complex behavioral phenotypes (period-lengthening after down-regulation and overexpression of p38, as well as no effects of null mutations in p38a and p38b) argue for the putative interaction of several kinases in PER phosphorylation at S661.

In summary, our results demonstrate direct effects of p38 on circadian rhythms in behavior as well as on the molecular clock. Besides affecting the phosphorylation degree and nuclear entry of PER, p38 may influence the clock machinery in several ways due
to its many putative targets in *Drosophila’s* clock neurons. As we show here, one of the major p38 targets may be PER itself. Altogether, this places p38 in the center of multiple pathways that can affect circadian rhythms. Regarding its known role in transmitting cellular stress responses, p38 MAPK may even act as a factor that integrates responses of the circadian clock and the acute stress system to external stimuli. However, future studies have to reveal the exciting connection between the two systems in more detail.

**Materials and Methods**

**Fly strains and constructs**

Flies were raised on a standard cornmeal/agar medium at 25°C in LD 12:12. To investigate locomotor activity in p38 mutant flies, we recorded two p38 knockout strains: w^{1118};+;p38a^{D1} and yw;+;p38b^{M5};+ (kindly provided by R. Cagan and A. Vrailas-Mortimer). The latter carries a 1065bp deletion in the coding region of p38b, while w^{1118};+;p38a^{D1} flies completely lack the p38a locus. In addition the precise excision line yw;p38bpex41;+ (a gift of A. Vrailas-Mortimer) served as control for yw;+;p38b^{D45};+ and w^{1118};+;p38a^{D1} flies served as control for the w^{1118};+;p38a^{D1} mutants. Two double mutant strains, p38b^{M5};p38a^{D1} and p38b^{AA2};p38a^{D1} (both provided by A. Vrailas-Mortimer; the latter exhibits a hypomorphic p38b allele) were used to knockout both p38 isoforms; but these turned out to be either lethal or only weakly viable in our hands. Therefore, we could not perform any statistical analysis of their locomotor activity rhythms. For studying p38 knockdown exclusively within the circadian clock, we used two different RNAi lines, w;+;UAS-p38a^{RNAi} (Vienna Drosophila RNAi Center; #52277) and w;UAS-p38b^{RNAi};+ (Vienna Drosophila RNAi Center; #108099), as well as a combination of both (w;UAS-
Figure 9. p38b phosphorylates PER \textit{in vitro}. To test whether p38b phosphorylates PER \textit{in vitro}, either non-radioactive kinase assays followed by urea-PAGE (A–D) or radioactive kinase assays with autoradiography (E–F) were performed. A–D: Non-radioactive kinase assays were conducted with poly-histidine tagged p38b (His$_6$-p38b) and two truncated GST-tagged PER isoforms, GST-PER$^{1-700}$ (A,B) and GST-PER$^{658-1218}$ (C,D). Samples were subsequently separated with urea-PAGE and visualized by Coomassie staining (A,C). To further confirm PER's position in the gel two samples of the same gel were additionally blotted onto nitrocellulose membrane and immunolabeled using an anti-PER antibody and a secondary fluorescent antibody (B,D). While GST-PER$^{1-700}$ without kinase did not shift within 60 minutes, the addition of His$_6$-p38b induced a downward shift of GST-PER$^{1-700}$ indicating phosphorylation of PER (A; dotted line). Immunoblots with anti-PER further confirmed the size as well as the shift of the GST-PER$^{1-700}$ band (B). In addition to GST-PER$^{1-700}$, GST-PER$^{658-1218}$ also displayed band shifts after incubation with His$_6$-p38b (C). This was most prominent after 60 minutes, when addition of His$_6$-p38b resulted in two distinct shifted bands (black arrowheads), which could be additionally confirmed by Western blots (D). Time scale below graphs represents minutes after addition of His$_6$-p38b, the “C” refers to control and represents substrate samples without kinase and ATP. (E) Radioactive \textit{in vitro} kinase assays were conducted with the indicated GST-PER fusion proteins and
GSTM-p38b. Control reactions were performed in the absence of GST-p38b or with GST in combination with GST-p38b. Coomassie staining proved loading of the indicated protein combinations. Below, phosphorylation of GST-PER proteins was detected by autoradiography. (F) For quantitative analysis five independent in vitro kinase assay experiments were performed and analyzed. For each reaction within a single experiment, autoradiography signal intensities were normalized to the corresponding Coomassie stained protein band. Values in the graph are shown as percentages of GST-PER58–1218 phosphorylation (100%; * p<0.05, ** p<0.005).

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p38bRNAi; UAS-p38bRNAi). To restrict RNAi-mediated gene silencing to specific subsets of clock neurons, RNAi lines were crossed to a w;tim(UAS)Gal4;+ (kindly provided by Michael W. Young) as well as a yw;Pdf-Gal4;+ driver line (kindly provided by Jeffrey C. Hall) and combined with a UAS-dicer2;++;+ line (Vienna Drosophila RNAi Center; #60012) to further strengthen RNAi knockdown. In addition yw;Pdf-Gal4;+ and w;tim(UAS)-Gal4;+ flies were used to specifically overexpress wildtype p38b (UASp38b+ kindly provided by T. Adachi-Yamada) as well as two non-functional p38b isoforms: a dominant-negative UAS-p38b transgene, UAS-p38bDN-S (donated by T. Adachi-Yamada), and an UAS-p38b kinase-dead transgene, UAS-p38bKD (a gift of A. Vrailas-Mortimer). The dominant-negative p38b allele was generated by replacing the Thr194 of the MAPKK target site with Ala leading to a complete loss of enzymatic activity [28]. The UASp38bKD transgenic line, however, was made by exchanging a Lys residue at 53 in the catalytic domain with Arg [27]. This single amino acid substitution still allows target binding, but blocks kinase activity (A. Vrailas-Mortimer, personal communication). Here we used flies with two UAS-p38bKD transgenes, a weaker UAS-p38bKD (kindly provided by T. Adachi-Yamada) and a stronger UAS-p38bKD (UAS-p38bKD/Cyo-GFP; UAS-p38bKD/TM3; Ser-GFP). For studying the expression pattern of p38 within the brain of Drosophila melanogaster a Canton S (CS) wildtype strain was chosen as wildtype control for immunohistochemistry. In addition a p38b-Gal4 enhancer trap line (kindly provided by A. Vrailas-Mortimer) was used in combination with w++; UAS-GFP [63T] (Bloomington Stock Center, #1522; donated by Karl Fischbach) to express green fluorescent protein (GFP) in the p38b-neurons revealing p38b expression in detail. To analyze PER cycling on Western Blots, we used a w;cry-Gal4;+ driver line (kindly provided by F. Rouyer) to impair p38b signaling in p38bDN-S flies.

For in vitro kinase assays, N-terminal hexa-histidine or GST-tagged p38b fusion proteins (His6-p38b and GST-p38b) were created by first PCR amplifying the full-length p38b open reading frame (ORF) using the cDNA clone as template and following primers: 5’-CCGGATC CAGAAGTTCGACGAAAGTAGGCGCAAA TGGCCTTCG-3’ and 5’-GGGCGCCGCGATCTTTCCTTTGGGC- AGGAGCTCA-3’. After digestion with PvuII and NotI, the PCR product was inserted into the multiple cloning site of E. coli expression vector pH6HTN His6HaloTag T7 (Promega) and further subcloned as an EcoRI/NotI fragment into the pGEX 4T3 vector (GE Life Sciences). In order to generate recombinant GST-PER fusion construct, two truncated sequences of per, either encoding amino acids 1–700 (PER700) or amino acids 658–1218 (PER658–1218), were subcloned into the pGEX 6P vector (GE Life Sciences). All constructs were confirmed by DNA sequencing before use.

GSTM-PER658–1218 (S661G; pGEX6P-per661G) and GST-PER658–1218 (S975G; pGEX6P-per975G) constructs were generated by mutagenesis PCR using pGEX6P-per [58–1218] as template. The primers 5’-CTCGTGGACGGGACCATGGGGCCCATGGG GCCCCTG-3’ and 5’-CAGTGGGGCAGTGGGGCCCATGGG CTCGGTCCAGGAGG-3’ were used to generate GST-pGE X6P-per661G; and the primers 5’-CTCTAGCAGCCACCCGGGC- CCACCCGGTTCACCGAG-3’ and 5’-GGAGAGCCGGTGCCGC CCGTGTTGGGACTAAAG-3’ were used for pGEX6P-per975G generation. To generate the double mutant GSTPER58–1218 (S661/S975G) we performed a second mutagenesis PCR using pGEX6P-per661G as template and the pGEX6P-per975G mutagenesis primers as described above.

Behavioral analysis

Locomotor activity of individual flies was recorded using the Drosophila Activity Monitoring (DAM) System (Trikinetics) as previously described [67]. Briefly, to investigate locomotor behavior 3–7 day old male flies were monitored in LD 12:12 for 7 days (with a light intensity of 100 lux in the light phase) followed by additional 14 days in constant darkness (DD). In case of p38bKD; p38aKD flies were entrained in LD 12:12 during pupal stage and monitored directly after eclosion in DD conditions. All recordings took place under constant 20°C in a climate–controlled chamber. Raw data of individual light beam crossings were collected in 1-minute bins and displayed as double-plotted actograms using Actogram [68], a freely available [Java plug-in of ImageJ (freely available at http://rsb.info.nih.gov/ij/)]. We generally excluded data of the first experimental day from analysis to exclude side effects of fly handling. For generating average daily activity profiles for single genotypes, first raw data of day 2–7 in LD were averaged for each single fly. Theretofore, single activity profiles were averaged across all entrained flies of each genotype and smoothed by applying a moving average of 11. For determining the individual free-running period (τ) of rhythmic flies, DD data from day 2–12 were analyzed using 2-periodogram analysis and average period length of each genotype was calculated. To analyze timing of evening activity, raw LD data were converted into 15 minutes bins and evening activity onset was determined after generation of average days for each single fly. Finally, data were averaged across the genotype and tested for statistically significance.

Immunohistochemistry

To investigate p38 expression and oscillations in nuclear PER in adult Drosophila brain, 5–10 days old male flies were entrained to LD 12:12 for at least 4 days and collected at indicated Zeitgeber Times (ZT; ZT0 indicates lights-on and ZT12 lights-off). To analyze nuclear PER and TIM localization under free-running conditions, flies were first entrained to LD 12:12 for 4 days followed by 4 days in constant darkness and collected 96 hours after lights-on (ZT1) of the last day LD every 4 hours. Time points of collections were afterwards converted into Circadian Time (CT) according to the onset of activity in free-running flies that were monitored in parallel under the same conditions. Hereby, the activity onset of the flies on day 4 in DD is defined as CT0 and their activity offset as CT12. For light pulse (LP) experiments flies were reared in LD12:12 for 4 days, subsequently transferred to DD and collected at CT6 and CT18 on day 1 in DD right before as well as after 15 minute light-pulse. Flies were fixed in 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (pH 7.4) with 0.1% Triton X-100 for 2.5 hours. For fixation of flies expressing GFP, no Triton X-100 was added. After fixation PFA solution and fixation time was increased for additional 30 minutes. The fixation step was carried out on a shaker at room temperature and, if necessary, in absence of any light. After fixation flies were rinsed five times for 10 minutes in PB. After dissection 5% normal goat serum (NGS) in PB with 0.5% Triton X-
flies were collected according to ZTs and quickly decapitated on ice. Total RNA from 5 fly heads per genotype and ZT was extracted using the Quick RNA Micro Prep Kit (Zymo Research). cDNA derived from this RNA (using Quantifect Reverse Transcription Kit from Qiagen) was used as a template for quantitative real-time PCR (qPCR) in combination with the SensiFAST SYBR No-Rox Mix (Bioline) and one of the following primers: 5′-GGCCCCGTAGACAAATGGGAGGA-3′ and 5′-TAACCTTAGCAGCTGTTGGG-3′ for p38a, 5′-GAGATGTCATTTACGGCGGT-3′ and 5′-AGGATCAATTTAGGAGGAGGAGG-3′ for p38b and 5′-TCCTGGATTGCGTCCTAAC-3′ and 5′-GGAATCGCTTACGCTTGTTGC-3′ for z-tubulin.

**Western blot analysis**

5–10 days old flies were entrained to LD 12:12 for at least 4 days and collected every 2 hours. To analyze PER cycling, 25 heads of male flies per ZT were homogenized in protein extraction buffer (20 mM HEPES pH 7.5; 100 mM KCl; 5% glycerol; 10 mM EDTA; 0.1% Triton X-100; 20 mM β-glycerophosphate; 0.1 mM Na2VO4, pH 11) containing a protease inhibitor cocktail (Complete Mini EDTA-free; Roche) and loaded onto a 6% gel. SDS-polyacrylamide gel electrophoresis and transfer to nitrocellulose paper were performed according to standard immunoblotting protocols. To minimize differences due to variations in gel electrophoresis and protein blotting, samples of flies with altered p38 levels and respective controls were run and blotted to membrane simultaneously and repeated 4 times. For visualizing daily PER cycling, membranes were incubated in primary and secondary fluorescent antibodies which were diluted in tris-buffered saline with 0.1% Tween-20 (TBST) as follows: rabbit anti-PER 1:1000 (kindly provided by R. Stanewsky), Alexa Fluor goat-antirabbit 680 1:5000 (Invitrogen). Fluorescent signals were detected using the Odyssey Imaging System (Li-cor Bioscience).

**Protein expression, purification and in vitro kinase assays**

To express His6-p38b, the expression construct was introduced into BI.21(DE3)pLYSs competent E. coli cells (Promega) and protein expression was induced at an optical density of ~0.5 (OD600) with 0.3 mM isopropyl-B-D-thiogalactopyranoside (IPTG) for 3 hours at 37°C. After induction cells were washed, pelleted, and resuspended in phosphate-buffered saline (PBS) and pelleted once again. Washed lysate was then solubilized in lysis buffer (50 mM NaH2PO4; 300 mM NaCl; 10 mM imidazole; 1 mM PMSF; 10 μg/ml leupeptin; pH 8.0) containing protease inhibitor cocktail (complete Mini EDTA-free; Roche) and sonicated 5 x 5 seconds with short pauses on ice. After sonication Triton X-100 was added to a final concentration of 1% and lysate was centrifuged at 10000 g for 30 minutes at 4°C. 4% SDS-polyacrylamide gel electrophoresis and transfer to nitrocellulose paper were performed according to standard immunoblotting protocols. To minimize differences due to variations in gel electrophoresis and protein blotting, samples of flies with altered p38 levels and respective controls were run and blotted to membrane simultaneously and repeated 4 times. For visualizing daily PER cycling, membranes were incubated in primary and secondary fluorescent antibodies which were diluted in tris-buffered saline with 0.1% Tween-20 (TBST) as follows: rabbit anti-PER 1:10000 (kindly provided by R. Stanewsky), Alexa Fluor goat-antirabbit 680 1:5000 (Invitrogen). Fluorescent signals were detected using the Odyssey Imaging System (Li-cor Bioscience).
EDTA; 1% Triton X-100; pH 7.5) supplemented with protease inhibitors (Roche Complete Cocktail and 1 mM PMSE). Resuspended cells were then lysed by sonication and the lysate was cleared by 30 minutes centrifugation at 15000 g and 4°C. After centrifugation, lysates were incubated at 4°C overnight on a rotary wheel with 1.5 mM glutathione sepharose 4B beads (GE Life Sciences) to bind the fusion proteins. The beads were then transferred to a 10 ml Polyprop column (Biorad) and washed once with lysis buffer and thrice with wash buffer (50 mM Tris; 100 mM EDTA; pH 7.5) for 30 minutes or 60 minutes after kinase addition by adding equal amounts of GST-PER fusion protein or GST as a control were incubated at 30°C indicated ATP-PER fusion protein or GST as a control were cut before Coomassie treatment and immunoblotted as described in the western blot section.

Figure S2 Expression of active p38 in DN1as at ZT21 in Canton S wildtype, p38b+/- and p38a+/- flies. Both p38 null mutants displayed a significant reduction of p-p38 to 50% of wildtype level (p<0.05). Colored bars represent p-p38 levels of the genotypes normalized to the wildtype level. Error bars show SEM. Significant differences (p<0.05) are indicated by *. (TIF)

Figure S3 Number of p-p38 positive DN1as per wildtype brain hemisphere in course of a day. Daily variations in p38 activity in DN1as is not solely attributed to decreased or increased total p-p38 levels, it’s additionally the oscillating number of p-p38 stained DN1as per hemisphere that contributes. Even if in some cases not all p-p38+ of a brain hemisphere showed p-p38 staining during the night (ZT13-21), the average number of p-p38 positive DN1as was significantly higher than during the day. Colored bars represent average p-p38 positive DN1a per hemisphere. Error bars show SEM. Significant differences (p<0.05) are indicated by *. (TIF)

Figure S4 p38a and p38b mRNA expression in w;UAS-p38bRNAi;da-Gal4 (A) and w;UAS-p38bRNAi;+;da-Gal4/+ (B) compared to respective controls. Expression data of three biological replicates were averaged within the genotype and normalized to wildtype level: A: Quantitative real-time PCR revealed a high significant reduction in p38a mRNA in w;+;UAS-p38bRNAi;da-Gal4, confirming the effectiveness of the p38bRNAi transgene (p<0.001). B: Furthermore, significant reduction of p38b mRNA to 50% of wildtype level in w;UAS-p38bRNAi;+;da-Gal4/+ additionally proved the effectiveness of the p38bRNAi transgene (p<0.05). Error bars show SEM. Significant differences (p<0.05) are indicated by *, highly significant differences (p<0.001) by **. (TIF)

Figure S5 Locomotor activity rhythms of flies expressing a UAS-p38b kinase-dead transgene (UAS-p38bKD) in Drosophila clock neurons and respective controls. In LD, both experimental lines, w;UAS-p38bKD;tim(UAS)-Gal4;UAS-p38bRNAi/++;upper panels in A) and w;UAS-p38bKD;Pdf-Gal4;UAS-p38bRNAi/++;upper panels in B), showed a diurnal activity pattern with activity bouts around night (ZT13-21), confirming the effectiveness of the p38bRNAi transgene (p<0.001). A: Quantitative real-time PCR revealed a high significant reduction in p38a mRNA in w;+;UAS-p38bRNAi;da-Gal4, confirming the effectiveness of the p38bRNAi transgene (p<0.001). B: Furthermore, significant reduction of p38b mRNA to 50% of wildtype level in w;UAS-p38bRNAi;+;da-Gal4/+ additionally proved the effectiveness of the p38bRNAi transgene (p<0.05). Error bars show SEM. Significant differences (p<0.05) are indicated by *, highly significant differences (p<0.001) by **. (TIF)

Figure S6 PER and TIM clock protein cycling in p38b knockdown flies in DD. Nuclear PER (red) and TIM (blue) staining intensity was evaluated on the 4th day in DD in the s-LN{s} after down-regulation of p38b with Pdf-gal4 (p38b RNAi=dicer2;UAS-p38bRNAi/Pdf-gal4;+ flies). UAS-p38bRNAi/+ flies served as control. Interestingly, immunostainings revealed that the molecular cycling still persists in dicer2;UAS-p38bRNAi/Pdf-gal4;+ flies. However, the phase of the clock protein oscillation...
was delayed, which is in line with the long free-running period of these flies. Grey bars on top of the graphs indicate the subjective day of the flies, that starts with their activity (act.) onset (≈Circadian Time (CT) 0). Black bars indicate the subjective night of the flies that begins with the activity offset (≈ CT 12). For better clarity, 12 hours before and after the measured day are repeated to the left and the right (dotted curves). Red and blue arrows point to peaks in nuclear PER and TIM, respectively.

**Figure S7** Locomotor activity rhythms of flies overexpressing wildtype p38b (p38b+) and respective controls. Flies overexpressing p38b either in TIM-positive (dicer2;tim(UAS)-Gal4/+;UAS-p38bRNAi+/+) or PDF-positive clock neurons (dicer2;Pdf-Gal4/+;UAS-p38aRNAi+/+) A) or PDF-positive clock neurons (dicer2;Pdf-Gal4/+;UAS-p38bRNAi+/+) and respective controls. Flies overexpressing p38aRNAi/ either in TIM-positive (A) or PDF-positive clock neurons (B) showed wildtype-like locomotor behavior in LD with activity bouts around lights-on and lights-off. However, evening activity onset of both lines was significantly delayed compared to controls (upper panels in A and B) and resulted in a prolonged free-running period after transfer to constant darkness. For recording and processing of activity data as well as for figure labeling see Figure 3.

**Figure S8** p38a (A) and p38b (B) mRNA expression in Canton S wildtype, p38aRNAi and p38aΔ1 heads. Expression data of three biological replicates per genotype were averaged within the genotype and normalized to wildtype level. Quantitative real-time PCR clearly confirmed our p38a null (A) and p38b null (B) phenotypes (p<0.05 and p<0.001 respectively). In addition there was no compensatory effect on the transcription of one p38 isoform, when the other was missing. Error bars show SEM. Significant differences (p<0.05) are indicated by *, highly significant differences (p<0.001) by **.

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**Figure S9** Drosophila PER contains two p38 consensus phosphorylation sites. Online research (http://www.kinexus.ca/pdf/graphics Charts/ProteinSerKinaseSpecificity.pdf) and amino acid sequence comparison revealed that Drosophila PER contains two predicted p38 consensus phosphorylation sites (PXSP4): Ser661 and Ser975. The latter has not been described as phosphorylation site so far. In contrast, there is evidence that a proline-directed kinases, a family also p38 belongs to, phosphorylates PER at Ser661 and thereby primes it for further phosphorylation at Ser657 by SGG. Black characters represent Drosophila PER amino acid sequence, red characters represent predicted p38 MAPK consensus phosphorylation sites and stars indicate previous identified PER phosphorylation sites.

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**Author Contributions**

Conceived and designed the experiments: VD CW TY CHF. Performed the experiments: VD PRS BM HH. Analyzed the data: VD PRS. Contributed reagents/materials/analysis tools: TY BM TR. Wrote the paper: VD CHF.
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