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Loss of Angiotensin-converting enzyme-related (ACER) peptidase disrupts night-time sleep in adult *Drosophila melanogaster*

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SUMMARY

***Drosophila Acer* (*Angiotensin-converting enzyme-related*) encodes a member of the angiotensin-converting enzyme family of metallopeptidases that have important roles in the endocrine regulation of blood homeostasis in mammals. *Acer* is expressed in the embryonic heart of *Drosophila* and expression in the adult head appears to be regulated by two clock genes. To study the role of *Acer* in development and in circadian activity, we have generated *Acer* null mutants by imprecise excision of a P-element and have compared their development and circadian behaviour with that of wild-type flies with the same genetic background. We show that *Acer* is not required for normal development, but that night sleep, which is clock regulated, is disrupted in adult flies lacking ACER. *Acer* null adults have reduced night-time sleep and greater sleep fragmentation, but normal levels of daytime sleep. The quality of night sleep in flies fed inhibitors of ACER is affected in a very similar manner. We have shown, using specific antibodies, that ACER is present in the adult fat body of the head and abdomen, and is secreted into the haemolymph. ACER might therefore have a role in cleaving regulatory peptides involved in metabolism and activity behaviour. There are similarities with mammals, where ACE peptidases are also expressed in adipose tissue and are thought to be part of a signalling system linking metabolism with sleep.**

Key words: angiotensin-converting enzyme, development, sleep quality, *Drosophila melanogaster*, sleep fragmentation.

INTRODUCTION

Angiotensin-converting enzyme (ACE) was the first member of the M2 family of zinc metallopeptidases to be characterised at both the biochemical and the molecular levels (Soubrier et al., 1993). It is a dipeptidyl carboxypeptidase and a key player in the renin–angiotensin system (RAS), responsible for the removal of the C-terminal dipeptide of angiotensin I to generate the powerful vasoconstrictor, angiotensin II (for reviews, see Corvol et al., 2004; Turner and Hooper, 2002). ACE is therefore crucially involved in the homeostatic regulation of blood pressure and electrolyte balance and is strongly linked with a number of cardiovascular and renal diseases (Bernstein et al., 2005; Mezzano et al., 2001; Shen et al., 2008b). Indeed, ACE inhibitors that block the formation of angiotensin II are used widely as antihypertensives and to treat congestive heart failure and renal disease (Hoogwerf, 2010; Slagman et al., 2010). ACE, as part of the RAS, has also been implicated in body fat deposition, glucose clearance and energy expenditure, and in the processing of MHC class I peptide epitopes (de Kloet et al., 2009; de Kloet et al., 2010; Jayasooriya et al., 2008; Nakagawa et al., 2000; Segura and Ruilope, 2007; Shen et al., 2008a; Weisinger et al., 2009). A second mammalian ACE, known as testicular or germinal ACE, is exclusive to male germ cells and is required for male fertility (Fuchs et al., 2005; Hagaman et al., 1998; Krege et al., 1995; Soubrier et al., 1993). Although germinal and somatic ACE are transcribed from a single gene, they are structurally distinct (Hubert et al., 1991). Somatic ACE is a larger protein comprising two active site domains in tandem, whereas germinal ACE has one domain, which is identical to the C-terminal part of somatic ACE, apart from a short N-terminal peptide encoded by a germline-specific exon (Corvol et al., 1995). No physiologically relevant peptide substrate for germinal ACE has yet been identified.

ACE2 is a relatively new addition to the ACE peptidase family and is now recognised as another enzymatic component of the mammalian RAS involved in the metabolism of angiotensin peptides (for reviews, see Eriksson et al., 2002; Lambert et al., 2008). ACE2, unlike somatic and germinal ACE, is strictly a carboxypeptidase, removing a single amino acid from the C-terminus of peptides. ACE2 can cleave a range of peptide substrates (Vickers et al., 2002), but is particularly efficient at the *in vitro* conversion of the vasoconstrictor angiotensin II to angiotensin(1-7), a peptide that is vasodilatory and antiproliferative, and therefore acts antagonistically to angiotensin II (Danilczyk and Penninger, 2006; Donoghue et al., 2000; Turner et al., 2002). Several laboratories have generated deletion mutants of the mouse *Ace2* gene and used these animals to provide additional evidence for the importance of ACE2 in attenuating the hypertensive actions of angiotensin II by metabolic inactivation and conversion to the counteracting angiotensin(1-7) (Crackower et al., 2002; Yamamoto et al., 2006). ACE2 is highly expressed in the kidney and is also found at lower levels in many other tissues including the heart, testis, liver and lung. ACE2 in the lung, where it is co-expressed with other components of the RAS, has been implicated in the progression of several pulmonary diseases and has been found to have a protective role in murine models of acute respiratory distress syndrome through negative regulation of angiotensin II signalling (Imai et al., 2005; Imai et al., 2008).

ACE2 expression in the heart suggested an important role for the peptidase in cardiac physiology, which was supported in a study showing that loss of ACE2 gives rise to a defect in cardiac contractility and thinning of the wall of the left ventricle that becomes progressively worse with age (Crackower et al., 2002).

However, ACE2 knockout mice generated by several other laboratories did not display this severe cardiac phenotype (Gurley et al., 2006; Gurley and Coffman, 2008; Yamamoto et al., 2006).

An ACE2 homologue in *Drosophila melanogaster*, known as ACER (ACE-related), is strongly expressed in the presumptive cardiac cells during stage 14 of embryogenesis, suggesting a developmental role for this peptidase in the insect heart (Taylor et al., 1996). The embryonic lethality and the accompanying disruption of cardiac progenitor cells observed in a *D. melanogaster* line with a P-element inserted in the 5′UTR of the *Acer* gene ($Acer^{k07704}$) appeared to confirm that ACER was vital for heart morphogenesis and suggested that ACER and ACE2 might have evolutionarily conserved functions in heart development (Crackower et al., 2002). However, insects do not possess angiotensin peptides and therefore any role for the insect enzyme in the development and functioning of the heart must involve systems distinct from the mammalian RAS.

ACER is one of two ACE-like peptidases in *D. melanogaster* and shares several enzymatic properties with mammalian somatic and germinal ACE, but not ACE2 (Coates et al., 2000; Houard et al., 1998). It is a dipeptidyl carboxypeptidase, but compared with mammalian ACE and other invertebrate ACE-like peptidases, it has more restricted substrate specificity (e.g. ACER is very inefficient at converting angiotensin I to angiotensin II) (Bingham et al., 2006; Houard et al., 1998; Siviter et al., 2002). *Acer* is strongly expressed in the head of the adult fly and, in a study of global circadian gene expression, *Acer* mRNA expression was shown to cycle and to be regulated by *Clock* (McDonald and Rosbash, 2001). These data suggested a possible role for ACER in circadian behaviour *via* the metabolism of biologically active peptides.

In order to investigate a possible role for *Acer* in both heart development and in circadian behaviour, we have generated two *Acer* null mutant lines. In contrast to the results of the previous study that led to the conclusion that *Acer* was vital during early embryonic development, we now show that *Acer* is not vital for embryogenesis. Indeed, flies lacking ACER develop normally to adulthood and are fertile. Although we found no evidence of a role for *Acer* in circadian locomotor rhythms during a 12 h light-dark cycle, adults lacking ACER, or treated with peptidase inhibitors, experience disrupted night-time sleep, which is under circadian control. In the head capsule and the abdomen, ACER protein is associated with fat body, an important tissue for storage and supply of nutrients. The sleep phenotype observed in *Acer* null mutants is possibly linked to impairment in the homeostatic mechanisms responsible for maintaining the supply of energy substrate to the brain.

MATERIALS AND METHODS

Fly culture

Wild-type and mutant strains were maintained on oatmeal–molasses–agar medium at 25°C.

S2 cell culture

Drosophila melanogaster (Meigen 1830) S2 cells were purchased from Invitrogen Ltd, Paisley, UK and were cultured according to the manufacturer's instructions.

Assay of locomotor activity and sleep

Unless otherwise stated adult males (4–8 days old) were placed in glass tubes plugged at one end with agar (2%) containing sucrose (5%) and at the other end with a ball of cotton wool. The pharmacological experiment was undertaken by adding fosinopril (Bristol-Myers Squibb Pharmaceutical Research Institute, New Jersey, USA) to the molten agar–sucrose to give a final concentration of 1 mg ml^{-1}. Tubes were placed in activity monitors (DAM2, Trikinetics Inc., Waltham, MA, USA) that use an infrared beam to detect movement as the fly walks along the glass tube. The number of beam breaks occurring in 5-min time-bins was recorded for individual flies and the data analysed using Microsoft Excel. A sleep period was defined as a 5-min time-bin with no locomotor activity. Monitors were kept at a constant temperature of 25±1°C in a 12 h:12 h light:dark cycle. Insects were entrained in the light–dark regime for 4 days before data were recorded. Statistical analysis was performed using the Student's *t*-test.

Generation of *Acer* null alleles

To generate P-element excisions, $Acer^{CB\text{-}0338\text{-}3}$ homozygous females were crossed with the transposase strain w^{1118}; *CyO/Sp; Sb Δ2-3/TM6*. Individual $Acer^{CB\text{-}0338\text{-}3}$/*CyO; Sb Δ2-3* males from the F1 generation were crossed with w^{1118}; CyO/ap^{Xa} females and single white-eyed progeny from the F2 generation back-crossed to w^{1118}; CyO/ap^{Xa} flies. Out of 162 fertile F1 males, 78 independent excision lines were established. These lines were screened by PCR for deletions that removed the 5′ end of the *Acer* gene. Using a forward primer specific for a region upstream of *Acer* and a reverse primer specific for a region within the second exon, a 1137 bp amplification product was expected from wild-type DNA. Two lines (Δ164 and Δ168) gave PCR products of approximately 800 bp that were sequenced to confirm the position of the deletion.

Antibodies

The production of antibodies recognising ACER has been described previously (Houard et al., 1998). Standard methods were used to prepare affinity-purified antibodies from rabbit serum using a column of immobilised ACER, generated by coupling recombinant ACER to cyanogen bromide-activated Sepharose 4B (Sigma-Aldrich, Poole, Dorset, UK).

Western blots

Adult males (1–3 days old) or embryos (0–24 h old) were homogenised in 20 mmol l^{-1} Hepes, pH 7.5, 100 mmol l^{-1} KCl, 5% (v/v) glycerol, 20 mmol l^{-1} β-glycerophosphate, 100 μmol l^{-1} Na_3VO_4, 10 mmol l^{-1} EDTA, 0.1% (v/v) Triton X-100, 1 mmol l^{-1} dithiothreitol, 0.5 mmol l^{-1} PMSF. After centrifugation, the protein concentration of the supernatants was determined and equal quantities (10 μg) loaded into wells of NuPAGE® 4–12% Bis-Tris SDS-polyacrylamide gels (Invitrogen Ltd, Paisley, UK). After electrophoresis in MOPS-SDS running buffer, proteins were transferred onto PVDF membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and developed using an ECL Advance™ Western Blotting Detection Kit (GE Healthcare) following the manufacturer's protocol with a 1:3000 dilution of polyclonal rabbit anti-ACER antiserum and a 1:100,000 dilution of horseradish-peroxidase-conjugated goat anti-rabbit IgG (Dako Cytomation, Dako, Ely, Cambridgeshire, UK). Following addition of substrate, blots were exposed to Hyperfilm™ ECL (GE Healthcare).

Immunofluorescence

All steps were carried out at room temperature, unless otherwise stated. Heads and abdomens were dissected from 3- to 5-day old adult Oregon R (wild-type) or $Acer^{Δ164}$ flies in phosphate-buffered saline (PBS). The head capsule was opened up to allow antibody access, but the brain was left *in situ*. Abdomens were opened up and internal organs removed, leaving fat body attached to the body wall. Tissues were fixed for 20 min in 4% paraformaldehyde in

PBS then washed 4× 5 min in PBS followed by a 1 h wash in PBS plus 0.5% Triton X-100 (PBTX). Tissues were then blocked in PBTX containing 5% normal goat serum (PBTN) for 1 h. The PBTN solution was removed and replaced with a 1:400 dilution of affinity-purified rabbit anti-ACER antibody in PBTN. Tissues were left in antibody solution overnight at 4°C. Excess antibody was removed by washing 4× 30 min in PBTX. Tissues were then left in PBTN for 1 h before adding a 1:400 dilution of Alexa-Fluor-488-conjugated goat anti-rabbit IgG antibody (Invitrogen) and incubating for 1 h. Tissues were then washed 4× 5 min in PBTX followed by 4× 30 min in PBTX and 1× 5 min in PBS. Tissues were mounted in Vectashield (Vector Laboratories, Peterborough, UK) and imaged using a Leica TCS SP2 scanning confocal microscope.

RESULTS

Imprecise excision of the RS P-element from CB-0338-3 flies generates deletion mutants ($Acer^{\Delta 164}$ and $Acer^{\Delta 168}$) that develop normally and are fertile

The P-element insertion strain P{RS3}CB-0338-3 (Ryder et al., 2004) has an RS3 element inserted within the 5′ untranslated region of the *Acer* gene at 2L:8521983, 295 nucleotides upstream of the ATG start codon. Western blot analysis of embryos and adults homozygous for this insertion revealed normal levels of ACER protein. Imprecise excisions were generated from P{RS3}CB-0338-3 (see Materials and methods section) providing two mutant lines ($Acer^{\Delta 164}$ and $Acer^{\Delta 168}$) with a 329 bp deletion encompassing most of the 5′ untranslated region and the first 12 codons of the *Acer* gene. Two of the excision strains ($Acer^{+2}$ and $Acer^{+13}$), which yielded a wild-type PCR product that was confirmed by sequencing, were used as precise excision controls for further studies. Western blots showed that $Acer^{\Delta 164}$ and $Acer^{\Delta 168}$ homozygous flies did not express ACER and that the control $Acer^{+2}$ *and* $Acer^{+13}$ had wild-type levels of ACER protein (Fig. 1). Both $Acer^{\Delta 164}$ and $Acer^{\Delta 168}$ embryos developed normally giving rise to apparently healthy adults with levels of fertility indistinguishable from the control lines $Acer^{+2}$ and $Acer^{+13}$ (data not shown).

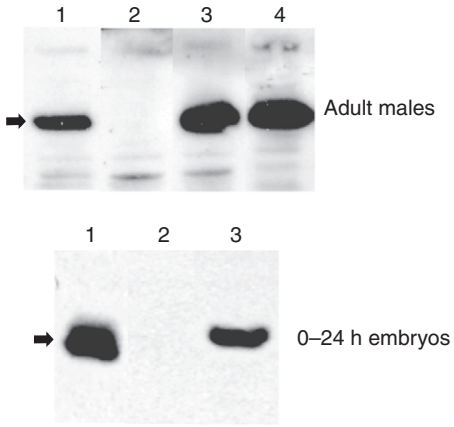

Fig. 1. Western blot analysis of ACER protein. (A) Adult males. Lane 1, Oregon R; lane 2, $Acer^{\Delta 164}$ homozygotes; lane 3, CB-0338-3 homozygotes; lane 4, precise excision homozygotes. The film was over-exposed to reveal non-specific bands and to ensure that no ACER was detectable in $Acer^{\Delta 164}$ homozygotes. (B) 0- to 24-h embryos. Lane 1, Oregon R; lane 2, $Acer^{\Delta 164}$ homozygotes; lane 3, precise excision homozygotes. The arrow in A and B indicates the position of the ACER band at 72 kDa.

Acer null mutants display increased night-time locomotor activity

Male adult flies were entrained in a 12 h light–dark cycle (lights on, 09.00 h and lights off, 21.00 h) at 25°C prior to the recording of locomotor activities of individuals. Both null mutant lines ($Acer^{\Delta 164}$ and $Acer^{\Delta 168}$) were two- to threefold more active than the control flies ($Acer^{+2}$ and $Acer^{+13}$) during the night-time period from midnight to 06.00 h, but not during the daytime quiescent period (12.00–18.00 h, Fig. 2A,B). The locomotor activity of $Acer^{\Delta 164}$ and $Acer^{\Delta 168}$ flies during wakeful episodes is no greater than wild-type control insects indicating that the null mutants are not hyperactive (Fig. 2C). $Acer^{\Delta 164}$ and $Acer^{\Delta 168}$ females also displayed higher locomotor activity during the night-time period (not shown).

Night-time sleep is disrupted in *Acer* null mutants

Locomotor activity was used to determine sleep patterns (number of 5-min time-bins with no activity). Null mutant lines ($Acer^{\Delta 164}$ and $Acer^{\Delta 168}$) slept around 15% less during the hours of darkness compared with wild-type flies (Fig. 3A,B). The longest period of sleep for individuals lacking ACER was shortened by around 50%

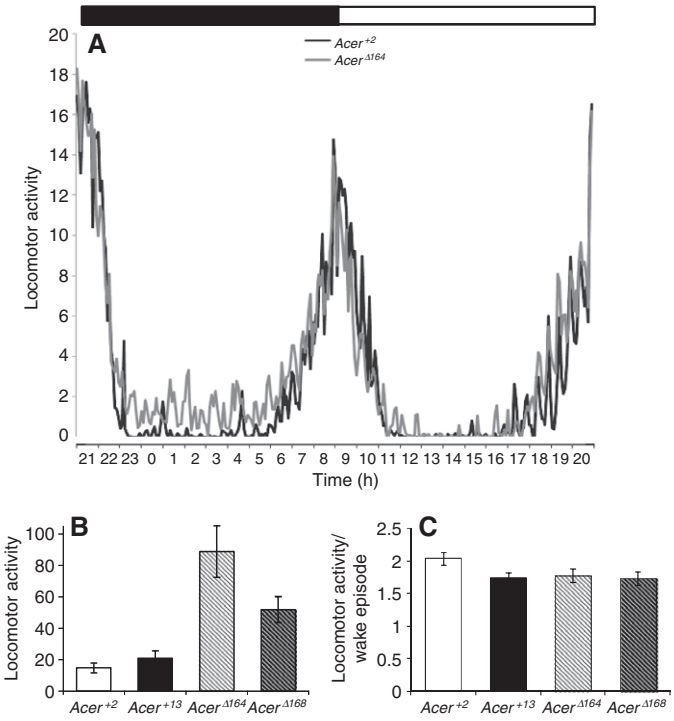

Fig. 2. Locomotor activity of wild-type ($Acer^{+2}$ and $Acer^{+13}$) and ACER null ($Acer^{\Delta 164}$ and $Acer^{\Delta 168}$) adult males. Flies (3-4 days old) were entrained in a 12 h:12 h light:dark cycle (lights on, 09.00 h; lights off, 21.00 h). Locomotor activity was monitored using Trikinetics DAM2 equipment and expressed as the number of beam breaks per 5 min (mean ± s.e.m., 20 flies of each genotype). (A) Representative activity profile for $Acer^{+2}$ and $Acer^{\Delta 164}$ showing peaks of locomotor activity at lights-on and lights-off, night-time quiescent period between 00.00 h and 06.00 h and day-time 'siesta' quiescence between 12.00 h and 18.00 h. White and black horizontal bars indicate day-time and night-time, respectively. (B) Sum of night-time locomotor activity occurring between 00.00 h and 06.00 h (mean ± s.e.m., 20 flies of each genotype). (C) Locomotor activity per wake episode during a 24 h day (mean ± s.e.m., 20 flies of each genotype). The difference between wild-type and ACER null flies is statistically significant (*P<0.002).

(Fig. 3C) and the number of sleep episodes experienced by the *Acer* mutants increased by 60% (Fig. 3D). Thus, flies with no ACER sleep less during the hours of darkness and have more fragmented night-time sleep.

An inhibitor of ACER activity fed to adult *D. melanogaster* disrupts sleep

Male adult flies were fed an inhibitor of ACER (fosinopril) incorporated into the agar–sucrose food, and the effects on circadian locomotor activity and sleep were recorded. Flies fed the inhibitor were more active during the night period of midnight to 06.00 h than control flies kept on untreated agar–sucrose, whereas the day-time quiescent period (12.00–18.00 h) was unaffected (Fig. 4A,B). Compared with controls, the inhibitor reduced the length of the longest sleep period by 35% and greatly increased (2.8-fold) fragmentation of night-time sleep (Fig. 4C,D).

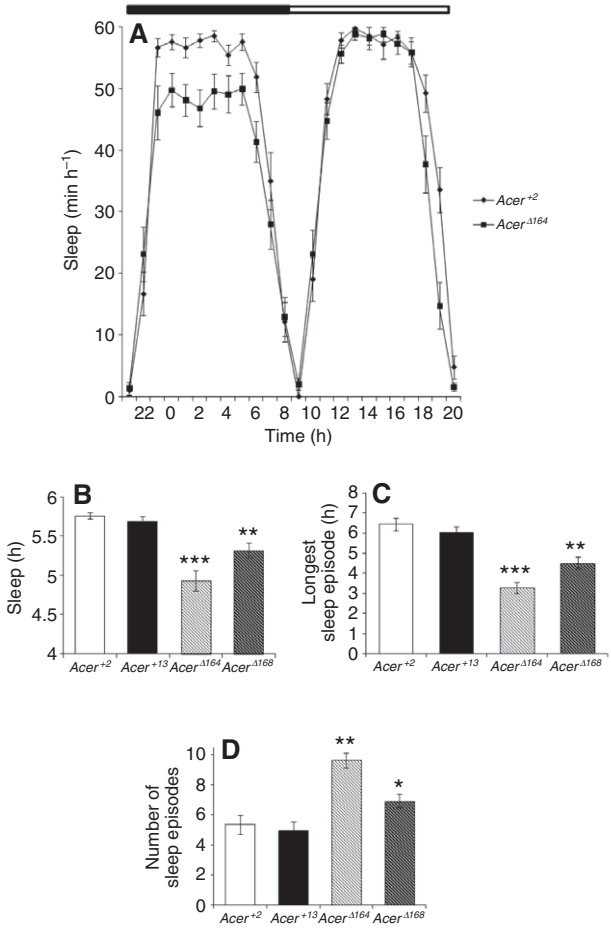

Fig. 3. Lack of ACER results in disrupted night-time sleep. (A) Representative sleep profiles for *Acer*$^{+2}$ and *Acer*$^{\Delta164}$ adult males. White and black horizontal bars indicate day-time and night-time, respectively. Sleep is expressed as minutes of sleep per hour. (B) Sleep between 00.00 h and 06.00 h for wild-type (*Acer*$^{+2}$ and *Acer*$^{+13}$) and ACER null (*Acer*$^{\Delta164}$ and *Acer*$^{\Delta168}$) adult male flies, expressed in hours. (C,D) Longest continuous period of sleep (C) and number of sleep episodes (D) of wild-type (*Acer*$^{+2}$ and *Acer*$^{+13}$) and ACER null (*Acer*$^{\Delta164}$ and *Acer*$^{\Delta168}$) adult male flies from lights-off (21.00 h) to lights-on (09.00 h). Values are means ± s.e.m., 60 flies of each genotype. ***P<0.0001 **P<0.001 and *P<0.05, statistical significance of the difference between wild-type and ACER null flies.

Acer is expressed in the adult fat body and is secreted into the haemolymph

Microarray analysis has shown that *Acer* is expressed in the adult head and that this expression may be under control of *Clk* (McDonald and Rosbash, 2001). The *Drosophila* adult gene expression atlas identifies the adult head, spermatheca and the fat body as tissues

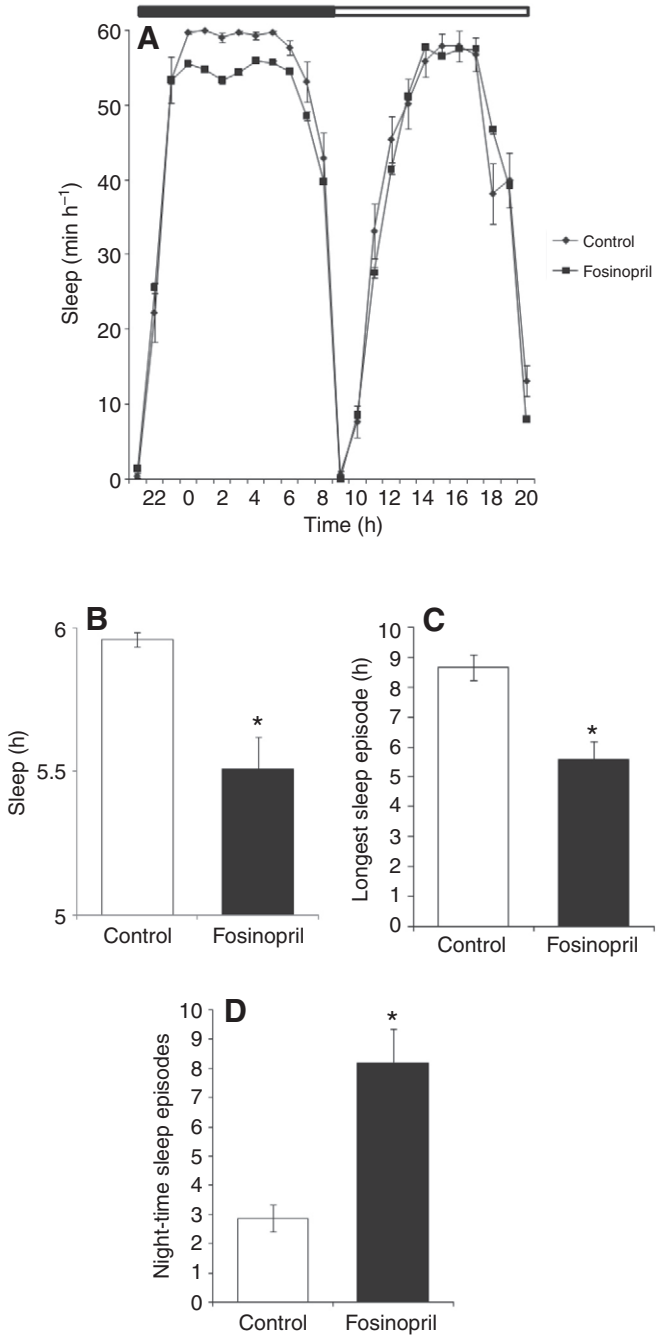

Fig. 4. Fosinopril disturbs night-time sleep. (A,B) Effect of feeding fosinopril (1 mg ml^{-1}) to flies on the amount they sleep between 00.00 h and 06.00 h. White and black horizontal bars indicate day-time and night-time, respectively. (C,D) Effect of fosinopril on longest sleep episode (C) and number of sleep episodes (D) during night-time (21.00–09.00 h) for wild-type male flies. Values are means ± s.e.m., 20 flies of each genotype. *P<0.005, statistical significance of the difference between control and inhibitor fed flies.

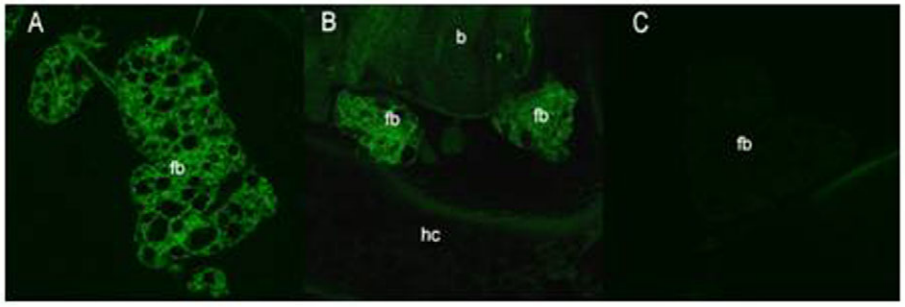

Fig. 5. Immunofluorescence staining for *Acer* in adipose tissue from adult *D. melanogaster*. (A) Abdominal fat body. (B) Head fat body. (C) Abdominal fat body from ACER null ($Acer^{\Delta 164}$) flies. b, brain; fb, fat body; hc, head capsule.

with high levels of *Acer* mRNA with greatest enrichment occurring in adipose tissue (Chintapalli et al., 2007). *In situ* hybridisation and immunofluorescence using ACER-specific antibodies did not detect any localised expression of *Acer* in the adult brain (data not shown), but did establish that ACER was strongly associated with the fat body in both the head and the abdomen (Fig. 5).

ACER is predicted to have a cleavable N-terminal secretion signal, suggesting that the enzyme is a soluble secreted protein. *Acer* is expressed naturally in *D. melanogaster* S2 cells and therefore these cells provided a convenient system to address the question of whether ACER is a cellular or secreted protein. Therefore, we cultured S2 cells in normal insect cell medium until 24 h before harvesting of the cells at confluence. During the last 24 h in culture, the medium was replaced with medium minus foetal calf serum. For western blot analysis using affinity-purified ACER antibody, the medium was dialysed and concentrated to the same volume as the cell homogenate, and equal volumes were loaded onto the SDS-PAGE gel (i.e. this result should reflect the distribution of secreted and cellular ACER). The results, which were reproducible in three separate experiments (three flasks of cells), showed that the majority of ACER was present in the culture medium. This result established that ACER from S2 cells is a secreted soluble protein (Fig. 6A). Western blotting also established that ACER is present as a soluble protein in adult haemolymph (Fig. 6B).

DISCUSSION

ACER is a member of the M2 family of zinc metallopeptidases that includes mammalian ACE and ACE2, two peptidases with important regulatory roles not only in the systemic RAS, but also in local tissue RAS, such as in the heart (Corvol et al., 2004; Turner et al., 2004). ACE2 appears to have a particularly important role in mammalian heart where it is found at high levels (Turner, 2008). The first phenotypic analysis of ACE2 knockout mice by Crackower et al. showed that mice lacking ACE2 developed heart contractility problems with age (Crackower et al., 2002). The fact that ACER is strongly expressed in the developing heart of *D. melanogaster* led to speculation that the insect M2 peptidase was also important for normal heart development and functioning (Crackower et al., 2002; Taylor et al., 1996). Indeed, Crackower et al. reported reduced numbers and disorganisation of heart progenitor cells in the lethal P-element insertion strain *l(2)k07704*, which has a P-element inserted in the *Acer* gene, 51 bp downstream of the inferred transcription start site, in the 5′ untranslated region (Crackower et al., 2002). We have studied the expression of *Acer* in *l(2)k07704* homozygous embryos and we arrived at a different conclusion to that presented by Crackower et al.: the P-element insertion does not alter levels of ACER protein relative to wild-type embryos, as determined by western blot analysis using an ACER-specific antiserum (A.D.S., unpublished results) Also, flies transheterozygous for *Df(2L)N22-14* and *l(2)k07704* are viable. The breakpoints of *Df(2L)N22*-14 have been mapped by microarray analysis (Yamamoto et al., personal communication to Flybase – FlyBase ID FBrf0207891) and *Acer* lies well within the deleted segment (Tweedie, 2009). Thus, there would appear to be another lethal mutation on the same chromosome as *l(2)k07704*, and the defects in heart development observed by Crackower et al. may be a consequence of this second mutation. We have now generated *Acer* null mutants by imprecise excision of a P-element from P[RS3}CB-0338-3 and show conclusively that ACER is not a vital protein. Flies lacking the enzyme develop normally and are fertile, however, it is possible that loss of ACER can result in undetected abnormal cardiac function. Indeed, *Acer* is strongly expressed in the adult heart and therefore it would be unwise to conclude that the lack of ACER peptidase in the heart has no detrimental effect.

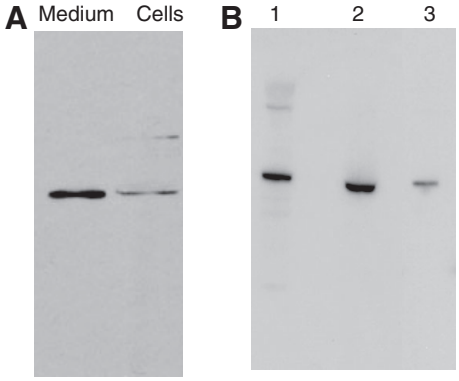

Fig. 6. Western blot analysis of secreted and cellular ACER protein from (A) cultured S2 cells and (B) adult haemolymph. In B: lane 1, adult male haemolymph; lane 2, adult female haemolymph; lane 3, whole fly extract.

The FlyAtlas project has shown that high levels of *Acer* mRNA are present in the adult, but not larval, fat body as well as the heart (Chintapalli et al., 2007). We now confirm, using specific antibodies, that ACER protein is enriched in the fat body of the head capsule and of the abdomen of adults and that ACER is a secreted soluble protein. Because of the relative mass of the adult fat body and heart, we propose that the fat body is the most likely source for much of the ACER that we found in the haemolymph of adults. The fat body of *D. melanogaster* is physiologically analogous to vertebrate liver and white adipose tissue, and is responsible for the metabolism and storage of proteins, carbohydrates and lipids (Arrese and Soulages, 2010; Roma et al., 2010). During normal growth, the fat body of the last larval instar synthesises glycogen, triacylglycerol and larval serum proteins, which can be converted to circulating trehalose, diacylglycerol and free amino acids, respectively, to provide energy and materials for

the construction of adult tissues during metamorphosis. Other functions of the adipose tissue include the synthesis of antimicrobial peptides, yolk proteins in adult females and the provision of energy substrates during periods of energetically demanding activity, such as flight (Arrese and Soulages, 2010).

The strong expression of ACER in the fat body is similar to the situation in mammals where ACE is expressed in adipocytes as part of a functional local RAS (for a review, see Engeli et al., 2003). There is increasing evidence that the adipocyte RAS is involved in the aetiology of obesity and insulin resistance, in addition to its well-established role in blood pressure and electrolyte homeostasis (de Kloet et al., 2009; Hennes et al., 1996; Jayasooriya et al., 2008; Segura and Ruilope, 2007; Zorad et al., 1995). The expression of ACE was found to be upregulated in primary human adipocytes from obese hypertensive subjects, and angiotensin II (AII), the main product of mammalian ACE activity, promotes adipocyte proliferation and differentiation (Gorzelniak et al., 2002; Ye et al., 2009). Furthermore, several studies have shown that blocking the formation of AII in ACE knockout ($ACE^{-/-}$) mice or by chronic administration of ACE inhibitors to rats leads to a large (around 50%) reduction in fat mass, together with increased glucose tolerance (Jayasooriya et al., 2008; Weisinger et al., 2009). The loss of body fat in $ACE^{-/-}$ mice was accompanied by elevated expression of liver genes involved in fatty acid metabolism, and a large reduction in circulating leptin titre (Jayasooriya et al., 2008). Recently it has been shown that mouse adipocytes also synthesise and secrete ACE2 and that levels of secretion are regulated by a high-fat diet (Gupte et al., 2008).

In *Drosophila,* energy and metabolic homeostasis is maintained by bidirectional signalling between the fat body and the neuroendocrine system, and involves neuropeptides such as *Drosophila* insulin-like peptides (DILPs) and adipokinetic hormone (AKH), a functional analogue of glucagon (Gronke et al., 2007; Isabel et al., 2005; Kim and Rulifson, 2004; Lee and Park, 2004; Slaidina et al., 2009). The insect fat body monitors the nutritional status of the fly and remotely regulates neuroendocrine secretion of brain DILPs that couple nutrient supply to growth (Geminard et al., 2009; Hong and Park, 2010). The humoral signal emanating from the adipocytes has recently been identified as DILP6, which is required at times of high nutritional demand, such as the growth of adult tissues in the non-feeding pupal stage (Okamoto et al., 2009). Although biochemical studies suggest that ACER is unlikely to be involved in the metabolism of either ILPs or AKH, it is possible that ACER has a role in the processing of other peptide hormones that are important for metabolic homeostasis (Houard et al., 1998; Siviter et al., 2002). Interestingly, ACER activity levels follow a similar pattern to DILP6 expression, rising from a low level at the transition from larval to pupal development (pupariation) to a broad peak of activity between pupal stages P6 and P11, suggesting an important role for the enzyme during metamorphosis (Houard et al., 1998).

The strong expression of ACER in the adult head fat body together with the report that *Acer* expression was regulated by a clock gene led us to investigate whether null mutants displayed abnormal wake–sleep rhythm. Adult *D. melanogaster* display a robust rhythm of locomotor activity when entrained in a light–dark cycle, with peaks of activity at lights-on and lights-off. In between, there are periods of quiescence that have been shown to be sleep-like states with many characteristics of mammalian sleep (e.g. increased arousal threshold, homeostatic response to sleep deprivation, robust circadian control of the sleep–activity cycle, age-related changes to sleep quality) (Andretic and Shaw, 2005). Although the rhythm of locomotor activity is maintained in flies lacking ACER, we noticed an increase in the level of activity during scotophase compared with wild-type flies with the same genetic background. This elevated activity resulted in a reduction in night-time sleep and greater sleep fragmentation. This change in night-time behaviour was reproduced by oral administration of an inhibitor of ACER. The reasons for the disturbed night-time sleep are at present unclear. However, we might speculate that the absence of a peptide-hormone-processing enzyme in the fat body and the haemolymph disrupts metabolic control, which in turn results in a change in behaviour and reduced night-time sleep. Recently, it has been shown that ablation of DILP-secreting neurons from the adult brain leads to a loss of night-time sleep under conditions of low sucrose, thus supporting a link between nutritional status and sleep in *D. melanogaster* (Broughton et al., 2010). In addition, Keene et al. have shown that starvation suppresses sleep and that a subpopulation of *Clk*-expressing dorsal neurons are responsible for the change in behaviour in response to caloric restriction (Keene et al., 2010).

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