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N-β-alanyldopamine metabolism, locomotor activity and sleep in *Drosophila melanogaster ebony* and *tan* mutants

FABIANA A. ROSSI, PABLO A. BOCHICCHIO, LUIS A. QUESADA-ALLUÉ and MARTÍN M. PÉREZ

Department of Biological Chemistry, FCEyN, University of Buenos Aires, Fundación Instituto Leloir and IIBBA-CONICET, Buenos Aires, Argentina

Abstract. *Drosophila melanogaster* Meigen mutants for N-β-alanyldopamine (NBAD) metabolism have altered levels of NBAD, dopamine and other neurotransmitters. The *ebony*^{1} mutant strain has very low levels of NBAD and higher levels of dopamine, whereas the opposite situation is observed in the *tan*^{1} mutant. Dopamine is implicated in the control of movement, memory and arousal, as well as in the regulation of sleep and wakefulness in *D. melanogaster*. *N*-β-alanyldopamine, which is best known as a cuticle cross-linking agent, is also present in nervous tissue and has been proposed to promote locomotor activity in this fly. The daily locomotor activity and the sleep patterns of *ebony*^{1} and *tan*^{1} mutants are analyzed, and are compared with wild-type flies. The *tan*^{1} mutant shows reduced locomotor activity, whereas *ebony*^{1} shows higher levels of activity than wild-type flies, suggesting that NBAD does not promote locomotor activity. Both mutants spend less time asleep than wild-type flies during night-time; *ebony* shows more consolidated activity during night-time and increased sleep latency, whereas *tan* is unable to consolidate locomotor activity and sleep in either phase of the day. The daily level of NBAD-synthase activity is measured *in vitro* using wild-type and *tan*^{1} protein extracts, and the lowest NBAD synthesis is observed at the time of higher locomotor activity. The abnormalities in several parameters of the waking/sleep cycle indicate some dysfunction in the processes that regulates these behaviours in both mutants.

Key words. Dopamine, *ebony*, locomotor activity, *N*-β-alanyldopamine, sleep, *tan*, wild-type.

Introduction

The fruit fly *Drosophila melanogaster* Meigen, which is an important model species for studying the molecular and genetic bases of complex behaviour patterns, is a significant resource in understanding the mechanism of sleep and locomotor activity. Circadian rhythms and sleep/wake patterns in this fly show many of the same properties as in mammals, and these patterns are homeostatically regulated (Hendricks *et al*., 2000; Shaw *et al*., 2000; Ho & Sehgal, 2005). Many of the genes identified in mammals and other animals as being important for the regulation of sleep/locomotor activity have close orthologues in *D. melanogaster* (Sandrelli *et al*., 2008; Rosbash, 2009). The sleep/wake phenotype can be monitored through assessment of locomotor and circadian activity.

Dopamine (DA) and genes involved in dopaminergic signalling play central regulatory roles controlling locomotor activity, sleep and arousal (Andretic *et al*., 2005, 2008; Kume *et al*., 2005; Seugnet *et al*., 2008, 2009; Wu *et al*., 2008; Riemensperger *et al*., 2011). Indeed, there appears to be a simple relationship between dopamine levels, locomotor activity and sleep: flies with high DA levels are hyperactive and sleep less than wild-type flies, whereas flies with low DA content behave in the opposite way (Andretic *et al*., 2005; Seugnet *et al*., 2008, 2009; Van Swinderen & Andretic, 2011).

Correspondence: Martín M. Pérez, Fundación Instituto Leloir, Patricias Argentinas 435, Buenos Aires 1405, Argentina. Tel.: +54 11 5238 7500; e-mail: mperez@leloir.org.ar

The *ebony* and *tan* genes in *D. melanogaster* encode for the synthesis of *N*-β-alanyldopamine synthase (NBAD-synthase or Ebony protein) and *N*-β-alanyldopamine hydrolase (NBAD-hydrolase or Tan protein), respectively. These genes and their expressed proteins constitute a scarcely explored system regulating the levels of the neurotransmitters DA and histamine, and possibly also serotonin (5-HT) and octopamine. The enzyme NBAD-synthase, which is found in glial cells, serves to conjugate DA and histamine with β-alanine, thus terminating the action of these neurotransmitters. By contrast, NBAD-hydrolase, present in neurones, hydrolyzes the β-alanyl conjugates liberating DA, histamine and β-alanine (Borycz *et al*., 2002; Pérez *et al.*, 2010). The *ebony* mutant is defective for the synthesis of N-β-alanyl derivatives (Wright, 1987; Pérez *et al*., 1997, 2002, 2004), the best known being *N*-β-alanyldopamine (NBAD) and N-β-alanylhistamine (carcinine). The *tan* mutants are unable to hydrolyze *N*-β-alanyl derivatives (Wright, 1987; True *et al*., 2005; Pérez *et al*., 2011). Both mutants have altered levels of neurotransmitters; the NBAD concentration is drastically reduced in homozygous *ebony* flies compared with wild-type, whereas DA levels are elevated approximately two-fold (Hodgetts & Konopka, 1973; Walter *et al*., 1996; Gruntenko *et al*., 2004). On the other hand, DA occurs in reduced amounts in *tan* relative to wild-type flies, whereas NBAD levels are increased approximately three-fold in the homozygous *tan* mutant (Konopka, 1972; Walter *et al*., 1996). As a consequence of the differences in neurotransmitters levels, these two mutants show neurological and behavioural defects. A large fraction of homozygote *ebony* flies are arrhythmic with respect to locomotor activity (Hotta & Benzer, 1969; Newby & Jackson, 1991) and their electoretinograms are abnormal, lacking on and off transients (Hotta & Benzer, 1969; Heisenberg, 1972). Flies lacking Tan function also exhibit abnormalities in vision (Benzer, 1967; Inoue *et al*., 1988; True *et al*., 2005) and $tan^{-/-}$ males display abnormal courtship behaviour (Cook, 1980; Tompkins *et al*., 1982).

Microarray-based studies have identified *D. melanogaster* transcripts that show rhythmic daily changes in abundance, with one of these being the *ebony* gene (Claridge-Chang *et al*., 2001). Moreover, *ebony* RNA exhibits diurnal (in Light/Dark) and circadian (in Dark/Dark) oscillations in levels in adult head tissues, with peak abundance occurring at the beginning of the subjective day (Suh & Jackson, 2007). Moreover, Suh & Jackson (2007) postulate that the production of NBAD serves as a bioactive compound to drive locomotor activity during daytime. Accordingly, $ebony^1$ mutants exhibit a selective daytime deficit in locomotor activity that appears to be consistent with this hypothesis (Suh & Jackson, 2007). These observations of reduced locomotor activity in $ebony^1$ mutants compared with wild-type flies during the daytime appear to disagree with previous reports on *ebony* activity (Kyriacou *et al*., 1978) and other studies regarding DA levels and locomotor activity (Andretic *et al*., 2005; Seugnet *et al*., 2008, 2009; Van Swinderen & Andretic, 2011), thus pointing to the necessity for clarifying this topic. Moreover, if NBAD promotes locomotion, then tan^1 flies, which have high NBAD levels, should be very active during daytime.

In the present study, locomotor activity and sleep phenotypes of *ebony* and *tan* mutants of *D. melanogaster* are reported. Monitoring of these behaviours provides a sensitive method for detecting neuronal dysfunctions. The aim is to determine whether abnormalities in the metabolism of β-alanyl derivatives affect the physiology of the nervous system. The correlation between N-β-alanyl derivatives, metabolism and locomotor activity is assessed, and the daily activity profile of brain NBAD-synthase are reported.

Materials and methods

Fly stocks

Drosophila melanogaster wild-type Canton S and mutant strains $ebony^1$ (e^1) and tan^1 (t^1) were obtained from the Bloomington Stock Center (Indiana University, Bloomington, Indiana). Flies were kept under an LD 12 : 12 h photocycle at 25 °C and 50% relative humidity and on standard sucrose, corn meal and yeast agar medium. Both mutants were isogenized to Canton S background, backcrossing them five times. Because both mutants are recessive, each mutant was first outcrossed to wild-type flies to obtain the F1, which is heterozygous and all individuals are phenotypically similar (wild-type phenotype). Next, the heterozygous flies were crossed to obtain the F2, from which after observing them under binocular microscope the mutant flies were selected (i.e. black flies in the case of $ebony^1$). The total number of crossings was 10, five backcrosses between Canton S and mutant flies, and five outcrosses between the heterozygous flies, to obtain the recessive phenotypes.

Monitoring locomotor activity and sleep

Six-day-old male flies were placed individually into 65-mm glass tubes containing the same standard food media as described above, to allow continuous recording of locomotor activity and sleep using the *Drosophila* Activity Monitoring (DAM) system (TriKinetics, Waltham, Massachusetts). Data were collected every 5 min over 5 days. The first day of recording was not considered. Sleep was considered as a 5-min period of inactivity, as described previously (Shaw *et al.*, 2000; Andretic & Shaw, 2005). Latency to sleep was defined as the time in minutes from the moment lights were turned off to the first sleep episode. The number of flies per group was: 138 wild-type, 128 $ebony^1$ and 139 tan^1.

Chemicals

All of the available biogenic amines and/or standards were from Sigma-Aldrich Chemical Co. (St Louis, Missouri). [^{14}C]β-Alanine (2.035 GBq mmol^{-1}) was from American Radiolabeled Chemicals Inc. (St Louis, Missouri). *N*-β-alanyldopamine (NBAD) and other standards were generously synthesized by Dr J. M. Aguirre (Luján University, Argentina), as described by Yamasaki *et al*. (1990), with slight modifications.

NBAD-synthase assay and product characterization

Adult male *D. melanogaster* were collected between 0 and 4 h post eclosion from *Zeitgeber* time (ZT) ZT-0 to ZT-3 and maintained over 6 days. Heads were dissected and homogenized in 50 mM sodium borate buffer, pH 8.2, saturated with phenylthiourea and containing 10 mM $MgCl_2$, 40 mM 2-mercaptoethanol, 2 mM dithiothreitol, 10% glycerol and a mixture of protease inhibitors (E-64, pepstatin and phenylmethanesulphonyl fluoride). Cell-free NBAD-synthase activity in head homogenates was measured every 2 h, from ZT-0 to ZT-23, as described previously (Pérez *et al*., 2002), by adding 3 μg of protein extract in each reaction. All of the experiments were performed using at least three different homogenates, and each assay within the experiment was made, as a minimum, in duplicate. Catecholamine derivatives were isolated using alumina columns and analyzed by reversed-phase high-performance liquid chromatography (Econosphere-C-18; Alltech, Deerfield, Illinois) as described previously (Pérez *et al*., 2002). Radioactivity in the samples was measured using a Rackbeta 1214 liquid scintillation counter (Pharmacia LKB Biotechnology AB, Sweden) with Optiphase 'Hisafe' 3 (Perkin Elmer, Waltham, Massachusetts) as scintillant. Protein concentration was determined using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, California) and bovine serum albumin as a standard.

Statistical analysis

Results are expressed as the mean ± SEM. Two-way analysis of variance (ANOVA) and repeated measures ANOVA models were used to test null hypotheses about the effects of the between-subjects factors (variables that divide the population into groups) and the within-subjects factors (variables measured several times on each experimental unit); these models were also used to investigate interactions between factors, as well as the effects of individual factors. Levene's test was used to evaluate the hypothesis of homogeneity of variance and Mauchly's test (Mauchly, 1940) was used to evaluate the assumption of sphericity. Bonferroni multiple comparison procedures were conducted to analyze all possible main effects and pairwise comparison. Profile plots (interaction plots) were performed to compare marginal means in the models. The Pearson product-moment correlation coefficient was used to measure the degree of linear dependence between two numerical variables. The alpha level of significance used for all statistical inferential tests was set at $P < 0.05$. All analyses were performed using SPSS, version 17.0 (SPSS Inc., Chicago, Illinois).

Results

Daily locomotor activity of ebony and tan mutants

The daily locomotor activity of *ebony* and *tan* mutants was analyzed and compared with wild-type activity. As expected, the activity profiles showed the typical bimodal pattern for *D. melanogaster*, with two main peaks during the dark to light and during light to dark transitions (ZT-0 and ZT-12) (Fig. 1A). During the light to dark transition (evening peak), the activity of $ebony^1$ was significantly higher than wild-type ($P < 0.001$). During the morning peak, the locomotor activity of $ebony^1$ was not significantly different from wild-type ($P > 0.05$). During the entire dark period, $ebony^1$ showed higher activity than wild-type, ($P < 0.001$). No significant differences were observed during the light phase (Fig. 1B). However, by comparing at each hour, it was observed that, from ZT-7 to ZT-10, the activity of $ebony^1$ was lower, which is in agreement with the findings reported by Suh & Jackson (2007) ($P < 0.005$) (Fig. 1A). The tan^1 mutant showed lower locomotor activity than wild-type and $ebony^1$ during the light and dark phases (Fig. 1B). This mutant was less active than wild-type and $ebony^1$ during both transitions, particularly during light to dark transition ($P < 0.001$) (Fig. 1A).

Flies alternate several periods of activity and quiescence during the day. The bout length is considered to be a measurement of consolidated activity or sleep. Activity bouts in $ebony^1$ were longer than in the other strains during either phase of the day (daytime and night-time). However, the dark period is the moment of main locomotor activity in $ebony^1$ ($P < 0.001$); bouts are longer during this period than daytime, indicating that it is a fly with nocturnal behaviour (Fig. 1C). On the other hand, tan^1 showed the shortest bouts duration (Fig. 1C) with similar length during daytime and night-time ($P > 0.05$). As expected, wild-type flies showed their longer activity periods during daytime ($P < 0.01$) (Fig. 1C). These results indicate that, compared with wild-type, *ebony* have more consolidated periods of wakefulness during daytime and, specifically during night-time, whereas *tan* is unable to consolidate activity into long bouts, either during daytime or night-time.

Measurement of total locomotor activity yields an estimate of the net amount of activity within a given period, although it cannot distinguish whether a fly is poorly active but spends most of the time awake, or is very active but sleeps most of the time in a given hour. To address this question, the locomotor activity for each waking minute was analyzed; this measure allows the difference between hypoactive and hyperactive flies to be distinguished, irrespective of sleep time. During the whole dark phase, $ebony^1$ showed higher activity per waking minute than wild-type ($P < 0.05$), whereas the opposite was observed during the light phase ($P < 0.001$) (Fig. 2, inset). Unexpectedly, the comparison at each hour revealed similar activity (count number/waking minute) in $ebony^1$ and wild-type during the dark phase, except at ZT-23 when $ebony^1$ locomotor activity was lower than wild-type and ZT-13 when it was higher ($P < 0.001$) (Fig. 2). During the light phase, between ZT-8 and ZT-11, the number of counts/waking minute was lower in $ebony^1$ compared with wild-type ($P < 0.05$). However, tan^1 showed significantly lower locomotor activity per waking minute than the other two strains [$P < 0.001$; except $ebony^1$ versus tan^1 ZT-6, 7 and 11 ($P < 0.05$) and ZT-8 and 10 (not significant)] (Fig. 2).

These results showed that $ebony^1$ is more active during the subjective night (dark period); total locomotor activity was almost 50% higher during night-time than during daytime, 1160 ± 58 min versus 805 ± 36 min, respectively ($P < 0.001$) (Fig. 1B). The activity per waking minute was also higher

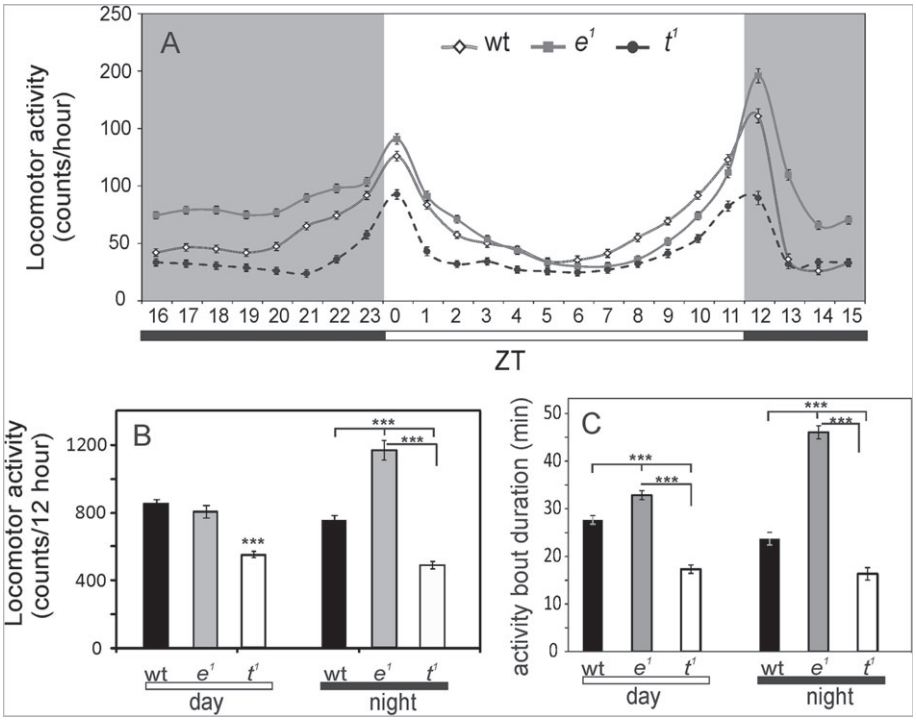

Fig. 1. (A) Profile plot of total locomotor activity (number of counts detected per hour) during the whole day in wild-type (wt), *ebony*^{1} (e^1) and *tan*^{1} (t^1) males of *Drosophila melanogaster*. (B) Total locomotor activity during daytime and night-time in wt, e^1 and t^1 males. During daytime, *tan*^{1} showed lower locomotor activity than wt and *ebony*^{1} (wt versus e^1; not significant). During night-time, *ebony*^{1} showed the highest locomotor activity, whereas *tan*^{1} showed the lowest activity. (C) Bar plot of activity bout duration in male flies during daytime or night-time. The *ebony*^{1} mutant showed the longest bouts duration, whereas *tan*^{1} showed the shortest, during both daytime and night-time (***$P < 0.001$, two-way analysis of variance). The bottom white and black bar indicates daytime (white segment) and night (black segment). ZT, *Zeitgeber* time.

during night-time, at 2.33 ± 0.028 counts per minute, whereas, during the daytime, it was 1.78 ± 0.018 counts per minute (Fig. 2, inset). On the other hand, *tan*^{1} showed similar levels of locomotor activity during daytime and night-time. Strikingly, the evening peak, which is the highest peak in wild-type and *ebony*^{1}, is reduced in *tan*^{1} and is similar to the morning peak. The activity of this mutant is evenly distributed during the whole day (Fig. 1B, C).

Daily sleep patterns in ebony and tan mutants

Because the analysis of locomotor activity showed clear differences between the three strains, it was possible that sleep patterns were also altered. To assess this question, the daily distribution of sleep was determined. Both mutants showed the maximum period of daytime sleep from ZT-5 to ZT-8 (Fig. 3). Indeed, this period represented the main period of sleep time during the entire day for *ebony*^{1} and *tan*^{1} (Fig. 3); however, the *tan*^{1} mutants slept for less time than wild-type and *ebony*^{1} from ZT-5 to ZT-7 ($P < 0.001$). During the dark period, which is the time when wild-type flies spent more time sleeping ($P < 0.001$), the *ebony*^{1} and *tan*^{1} mutants exhibited different behaviours. Night-time sleep was reduced in both mutants compared with wild-type ($P < 0.001$). From ZT-19 to ZT-23, *ebony*^{1} slept less time than the other two strains ($P < 0.001$; *ebony*^{1} versus *tan*^{1} at ZT-19, $P < 0.05$). From ZT-21 to ZT-23 there was no difference in wild-type and *tan*^{1} sleeping behaviour (Fig. 3). Over the whole day, *ebony*^{1} flies slept less time than wild-type and *tan*^{1} ($P < 0.001$ and $P < 0.01$, respectively) and *tan*^{1} slept less time than wild-type flies ($P < 0.001$). Daily total sleep was 603.8 ± 20.2 min for *ebony*^{1}, 673.15 ± 19 min for *tan*^{1} and 789 ± 19.45 min for wild-type (Fig. 4A). Moreover, *ebony*^{1} spent much less time sleeping during night-time than daytime ($P < 0.001$), which agrees with the longer bouts of locomotor activity observed in this mutant during the dark period. Indeed, the reduced total sleep of *ebony*^{1} is a result of the reduction in night-time sleep. On the other hand, *tan*^{1} slept the same during both daytime and night-time. The day versus night sleep ratio was 0.82 in wild-type, 1.8 in *ebony*^{1} and 1.14 in *tan*^{1} (Fig. 4B).

Sleep bouts and sleep latency

In addition to daily sleeping patterns, other sleep parameters were evaluated, such as sleep duration, number of sleep bouts, longest sleep bout duration and sleep latency. Each of these parameters provided an insight into the sleep regulatory processes. The analysis of sleep bouts showed that the mean sleep bout duration of wild-type and *ebony*^{1} did not differ during daytime ($P > 0.05$), whereas *tan*^{1} showed the shortest bouts of sleep ($P < 0.001$). During night-time, wild-type flies increased

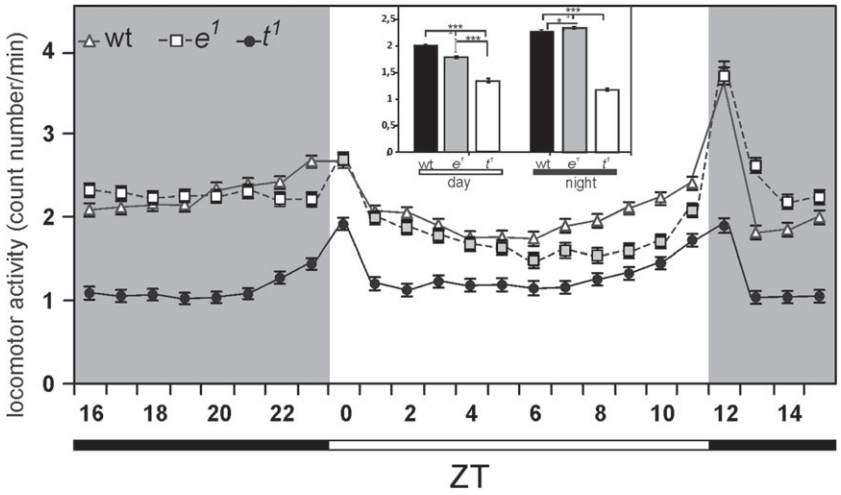

Fig. 2. Profile plot of activity per waking minute (number of counts detected per minute) during the whole day in wild-type (wt), *ebony*^{1} (*e*^{1}) and *tan*^{1} (*t*^{1}) males of *Drosophila melanogaster*. Inset: mean activity per waking minute during daytime and night-time (***$P < 0.001$; *$P < 0.05$, two-way analysis of variance). The bottom white and black bar indicates daytime (white segment) and night-time (black segment). ZT, *Zeitgeber* time.

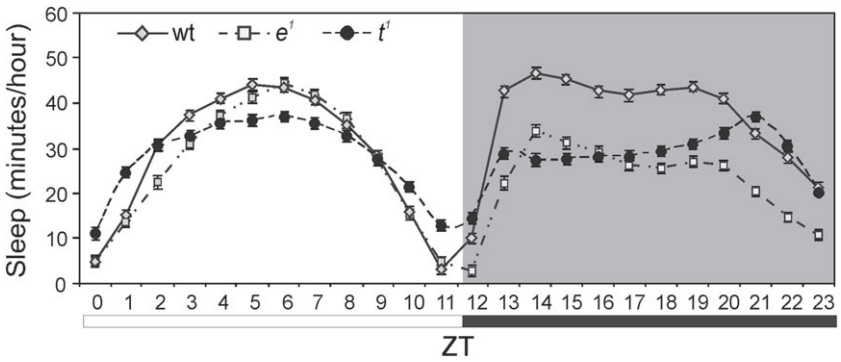

Fig. 3. Daily distribution of sleep in wild-type (wt) and *ebony*^{1} (*e*^{1}) and *tan*^{1} (*t*^{1}) males of *Drosophila melanogaster*. The bottom white and black bar indicates daytime (white segment) and night-time (black segment). ZT, *Zeitgeber* time.

the time they slept, showing longer sleep bouts than *ebony*^{1} and *tan*^{1} ($P < 0.001$). Comparing daytime and night-time sleeping patterns in each strain, wild-type slept more time during the night ($P < 0.001$); *ebony*^{1} slept for less time during this period ($P < 0.001$), thus showing sleep bouts of shorter duration than during daytime. The *tan*^{1} mutant showed the same sleeping time during night-time and daytime (not significant) (Fig. 4C), with bouts of shorter duration than the other strains. The mean sleep bout duration was approximately 17 min in *tan*^{1} during either phase, whereas, in wild-type, it was approximately 29 min during daytime and 43 min during night-time; for *ebony*^{1}, the sleep bouts were 32 min during daytime and 25 min during the night (Fig. 4C). Regarding the number of sleep bouts, these were similar for wild-type and *ebony*^{1} during daytime and night-time but very different in *tan*^{1}, in which the number of sleep episodes increased almost two-fold during both dark and light periods (Fig. 4D). The longest sleep bout of the day in wild-type occurred at night ($P < 0.001$); this was almost two-fold longer than the longest sleep bout in *ebony*^{1} and *tan*^{1} (Fig. 4E). The longest sleep bout in *ebony*^{1} occurred during the light period ($P < 0.001$). There were no differences in the longest sleep bout between daytime or night-time in *tan*^{1} (not significant) (Fig. 4E). The sleep parameters in *ebony*^{1} and *tan*^{1} are in concordance with the locomotor activity data reported above. The *ebony*^{1} mutant strain showed more consolidated activity during night-time and less consolidated sleep during this phase. On the other hand, *tan*^{1} is not only unable to consolidate activity into long bouts, but also is unable to consolidate sleep, either during daytime or night-time. The two-fold increment in bouts of sleep, together with their short duration, indicates that *tan*^{1} cannot get restorative sleep and tries to overcome this by initiating more sleep episodes.

The last parameter to be assessed was sleep latency. The wild-type and *tan*^{1} flies began to sleep during the first hour of darkness showing the same sleep latency, whereas *ebony*^{1} flies started to sleep more than 2 h after the light turned off, showing long sleep latency ($P < 0.001$) (Fig. 4F). This indicates that either wild-type and *tan*^{1} and ready to start sleeping when the lights off, whereas *ebony*^{1} showed deficits in the mechanism underlying sleep initiation.

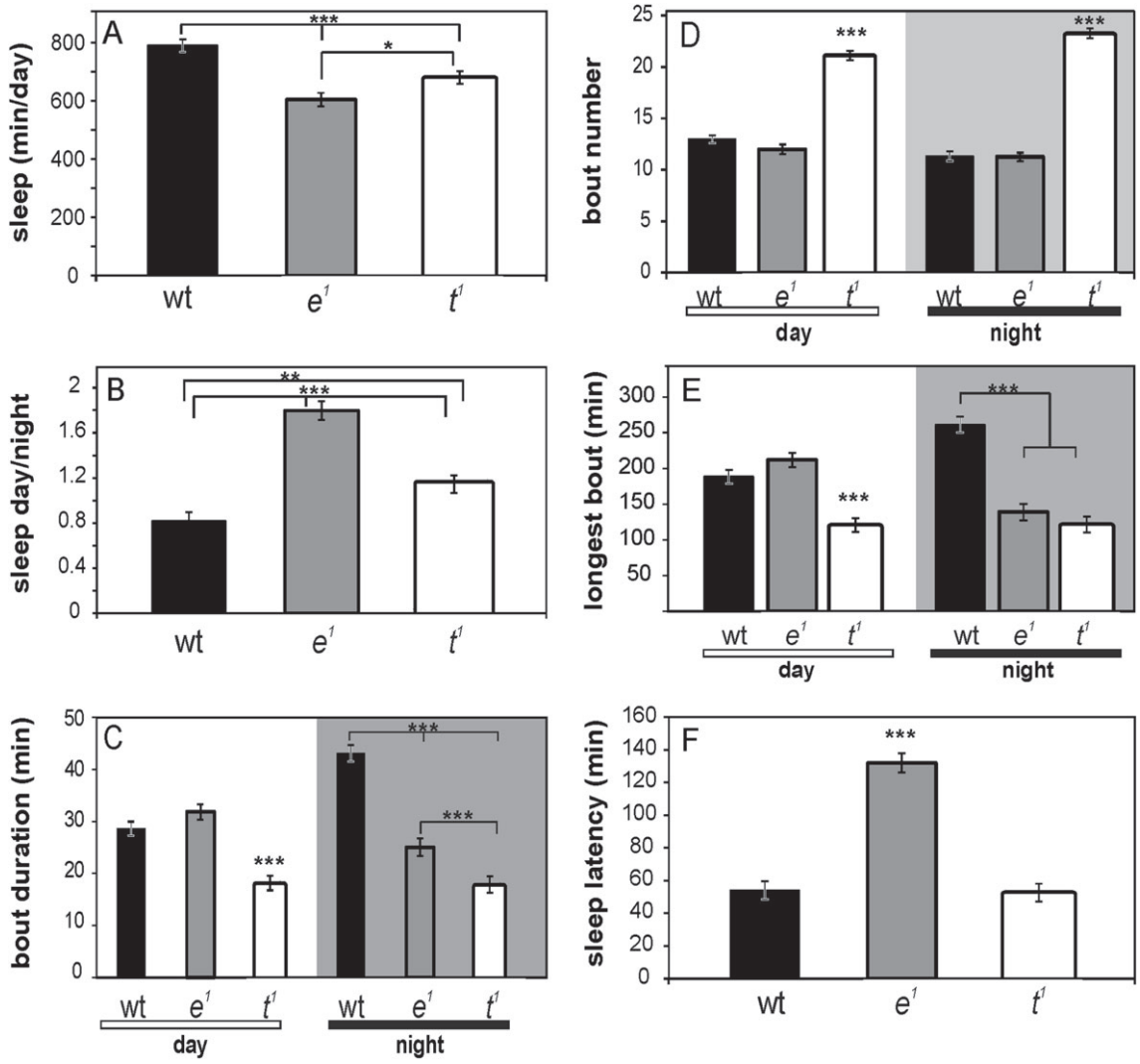

Fig. 4. Sleep parameters in wild-type (wt), *ebony*^{1} (e^1) and *tan*^{1} (t^1) males of *Drosophila melanogaster*. (A) Total sleep time during 24 h expressed in minutes. Both mutants sleep less than wt; *ebony*^{1} sleep less than *tan*^{1} [$^{***}P < 0.001$; $^{*}P < 0.05$; two-way analysis of variance (ANOVA)]. (B) Relationship between daytime and night-time sleep in the three strains. Both mutant strains sleep more time during daytime than during night-time compared to wt ($^{***}P < 0.001$;$^{**}P < 0.01$; two-way ANOVA). (C) Sleep bout duration is similar in wt and *ebony*^{1} and shorter in *tan*^{1} during daytime; during night-time sleep bout duration is reduced in *ebony*^{1} and *tan*^{1} versus wt flies, in *tan*^{1} sleep bouts are shorter than in *ebony*^{1} ($^{***}P < 0.001$; $^{**}P < 0.01$; repeated measures ANOVA). (D) Number of sleep bouts during daytime and night-time are increased in *tan*^{1} ($^{***}P < 0.001$; repeated measures ANOVA). (E) Longest sleep bouts are similar in wt and *ebony*^{1} and shorter in *tan*^{1} during daytime. During night-time *ebony*^{1} and *tan*^{1} longest bouts are shorter than wt ($^{***}P < 0.001$; repeated measures ANOVA). (F) Sleep latency is increased in *ebony*^{1} ($^{***}P < 0.001$; two-way ANOVA).

NBAD-synthase activity profile during the whole day

NBAD-synthase activity was measured in wild-type and *tan*^{1} heads every 2 h during the whole day (*ebony* activity is null and so it cannot be measured). The wild-type enzymatic activity showed a maximum at night, at ZT-16 ($P < 0.05$, except with ZT-2 and ZT-18, not significant), after the evening peak of locomotor activity. The other peak of NBAD-synthase activity was observed at ZT-2, after the morning peak of locomotor activity. The minimum enzymatic activity was observed at ZT-12, which corresponds to the evening peak of locomotor activity. These maxima were observed in the subjective night when the flies are quiescent and during the mid-morning when the locomotor activity is decreasing (Fig. 5A). On the other hand, the pattern of NBAD-synthase activity in *tan*^{1} showed some differences from that of wild-type. Instead of two peaks, one at ZT-2 and other at ZT-16, as in wild-type, *tan*^{1} NBAD-synthase activity showed an increased at midday (from ZT-4 to ZT-8) and another during the night (from ZT-14 to ZT-22). As with the pattern of NBAD-synthase activity in wild-type flies, the increases in enzymatic activity in *tan*^{1} were observed after the morning and evening peaks of locomotor activity. However, despite the fact that ZT-20 appears to be the higher activity peak, there were no significant differences between the different ZT

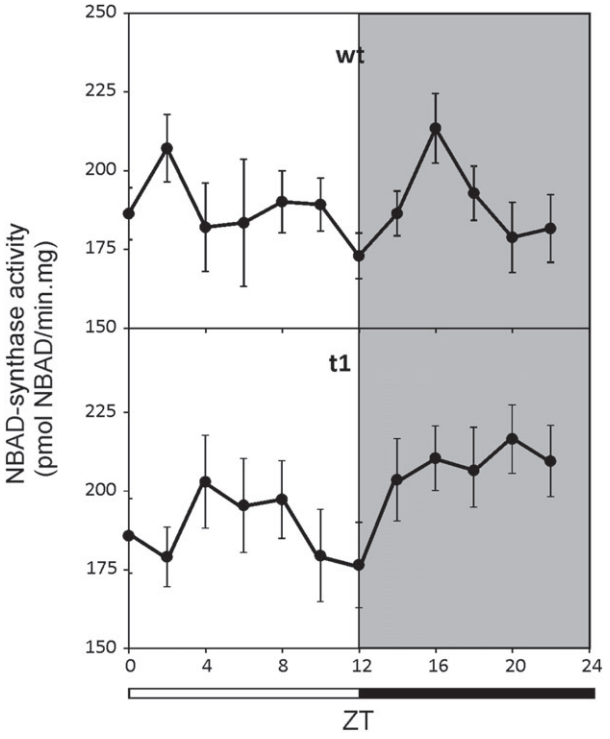

Fig. 5. Daily *N*-β-alanyldopamine-synthase activity profile in heads of (A) wild-type (wt) and (B) tan^1 (t^1) mutant *Drosophila melanogaster*. The bottom white and black bar indicates daytime (white segment) and night-time (black segment). ZT, *Zeitgeber* time.

analyzed during night (ZT-14 to ZT-22) or during midday (ZT-4 to ZT-8); thus, *tan1* enzymatic activity during these two periods of the day should be considered as steady (Fig. 5B).

Discussion

Dopamine and NBAD metabolism appears to be very important in the regulation of locomotor activity in flies (Suh & Jackson, 2007; Van Swinderen & Andretic, 2011). The $ebony^1$ and tan^1 mutants, which lack NBAD-synthase and NBAD-hydrolase activity, respectively, have altered levels of biogenic amines that make them excellent models for determining the role of NBAD (and other β-alanyl derivatives) in the locomotor and sleep physiology.

The mutant strains analyzed in the present study show the two daily peaks of locomotor activity reported for wild-type (Helfrich-Förster, 2000). However, there are differences in their activity profiles (Fig. 1). Total locomotor activity profile shows that $ebony^1$ is much more active than wild-type during the night. Total locomotor activity yields an estimate of the net amount of activity within a given hour, without taking into account the minutes that the flies are resting or quiescent. This measure cannot distinguish between a poorly active but awake fly and a very active fly that sleeps for a large portion of a given hour. By contrast, the measurement of counts per waking minute distinguishes between hypoactive and hyperactive flies irrespective of sleep time. Moreover, the latter measure provides a better indicator of the health of a fly because a reduction in the intensity of activity is suggestive of impairment (Andretic & Shaw, 2005). Thus, in addition to the total amount of locomotor activity, the intensity of activity is also evaluated (which is the mean total number of counts per waking minute; Fig. 2). This parameter also shows that $ebony^1$ is more active than wild-type during the dark phase. However, the difference from wild-type activity is smaller when total activity is analyzed. The comparison of the activity per waking minute between strains at each hour shows that the higher activity in $ebony^1$ is a result of the sharp decrease in locomotor activity of wild-type flies during the first hours of the dark period. At this time, wild-type flies start sleeping, whereas *ebony* flies are still very active. Finally, tan^1 not only shows a very low total locomotor activity, but also the lowest activity per waking minute, therefore indicating that this strain is hypoactive. Strikingly, the evening activity peak is similar to the morning peak, whereas, in wild-type and $ebony^1$ (and generally in male flies), it is higher (Figs 1A and 2). The tan^1 mutant is also unable to consolidate activity into long bouts (Fig. 1C).

The analysis of sleep behaviour patterns shows a different distribution between the two mutants compared with wild-type flies. The main period of sleep for wild-type was during the night-time, whereas $ebony^1$ sleeps more during daytime and tan^1 sleeps for almost the same total time during both phases (Fig. 4B). Daytime sleeping is similar within the three strains. During the dark hours, $ebony^1$ spends less time sleeping than wild-type; the duration of sleep bouts decreases in $ebony^1$ and increases in wild-type at night compared with the daytime. The tan^1 mutant shows sleep bouts of similar length during both daytime and night-time, and these are much shorter than the sleep bouts of the other two strains. The number of sleep episodes is similar in wild-type and $ebony^1$ but, in tan^1, the sleep episodes increment by two-fold, both during the light and dark hours (Fig. 4D). The longest sleep episode in wild-type occurs during night-time, whereas, in $ebony^1$, it is during daytime. There are no differences in the duration of the longest sleep episode in tan^1, which shows shorter sleep episodes than the other strains (Fig. 4E). Another sleep parameter measured in the present study is sleep latency, which is an indication of how ready is a fly to start sleeping. The $ebony^1$ mutant shows a long latency of more than 2 h to initiate sleep, whereas tan^1 and wild-type start sleeping during the first hour (Fig. 4F). This longer sleep latency in $ebony^1$ might be a result of the inability of this mutant to terminate the excitatory action of DA or to circadian clock malfunction. As noted previously, homozygous $ebony^1$ mutants are arrhythmic (Newby & Jackson, 1991). Sleep is regulated by both circadian and homeostatic processes and the abnormalities shown by $ebony^1$ mutants suggests either that homeostatic mechanisms are altered, or that sleep and/or wake promoting signals from the circadian clock are modified (Andretic & Shaw, 2005). With regard to tan^1, this mutant shows a disruption in the cycle length and sleep bout number, which are indicative of deficits in the mechanisms regulating sleep timing (Andretic & Shaw, 2005); however, the sleep latency appears to be normal. The increment in the sleep bout number in tan^1 is indicative that this strain cannot consolidate the sleep episodes. Therefore, the tan^1 mutant is unable to obtain restorative sleep

and, as previously noted, tries to overcome this by initiating more sleep episodes.

It is suggested that NBAD may promote locomotor activity in flies. From measurements of RNA synthesis, Suh & Jackson (2007) conclude that NBAD is synthesized mainly during daytime to drive locomotor activity. However, the results reported in the present study show that NBAD does not promote locomotor activity in flies; the fact that *tan*^{1} mutants have higher levels of NBAD than the other flies studied but are hypoactive supports this idea. Despite the scarce levels of NBAD, *ebony*^{1} shows higher locomotor activity than *tan*^{1} and wild-type. Kyriacou *et al*. (1978) report that *ebony* mutants are hyperactive, which is in agreement with the results of the present study. Furthermore, NBAD-synthase in the head (= brain expressed) shows the highest enzyme activity at night during the quiescent period and in the mid-morning, indicating that synthesis of NBAD occurs mainly when locomotor activity is reduced (Fig. 5A). The lowest NBAD-synthase activity occurs during the evening bursts of locomotor activity. The activity profile of brain expressed NBAD-synthase indicates that this enzyme plays a role inactivating the excitatory action of DA that occurs during the waking period, instead of promoting locomotor activity. The low point of NBAD-synthase activity at ZT-12 correlates with the requirement of DA to drive bouts of locomotor activity. NBAD-synthase activity is required within glia for the clock-based control of locomotor activity. Ebony containing glia cells are positioned near DA and 5-HT neurones, suggesting a role for NBAD-synthase in terminating the action of DA and 5-HT (Suh & Jackson, 2007). The activity of NBAD-synthase may result in a circadian modulation of DA action (also production and release of NBAD) contributing to decrease the excitatory drive behaviour of this neurotransmitter.

On the other hand, NBAD-synthase activity in *tan*^{1} shows a different pattern. There is no peak at ZT-16; instead, the activity increases after ZT-12 and remains steady during the whole of the dark period. During daytime, the increase at ZT-2 observed in wild-type disappears; the activity augments at ZT-4 and remains steady until ZT-8. As observed for NBAD-synthase activity in wild-type, the activity of NBAD-synthase in *tan*^{1} drops at ZT-12. Ebony expression is regulated by clock proteins (Suh & Jackson, 2007). The activity of NBAD-synthase is cyclic in *tan*^{1} mutants but, because there is much NBAD and low levels of DA, it might be possible that the activity of this enzyme is also being modulated by the lack of substrate or excess of product.

Because DA can be metabolized by other enzymes, the relevance of NBAD metabolism to the physiology of dopaminergic systems has not been properly taken into account. The different physiological defects shown by *ebony*^{1} and *tan*^{1} demonstrate that NBAD metabolism is more important than previously known for the homeostasis and the normal function of the nervous system. Moreover, NBAD-synthase can metabolize other biogenic amines such as octopamine, tyramine, histamine and 5-HT, which are also involved in the regulation of locomotor activity and sleep in flies (Yuan *et al.*, 2006; Crocker & Sehgal, 2008; Chen *et al*., 2013; Oh *et al.*, 2013). The NBAD-hydrolase enzyme is also able to hydrolyze other β-alanyl derivatives (True *et al.*, 2005; Pérez *et al*., 2011). The possibility that β-alanyl derivatives of these amines might be simultaneously synthesized and hydrolyzed by the NBAD-synthase and NBAD-hydrolase enzymes, respectively, cannot be excluded and therefore should be explored in future studies. The results of the study by Simon *et al.* (2009) show that mutant flies lacking synaptic vesicles are able to survive to the adult stage and demonstrate normal responses to certain environmental stimuli, suggesting that the recycling of neurotransmitters by NBAD-synthase/NBAD-hydrolase may be crucial for maintaining the low levels of neurotransmitters in these flies and sustaining the normal function of central nervous system physiology. The possibility that NBAD *per se* (or other β-alanyl derivatives) might have some activity in the central nervous system must be explored. Furthermore, it is well known that NBAD has diverse roles in epidermis, both as a cross-linking precursor in cuticle sclerotization (Hopkins & Kramer, 1992) and as effector of the innate immune response (Schachter *et al*., 2007).

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