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A neuropeptide hormone cascade controls the precise onset of post-eclosion cuticular tanning in *Drosophila melanogaster*

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A neuropeptide hormone-signalling pathway controls events surrounding eclosion in *Drosophila melanogaster*. Ecdysis-triggering hormone, eclosion hormone and crustacean cardioactive peptide (CCAP) together control pre-eclosion and eclosion events, whereas bursicon, through its receptor rickets (RK), controls post-eclosion development. Cuticular tanning is a convenient visible marker of the temporally precise post-eclosion developmental progression, and we investigated how it is controlled by the ecdysis neuropeptide cascade. Together, two enzymes, tyrosine hydroxylase (TH, encoded by *ple*) and dopa decarboxylase (DDC, encoded by *Ddc*), produce the dopamine that is required for tanning. Levels of both the *ple* and *Ddc* transcripts begin to accumulate before eclosion, coincident with the onset of pigmentation of the pharate adult bristles and epidermis. Since DDC activity is high before the post-eclosion onset of tanning, a different factor must be regulated to switch on tanning. Transcriptional control of *ple* does not regulate the onset of tanning because *ple* transcript levels remain unchanged from 24 hours before to 12 hours after eclosion. TH protein present before eclosion is degraded, and no TH activity can be detected at eclosion. However, TH protein rapidly accumulates within an hour of eclosion and we provide evidence that CCAP controls this process. Furthermore, we show that TH is transiently activated during tanning by phosphorylation at Ser32, as a result of bursicon signalling. We conclude that the ecdysis hormone cascade acts as a regulatory switch to control the precise onset of tanning by both translational and activational control of TH.

KEY WORDS: *Drosophila melanogaster*, Tyrosine hydroxylase, Tanning, Eclosion

INTRODUCTION

Hormones play a central role in regulating temporal and spatial patterns of gene expression during the development of multicellular organisms. In the fruitfly, *Drosophila melanogaster*, the steroid hormone 20-hydroxy ecdysone (hereafter referred to as ecdysone) controls all developmental transitions as the organism develops from a fertilised egg to an adult fly (Riddiford, 1993). Ecdysone is released before the larval moults, pupation and eclosion, and initiates a hormonal cascade that ultimately leads to shedding of the cuticle, a process known as ecdysis.

The precise timing of ecdysis occurs in response to the neuronal release of peptide hormones, which act on ecdysone-primed tissues (Fig. 1). In *Manduca sexta*, if ecdysone titres do not decline, ecdysis is blocked (Curtis et al., 1984), suggesting that a decline in steroid hormone titre signifies a readiness to shed the cuticle (Truman, 1996). The first hormone to be released in response to decreasing ecdysone levels is ecdysis-triggering hormone (ETH) (Park et al., 2002). This triggers an increase in eclosion hormone (EH) levels (Clark et al., 2004; Horodyski et al., 1993) and, in fact, EH acts in a positive-feedback loop to cause further release of ETH (Ewer et al., 1997; Kingan and Adams, 2000; Kingan et al., 2001). Together ETH and EH are responsible for the preparatory behaviour called pre-ecdysis (Baker et al., 1999). EH causes the release of crustacean cardioactive peptide (CCAP) (Ewer and Truman, 1996; Gammie and Truman, 1999). CCAP is thought to control the ecdysis motor response by shutting off pre-ecdysis and turning on the ecdysis motor program (Gammie and Truman, 1997; Horodyski et al., 1993; Park et al., 2003). In fact, the timing and organisation of ecdysis are severely disrupted in organisms carrying CCAP ablation knockouts (Park et al., 2003). CCAP causes activation of protein kinase A (PKA) to initiate ecdysis behaviour (Luan et al., 2006). The post-eclosion hormone, bursicon, a heterodimeric neuropeptide whose α and β subunits are encoded by the *bursicon* (*burs*) and CG15284 genes respectively, colocalises with most CCAP neurons (Dewey et al., 2004; Luan et al., 2006; Luo et al., 2005; Mendive et al., 2005). Bursicon is released into the haemolymph following eclosion and acts through its receptor, encoded by *rickets* (*rk*) (Baker and Truman, 2002; Dewey et al., 2004; Luo et al., 2005; Mendive et al., 2005). RK is a G-coupled glycoprotein hormone receptor and its activation causes an increase in cAMP (Kimura et al., 2004). Wing expansion, and probably cuticular expansion and tanning, occur in response to cAMP activation of PKA.

Wing and cuticle expansion are complete within 20 minutes of eclosion, whereas tanning of the cuticle takes 3 hours. Neck-ligation of flies at eclosion prevents tanning, whereas flies neck-ligated 30 minutes after eclosion tan normally (Fraenkel and Hsiao, 1962). Bursicon is released within 20 minutes of eclosion, and this process is disrupted in flies neck-ligated at eclosion (Fraenkel et al., 1966). Tanning in such flies is rescued by injection of 8-Br-cAMP or haemolymph extracted from flies 20 minutes after eclosion (Baker and Truman, 2002; Fraenkel et al., 1966; Luo et al., 2005; Mendive et al., 2005). We are interested in how the precise timing of the developmental events following eclosion is controlled by the ecdysis neuropeptide cascade.

Flies without EH or CCAP (EH-KO and CCAP-KO, respectively) can be generated by ablating cells that produce these neuropeptides. This is achieved by triggering cell-specific apoptosis using the Gal4-

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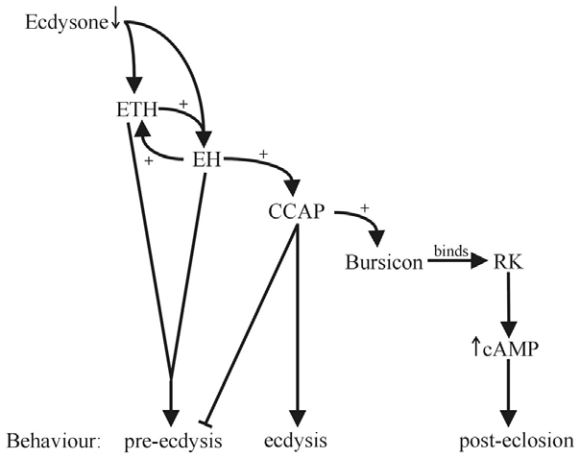

Fig. 1. The neuropeptide signalling pathway at eclosion. In response to decreasing levels of ecdysone, ecdysis-triggering hormone (ETH) is released, triggering the release of eclosion hormone (EH). These hormones act together in a positive-feedback loop, increasing the release of one another and regulating pre-ecdysis behaviour. EH causes the release of crustacean cardioactive peptide (CCAP), which shuts off pre-ecdysis and turns on the ecdysis motor program. At eclosion, CCAP causes release of bursicon, which binds to its receptor RK, and induces post-eclosion events by elevating levels of cAMP and causing tanning. This figure is modified from Clark et al. (Clark et al., 2004) and McNabb et al. (McNabb et al., 1997).

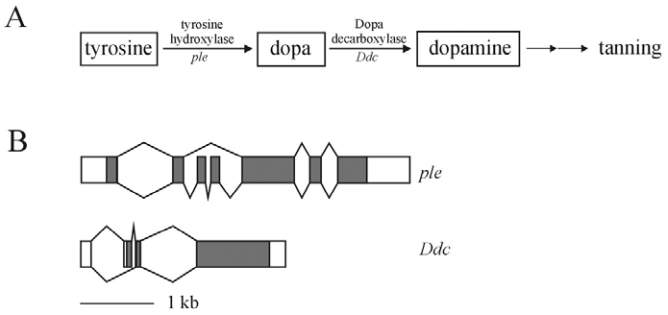

Fig. 2. Critical steps in the tanning pathway. (**A**) Metabolites of dopamine are required for tanning. Tyrosine hydroxylase (encoded by *ple*) converts tyrosine into dopa and dopa decarboxylase (encoded by *Ddc*) catalyses the conversion of dopa to dopamine. (**B**) Alternative splicing of *ple* and *Ddc*. Both *ple* and *Ddc* are alternatively spliced to produce neural-specific (shown above exon boxes) and epidermal-specific (shown below exon boxes) transcripts. The coding sequence is shaded in grey.

UAS system to express the gene, *reaper*, under the control of an EH or CCAP driver. EH-KO and CCAP-KO flies have a similar adult mutant phenotype to *burs* and *rk* mutants (Baker and Truman, 2002; Dewey et al., 2004; McNabb et al., 1997; Park et al., 2003). Such adults fail to expand their wings and exhibit a delay in tanning of their cuticle. This phenotype is mirrored in organisms that express a dominant negative form of the ecdysone receptor under the control of an EH driver, demonstrating the importance of ecdysone as a trigger for this hormonal cascade (Cherbas et al., 2003). Unlike *burs* and *rk* mutants, EH-KO and CCAP-KO flies also have severe defects at the larval and pupal ecdyses that result in reduced viability (Baker and Truman, 2002; Gammie and Truman, 1997; McNabb et al., 1997; Park et al., 2003).

The failure of EH-KO, CCAP-KO, *burs* and *rk* flies to properly undergo post-eclosion wing expansion and tanning indicates a loss of signalling that controls these developmental events. Tanning commonly refers to the colouration that accompanies the hardening of the cuticle. In *Drosophila melanogaster*, the mature cuticle is light brown over most of the organism, and therefore appropriately described as tanned; however, the abdominal tergites are black where melanisation occurs. Since all the mutants we are aware of that reduce overall colouration also reduce the black pigmentation in the tergites, we chose to include both processes in our use of the word tanning. Tanning requires metabolites of dopamine (DA) (Wright, 1987). The first step in the synthesis of DA is the rate limiting conversion of tyrosine to dihydroxyphenylalanine (dopa) by tyrosine hydroxylase (TH), which is encoded by the *pale* (*ple*) locus (Fig. 2A) (Levitt et al., 1965; Nagatsu et al., 1964; Neckameyer and White, 1993). The conversion of dopa to DA is catalysed by dopa decarboxylase (DDC) (Livingstone and Tempel, 1983), encoded by *Ddc* (Hirsh and Davidson, 1981). Similar to its role in vertebrates, dopamine plays a critical role in the *Drosophila* CNS; however, only 6% of DDC activity is present in the brain of third instar larvae (Scholnick et al., 1983). The remaining activity is present in the epidermis, demonstrating the importance of epidermal DA production. In addition to the requirement for DA following eclosion, DA is needed for tanning of the puparium at pupariation and for pigmentation of the pharate adult bristles and epidermis.

Both *Ddc* and *ple* mRNAs are alternatively spliced to produce neural- and epidermal-specific transcripts in *Drosophila* (Fig. 2B) (Birman et al., 1994; Morgan et al., 1986). The *Ddc* epidermal transcript lacks the neural-specific second exon, whereas the *ple* epidermal transcript contains the third and fourth exons, which are not included in the mRNA for the neural form of the enzyme. Both *Ddc* and *ple* null mutants are homozygous lethal, and rare *Ddc* mutant 'escaper' flies exhibit defects in cuticular tanning and die within 24 hours of eclosion (Neckameyer and White, 1993; Wright et al., 1976). Using transgenes to supply the respective epidermal, but not neural, forms of the enzymes to *Ddc* mutants (Morgan et al., 1986) or to *ple* mutants (Friggi-Grelin et al., 2003) rescues lethality, illustrating the importance of epidermal DA production. In vertebrates, TH activity is regulated at a variety of levels, including transcriptional regulation, alternative splicing, RNA stability, DA feedback inhibition and protein kinase activation (Dunkley et al., 2004; Hufton et al., 1995; Kumer and Vrana, 1996; Zigmond et al., 1989). Both transcriptional regulation and alternative splicing regulate TH activity in *Drosophila* (Birman et al., 1994). The additional exons included in the epidermal *ple* transcript encode a very acidic segment of 71 amino acids within the regulatory domain of this enzyme that reduces the sensitivity of TH to DA inhibition in vitro (Vie et al., 1999). Furthermore, *Drosophila* TH is activated in vitro by phosphorylation at Ser32, a site homologous to vertebrate Ser40 (Vie et al., 1999), the major site of PKA phosphorylation and activation (Dunkley et al., 2004; Kumer and Vrana, 1996). Phosphorylation of Ser32 has been proposed to increase the activity of the epidermal isoform of TH by reducing the ability of the protein to bind to inhibitory DA, and by increasing the rate of binding of the essential cofactor tetrahydrobiopterin (Vie et al., 1999). Interestingly, both CCAP and bursicon activate PKA by elevating cAMP levels (Baker and Truman, 2002; Luan et al., 2006), suggesting that either of these neuropeptides may be involved in activation of TH to regulate the onset of tanning.

In this paper, we set out to determine how the ecdysis neuropeptide hormone cascade regulates the precise timing of developmental events following eclosion. To achieve this, we focused on tanning because its biochemical basis is known, and it is a readily scored marker of post-eclosion development. We find that the onset of tanning does not depend on the regulation of *Ddc*. Rather, it is the regulation of TH protein accumulation and activity, elicited by the ecdysis neuropeptide hormone cascade that precisely controls tanning. Specifically, post-eclosion accumulation of TH from the *ple* transcripts that are already present in pharate adults is regulated by CCAP, whereas TH is activated by PKA as a result of bursicon signalling through RK. Regulation of TH transcription plays no part in the switch, effected by the ecdysis hormone cascade, that triggers post-eclosion tanning.

MATERIALS AND METHODS

Fly stocks and crosses

All stocks were maintained on a standard cornmeal-molasses medium at 25°C in a 12 hour light:12 hour dark cycle. The *yw;CCAP-Gal4* line was a gift from Douglas Allen (University of British Columbia, Vancouver, Canada) and the $burs^{Z1091}$ and $burs^{Z5569}$ lines were a gift from Susan McNabb (University of Washington, Seattle, WA). The *EH-Gal4*, *w UAS-rpr*, *w;UAS-rpr*, rk^{1} and rk^{4} flies were obtained from the Bloomington Stock Center. The mutation contained in the $burs^{Z1091}$ allele causes a Cys82 to Tyr conversion and the $burs^{Z5569}$ mutation results in a conversion of Gly115 to Cys (Dewey et al., 2004). The mutations in rk^{1} and rk^{4} flies introduce a premature stop codon in the transmembrane domain of this receptor (Baker and Truman, 2002). To obtain EH-KO flies, we crossed *w UAS-rpr* females to *EH-Gal4* males. CCAP-KO flies have severe defects at both the larval and pupal ecdyses, rarely yielding viable adult flies. To obtain a sufficient number of viable CCAP-KO adult flies for analysis, we crossed *yw;CCAP-Gal4* females to *w;UAS-rpr* males. In each experiment, *yw* flies were used as a wild-type control.

Neck ligations and CCAP and 8-Br-cAMP injections

Pupae that were within 6 hours of eclosion, according to previously defined cues (Kimura and Truman, 1990), were mounted on double-sided tape on a slide, and the operculum was removed. As each fly emerged from the pupal case, it was immobilised on an ice-cooled Petri dish and was neck-ligated using a strand of hair. When injected, approximately 300 nl of 0.1 mM CCAP (Bachem) or 0.1 M 8-Br-cAMP was delivered into the thorax through a fine glass capillary needle. Flies were kept in a humidified chamber until 2.5 hours after eclosion and frozen at –80°C for subsequent analysis.

RNA manipulations and RT-PCR

RNA was extracted from pools of organisms using TRIzol, treated with amplification grade DNaseI, and reverse transcribed using Superscript II according to the manufacturer's instructions (Invitrogen). Table 1 shows the sequence of all primers used in these experiments. Synthesis of the *Ddc*- and *ple*-specific cDNAs was initiated using the DDC1 and PLE1 primers, respectively, on 150 ng isolated RNA. First-strand reactions were co-reverse transcribed with RPL32-1, a primer specific for the ribosomal gene *RpL32*, which serves as a loading control. A 3 μl aliquot of the resulting cDNA mixture was combined either with *ple*-specific primers (PLE-F and PLE-R) and amplified for five cycles or with *Ddc*-specific primers (DDC-F and DDC-R) and amplified for three cycles. The program was then stopped, *RpL32* gene-specific primers were added, and the program was allowed to continue for 23 more cycles. After an initial denaturing step of 2 minutes, the final PCR conditions for *ple* consisted of 28 cycles of 95°C for 1 minute, 60°C for 1 minute and 73°C for 1 minute. The conditions were the same for *Ddc*, except the reaction ran for a total of 26 cycles.

Table 1. PCR primers used in this study

Primer	Orientation	Sequence (5′-3′)
PLE-1	Reverse	CGGGCTCGTACTTGGTCATC
PLE-F	Forward	TCCCTCCGAGCAAGATGTGG
PLE-R	Reverse	ACCACGGAGCCTTGACATTC
DDC-1	Reverse	AATCGCTCCACTCAGCATGT
DDC-F	Forward	GCCAAGCGCACAGCAATCAG
DDC-R	Reverse	ATGACTCGCTCGATGTCCTG
RPL32-1	Reverse	CTTCTTGAGACGCAGGCGA
RPL32-F	Forward	AGCATACAGGCCCAAGATCG
RPL32-R	Reverse	AGTAAACGCGGGTTCTGCAT

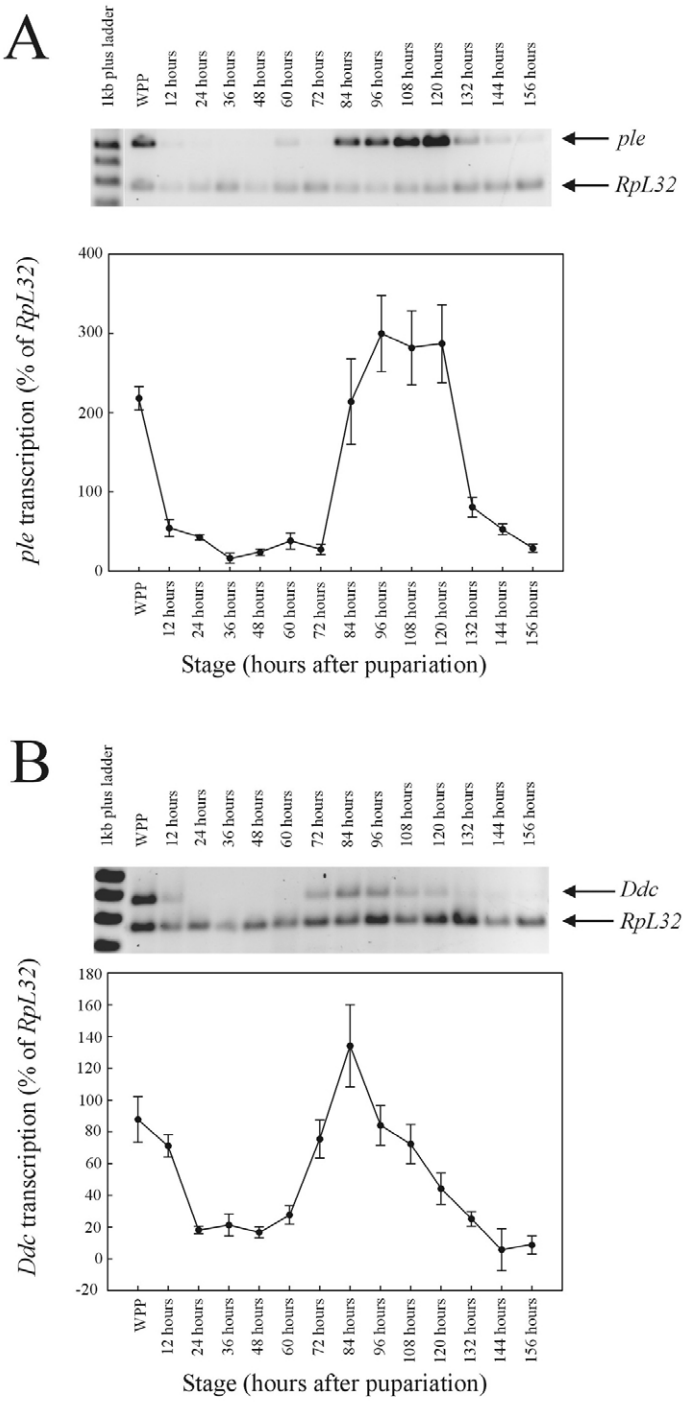

Fig. 3. Quantitative RT-PCR of *ple* and *Ddc* transcripts following pupariation. (**A**) *ple* or (**B**) *Ddc* transcripts are shown with an *RpL32* loading control. Bands from at least four different extracts for each time point were quantified and averaged, and expressed as a percentage of the loading control band to produce the graphs. Error bars indicate s.e.m.

Under these conditions, the amount of each product was proportional to the mass of RNA when 50-200 ng RNA was added to the first strand reaction. Using 150 ng input RNA, the amount of *Ddc* product was proportional to number of cycles for 24-27 cycles, the amount of *ple* product was proportional to the number of cycles for 26-29 cycles, and the amount of *RpL32* product was proportional to the number of cycles for 22-25 cycles. The amount of DNA present in each band was quantified using Image J (Abramoff et al., 2004).

Western blots

Organisms were macerated in running buffer (0.125 M Tris-HCl pH 6.8, 12.5% glycerol, 2.5% SDS, 1.25% β-mercaptoethanol and 0.025% Bromophenol Blue), boiled for 10 minutes, and 0.5 (TH detection), 1 (phospho-Ser32 TH detection), or 0.25 (actin detection) organism equivalents were loaded and separated on 8% denaturing acrylamide gels. Proteins were transferred to a nitrocellulose membrane (Amersham Biosciences Hybond-ECL). Membranes were blocked in Tris-buffered saline with 0.1% Tween (TBS-T) and 1% skimmed milk powder. Membranes were then incubated with either rabbit anti-TH antibody (1:3000 dilution, 3 hours, room temperature), rabbit anti-phospho-TH (Ser40) (1:2000 dilution, overnight, 4°C), or mouse anti-actin (1:10,000 dilution, 1 hour, room temperature). The rabbit anti-TH antibody was a gift from Wendi Neckameyer (St Louis University, St Louis, MO), the rabbit anti-phospho-TH (Ser40) antibody was purchased from Zymed Laboratories, and the mouse anti-actin (JLA-20) antibody developed by Jim Jung-Ching Lin was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences. The membranes were then washed with TBS-T and incubated with either a goat anti-rabbit IgG (H + L)-HRP Conjugate (Bio-Rad) or an ECL anti-rabbit IgG HRP-linked species-specific whole antibody (from donkey) (Amersham Biosciences), or a goat anti-mouse IgG (H+L)-HRP conjugate (Invitrogen) (1:1000 dilution, 1 hour, room temperature). Membranes were washed with TBS-T and developed using Supersignal West Pico Chemiluminescent Substrate (Pierce) according to the manufacturer's instructions.

Tyrosine hydroxylase activity assays

Except where indicated, all reagents were obtained from Sigma. Pools of organisms were macerated in 1× phosphate buffered saline containing 1× Complete, Mini, EDTA-free Protease Inhibitor Cocktail (Roche) (25 μl per organism). The extract was cleared by centrifugation, and a 25 μl aliquot of the supernatant was added to the reaction mixture (50 mM PIPES, pH 8.0, 25 μM L-tyrosine, 5 mM DTT, 10 μM $Fe(NH_4)_2(SO_4)_2$, 40 μg/ml catalase and 1 μCi L-[3,5-^{3}H]tyrosine (Amersham), in a total volume of 49 μl. The reaction mixture was equilibrated to 27°C for 3 minutes, and then 1 μl of 100 μM tetrahydrobiopterin or H_2O (for blanks) was added to start the reaction. The reaction was incubated at 27°C for 15 minutes. Residual L-[3,5-^{3}H]tyrosine was removed with 500 μl of 7.5% activated charcoal in 1 M HCl. Finally, 100 μl supernatant was combined with 10 ml scintillation fluid and counted.

RESULTS

Both *ple* and *Ddc* transcripts accumulate before eclosion

To determine whether control of tanning is regulated at a transcriptional level, we examined the transcriptional profile of epidermal *ple* and *Ddc* after pupariation by semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) (Fig. 3). For each indicated time point, isolated RNA was co-reverse transcribed with a *ple*- or *Ddc*-specific primer and an *RpL32*-specific primer to serve as a loading control. For *ple*, the forward amplification primer lies within the first exon, and the reverse primer is anchored within the epidermal specific third exon (Fig. 2B), so the product represents only the epidermal transcript. The *Ddc* forward and reverse primers are anchored in the first and fourth exons respectively, which are common to both epidermal and neural transcripts (Fig. 2B); however, the PCR conditions only allowed us to amplify the shorter epidermal transcript. The *yw* white pre-pupae (WPP) were collected and sampled every 12 hours until 156 hours after puparium formation (APF), approximately 2 days after eclosion occurs. Under our culture conditions, eclosion occurred, on average, at 108 hours APF. At this time, approximately half the flies had eclosed from the sample collected as WPP. Both pharate adults and eclosed adults collected at 108 hours APF were combined for RNA extraction, as we saw no difference in *ple* or *Ddc* transcript levels in these populations (data not shown). Both *ple* and *Ddc* transcript levels were high in WPP. After this stage, the profiles of transcription of each gene differed substantially: *ple* transcript levels dropped within 12 hours of pupariation, and remained low until 72 hours APF when they rose dramatically (Fig. 3A). Transcript levels remained high until 120 hours APF, approximately 12 hours after eclosion. *Ddc* transcript levels fell slowly after pupariation (Fig. 3B), reaching minimal levels 24 hours APF. Levels began to rise 60 hours APF, peaked 24 hours before eclosion and decreased thereafter.

Fig. 4. Tyrosine hydroxylase protein and activity profiles in *yw*, EH-KO, CCAP-KO, *burs^{Z1091}* and *rk^4* flies following puparium formation. Times are measured from pupariation. (**A**) Western blot of epidermal tyrosine hydroxylase (TH) from *yw* flies. An actin loading control is shown below the TH blot in this and subsequent figures. (**B**) TH activity in *yw* organisms. TH activity in this and subsequent figures is expressed as a percentage of the *yw* white pre-pupae (WPP) value. (**C**) Western blots of epidermal TH from EH-KO, CCAP-KO, *burs^{Z1091}* and *rk^4* flies. (**D**) TH activity in the same organisms as C. In B and D, black circles represent organisms within the pupal case, white circles represent adult flies. Values are means±s.e.m. of a minimum of three separate experiments.

TH protein and activity levels are high in pharate adults, drop before eclosion and rise thereafter

Transcriptional control of *ple* and *Ddc* cannot regulate the precise timing of tanning following eclosion because transcripts of both genes accumulate before eclosion. DDC has never been shown to be regulated at a translational or post-translational level, and high levels of the active enzyme are present at eclosion (Kraminsky et al., 1980). However, TH is known to be under translational and activational control in mammalian systems (Dunkley et al., 2004; Kumer and Vrana, 1996). For this reason, we looked at TH protein accumulation from pupariation until after eclosion (Fig. 4A). We staged flies as WPP and sampled the collection every 12 hours. We also separated the 108-hour APF organisms into pharate adult and eclosed adults, to examine the differences in these distinct samples. The TH antibody was not epidermal specific; however, identification of the epidermal isoform of TH was easily facilitated as it migrated much more slowly than the neural isoform. To simplify, only the epidermal isoform of TH is shown. The high level of TH in WPP decreased to an undetectable amount by 12 hours APF, and rose again 84 hours APF (Fig. 4A). Protein levels dropped before eclosion but rose sharply thereafter, remaining high for about 12 hours.

We also examined the activity profile of TH in *yw* organisms (Fig. 4B). We found that TH activity (expressed as a percentage of WPP activity) closely parallels the protein profile of TH. Enzyme activity could still be detected 132 hours APF although protein levels are below the limits of detection.

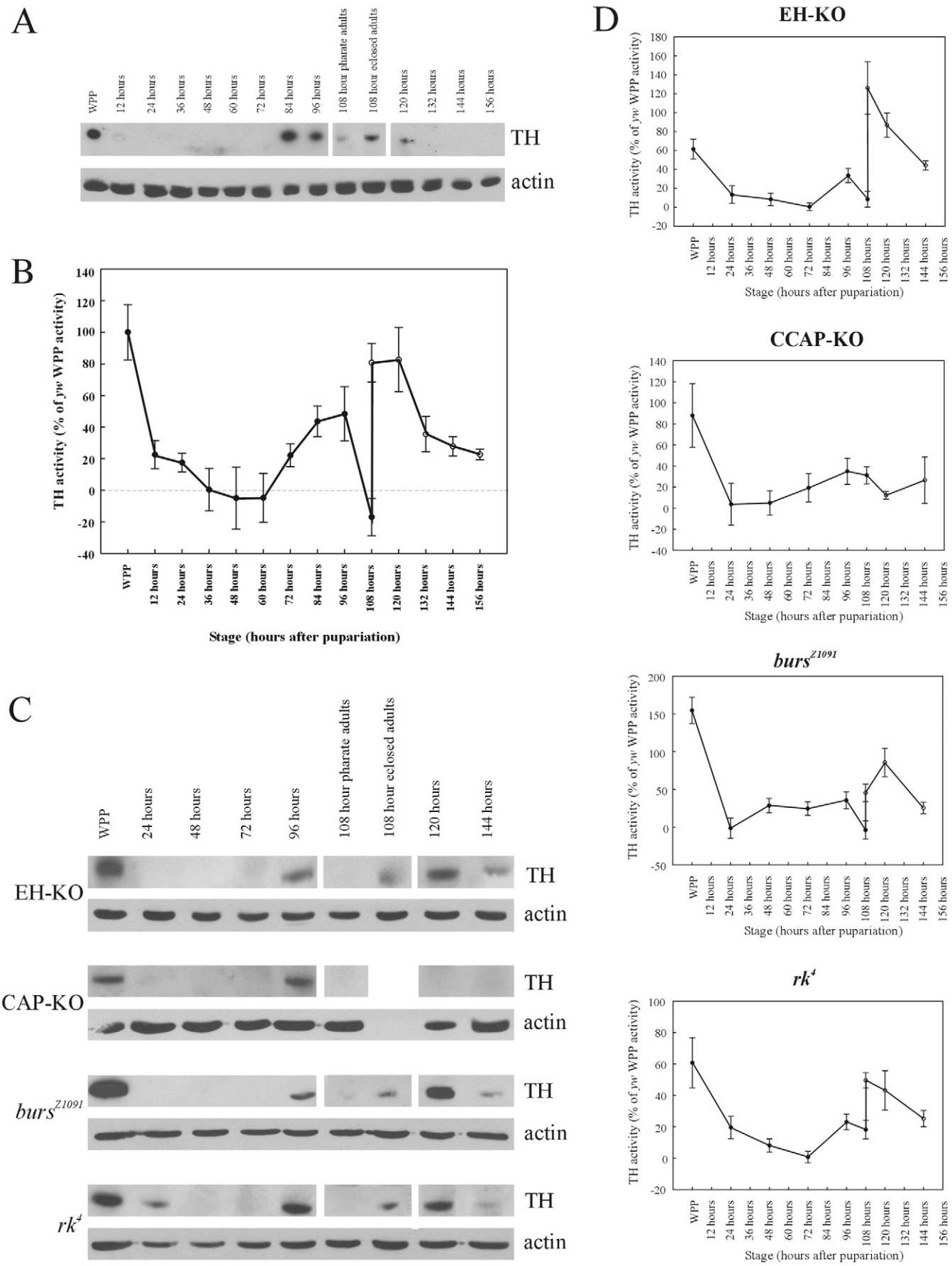

Fig. 4. See previous page for legend.

TH protein and activity levels are reduced after eclosion in CCAP-KO flies

EH-KO, CCAP-KO, *burs* and *rk* flies show defects in tanning after eclosion. To determine whether the ecdysis neuropeptide-signalling pathway (Fig. 1) plays a role in the regulation of TH, we examined the transcriptional, translational and activity profiles of TH in these flies (Fig. 4C,D). Organisms were staged as WPP and sampled every 24 hours until 144 hours APF. Again, the 108-hour time point was separated into pharate adults and eclosed adults. No CCAP-KO flies eclose by 108 hours, so this time point is absent in Fig. 4C. In the original description of EH-KO flies, it was noted that the proportion of flies that failed to expand their wings varied depending on the UAS-*rpr* line used (McNabb et al., 1997). Under our conditions, 58% (n=746) of the flies failed to expand their wings. For the experiments shown, only flies that failed to expand their wings were included. For most of our analysis, we used $burs^{Z1091}$ and rk^{4} homozygous flies, although we did some complementary heteroallelic studies using the $burs^{Z5569}$ and rk^{1} alleles. Although rk^{1} and rk^{4} mutations are null mutants because of a truncation of the receptor in the critical transmembrane domain (Baker and Truman, 2002), both $burs^{Z1091}$ and $burs^{Z5569}$ mutations are probably hypomorphic owing to single amino acid conversions that fail to completely eliminate protein function (Dewey et al., 2004). *ple* transcript levels in the EH-KO, CCAP-KO, $burs^{Z1091}$ and rk^{4} flies mimicked those exhibited by the *yw* flies (data not shown). Similar to control organisms (compare Fig. 4C with 4A), the mutants and ablation knockout flies had high levels of TH protein in WPP, which decreased to undetectable levels in the next 24-48 hours. Protein accumulated again at 96 hours but dropped in all four lines before eclosion (see Fig. 4C, 108 hour pharate adults). Protein appeared following eclosion in EH-KO, $burs^{Z1091}$ and rk^{4} flies and detectable levels persisted for longer than in the control flies. Importantly, TH protein failed to accumulate in CCAP-KO flies following eclosion.

All four mutants showed relatively normal profiles of enzyme activity during metamorphosis (Fig. 4D). EH-KO, $burs^{Z1091}$ and rk^{4} flies exhibited the rapid increase in activity seen after eclosion. By contrast, CCAP-KO flies showed severely reduced TH activity following eclosion compared with controls.

TH protein is translated within an hour of eclosion and transiently phosphorylated and activated thereafter

Two lines of evidence suggest that TH protein accumulation following eclosion is regulated: (1) TH protein levels dropped near eclosion in control flies (Fig. 4A) and rose again following eclosion, whereas *ple* transcript levels did not fluctuate during this period (Fig. 3A); (2) TH protein was absent after eclosion in CCAP-KO flies (Fig. 4C), although *ple* was transcribed normally (data not shown), indicating that CCAP may be required for TH accumulation. To determine precisely when TH protein first appears, western blotting was carried out on organisms collected at eclosion and every 30 minutes thereafter (top line, Fig. 5A). TH was absent in flies at eclosion, and was first detectable 1 hour later. Protein levels reached a maximum 2 hours after eclosion, and remained elevated until at least 12 hours after eclosion (Fig. 4A). We attribute the presence of TH in the 108-hour eclosed flies sample in Fig. 4A to organisms older than 1 hour.

The major site of activational control of *Drosophila* TH is the PKA phosphorylation site at Ser32 (Vie et al., 1999), and both CCAP and RK signalling lead to activation of PKA (Kimura et al., 2004; Luan et al., 2006). The Ser32 residue is homologous to the rat TH Ser40, so we were able to use a monoclonal antibody specific to rat phospho-Ser40-TH to determine the phosphorylation state of Ser32 (bottom line, Fig. 5A). Phosphorylated TH was detected only during a very short interval 1.5-3 hours after eclosion.

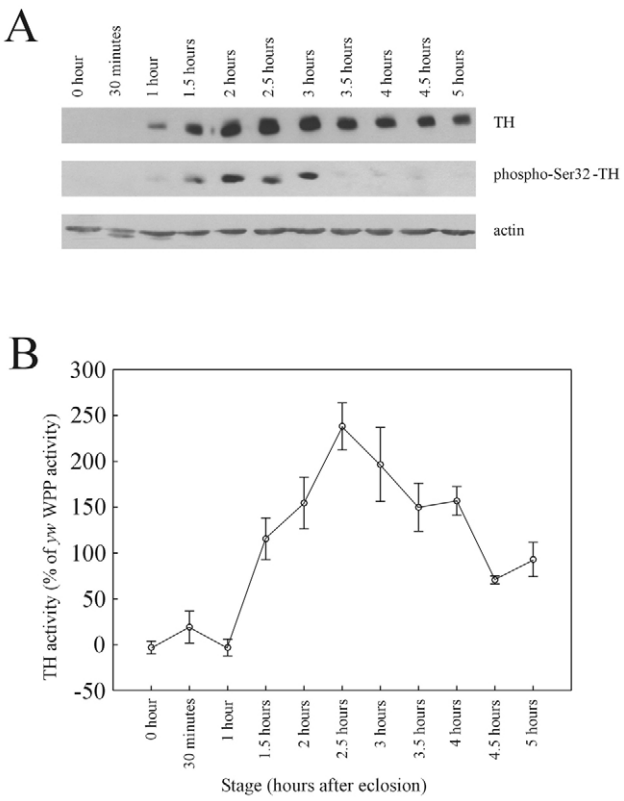

Fig. 5. TH protein and activity in *yw* flies after eclosion. (**A**) Western blots of epidermal TH protein and phospho-Ser32-TH. (**B**) TH activity in the same organisms. Values are means±s.e.m. of a minimum of three independent trials.

TH activity was low in flies at eclosion, and began to increase within 1.5 hours, peaking at 2.5 hours. This peak of activity corresponds to the time of phosphorylation and tanning.

TH phosphorylation and activity are eliminated in flies neck-ligated at eclosion

Flies neck-ligated at eclosion fail to tan, whereas those neck-ligated 30 minutes after eclosion tan normally (Fraenkel and Hsiao, 1962). The lack of tanning is due to the prevention of bursicon release (Baker and Truman, 2002; Fraenkel et al., 1966; Luo et al., 2005; Mendive et al., 2005) and can be rescued by injection of 8-Br-cAMP (Baker and Truman, 2002), a membrane permeable analog of cAMP. We sought to determine whether the rescue by 8-Br-cAMP injection was a result of the activation of the epidermal form of TH. Flies were neck-ligated at eclosion or 30 minutes following eclosion, and those treated with 8-Br-cAMP were injected immediately. All flies were aged 2.5 hours before TH western blots and activity measurements were carried out (Fig. 6A). We found that no TH protein was present at eclosion in control flies, but protein was detectable 2.5 hours later, in agreement with Fig. 5A. In the flies ligated at eclosion, less protein was apparent than in control flies, and phosphorylation of Ser32 does not occur (Fig. 6A). When flies are neck-ligated 30 minutes after eclosion, they accumulate and phosphorylate TH. TH phosphorylation is restored in flies neck-ligated at eclosion by injection of 8-Br-cAMP.

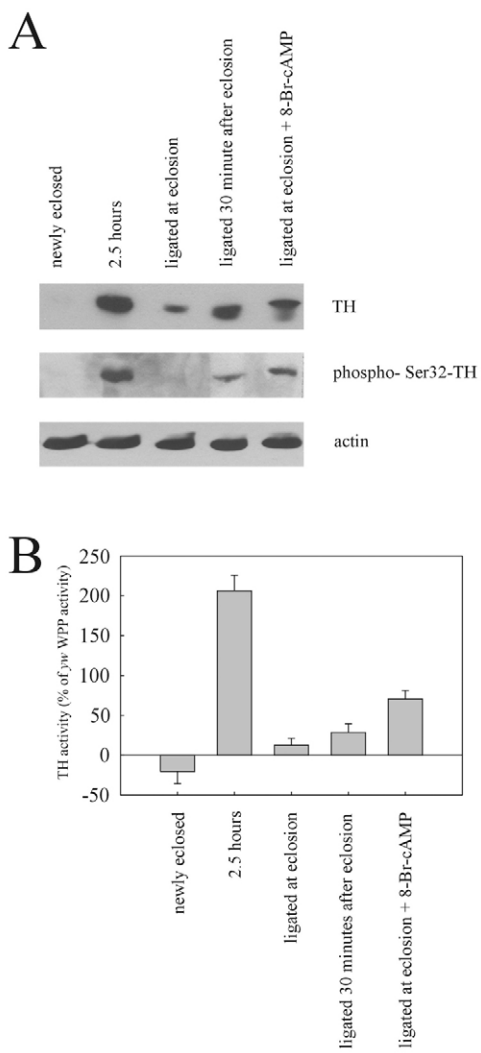

Fig. 6. TH protein, phosphorylation and activity in neck-ligated flies. (**A**) Western blots showing TH and phospho-Ser32-TH in *yw* flies neck-ligated at eclosion or 30 minutes thereafter and rescue by injection of 8-Br-cAMP. (**B**) TH activity in the same organisms. Each value is the mean+s.e.m. of a minimum of three independent experiments.

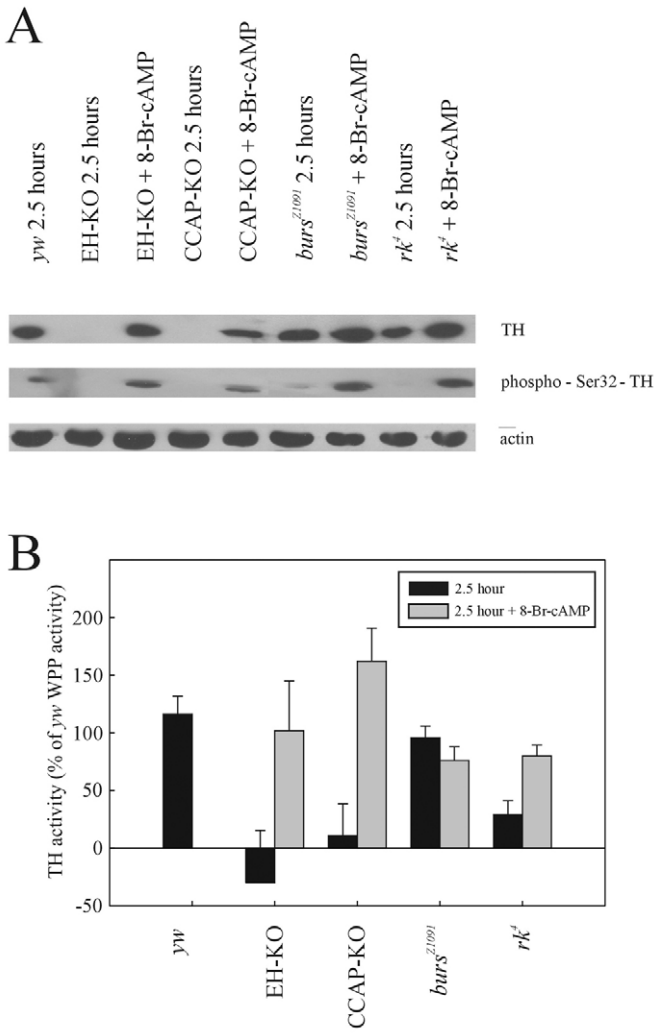

Fig. 7. Effects of 8-Br-cAMP on TH protein accumulation, phosphorylation and activity in peptide hormone mutants. (**A**) Western blots showing epidermal TH and phospho-Ser32-TH levels 2.5 hours after eclosion in untreated flies or flies injected with 8-Br-cAMP at eclosion. (**B**) TH activity in the same organisms. Each value is the mean+s.e.m. of a minimum of three independent experiments.

Neck-ligation of flies at eclosion reduces TH activity to 6% of the level in control flies (Fig. 6B). Flies neck-ligated 30 minutes after eclosion had double the activity of those neck-ligated at eclosion, and flies injected with 8-Br-cAMP had nearly sixfold higher activity than the flies neck-ligated at eclosion.

Injection of 8-Br-cAMP rescues translation, phosphorylation and activity defects in EH-KO, CCAP-KO, *burs* and *rk* flies

The absence of TH following eclosion in CCAP-KO flies (Fig. 4C), although *ple* is transcribed normally (data not shown), and the tanning defects in EH-KO, CCAP-KO, *burs* and *rk* mutants prompted us to examine whether the injection of 8-Br-cAMP affected TH levels and phosphorylation states in these mutants (Fig. 7A). We also injected CCAP-KO flies with CCAP in an attempt to rescue the defect in TH accumulation (see Fig. S1 in the supplementary material). Flies were collected 2.5 hours after eclosion, a time when TH protein, phosphorylation and activity levels are high in control organisms (Fig. 5). When EH-KO, CCAP-KO and rk^4 mutant flies were injected with 8-Br-cAMP, tanning of the adult cuticle occurred, in contrast to that observed in uninjected controls (Baker and Truman, 2002) (data not shown). We found that TH is absent in EH-KO and CCAP-KO flies, and consequently no phosphorylated protein was detected (Fig. 7A, lanes 2 and 4). This absence of TH in EH-KO flies appears to contradict the accumulation of TH evident in the 108-hour time point shown in Fig. 4C. We assume the 108-hour sample included organisms older than 2.5 hours. TH levels and phosphorylation were restored in both EH-KO and CCAP-KO flies injected with 8-Br-cAMP (Fig. 7A, lanes 3 and 5), and injection of CCAP into CCAP-KO flies caused accumulation, but not phosphorylation, of TH (see Fig. S1 in the supplementary material). Both $burs^{Z1091}$ and rk^4flies have normal levels of TH protein (Fig. 7A, lanes 6 and 8), but Ser32 phosphorylation was reduced in $burs^{Z0191}$and absent in rk^4 flies. We ruled out second-site mutation effects in the homozygous lines by showing that TH

protein levels were normal in $burs^{Z1091}/burs^{Z5569}$ and rk^{1}/rk^{4} heterozygotes, although Ser32 phosphorylation was absent (see Fig. S2 in the supplementary material). The phosphorylation defect in $burs^{Z1091}$ and rk^{4} mutants was rescued by 8-Br-cAMP injection (Fig. 7A, lanes 7 and 9).

Loss of TH protein in EH-KO and CCAP-KO flies corresponded with a loss of activity (Fig. 7B). Injection of 8-Br-cAMP restored TH activity. Activity is only slightly reduced in $burs^{Z1091}$ mutants and unchanged by injection of 8-Br-cAMP. The reduced activity in rk^{4} flies is restored by 8-Br-cAMP injection.

DISCUSSION

In *Drosophila*, the onset of tanning of the puparium occurs within 1 hour after the wandering larva becomes sessile. This requires metabolites of DA, the production of which is dependent on the actions of TH and DDC. Transcripts levels of both genes, and TH protein and activity levels, are all high in WPP (Figs 3, 4). Unlike at eclosion, TH does not appear to be activated by PKA phosphorylation for the rapid tanning of the pupal case at pupariation (data not shown). This is not unexpected because the ecdysis neuropeptides are not released until a full 12 hours APF. During the late third instar, the relatively insoluble tyrosine, which is indispensable for tanning, is stored as a more soluble derivative, tyrosine-*O*-phosphate (tyr-P) (Lunan and Mitchell, 1969). Tanning at pupariation is probably controlled by the release of tyrosine from tyr-P. No appreciable accumulation of tyr-P occurs before eclosion (Bel et al., 1992; Lunan and Mitchell, 1969), suggesting that post-eclosion tanning is switched on by a different mechanism.

We established a role for the ecdysis neuropeptide cascade in post-eclosion tanning by examining the regulation of two genes, *ple* and *Ddc*, which encode two enzymes with critical roles in tanning. We used semi-quantitative RT-PCR to examine the profile of transcription after puparium formation. Levels of both transcripts are high in WPP (Fig. 3), but they drop and then rise again before eclosion. *Ddc* levels begin to increase 60 hours APF, reach their peak 84 hours APF, and decline thereafter. DDC enzyme activity is required before eclosion for pigmentation of the pharate adult bristles and epidermis and after eclosion for tanning of the adult cuticle, and reaches a peak at eclosion (Kraminsky et al., 1980). This indicates that *Ddc* is transcribed and translated before eclosion to ensure enzyme activity is present when substrate becomes available. We investigated whether the control of substrate availability, and therefore the control of tanning, was effected by the transcriptional, translational, or post-translational regulation of TH.

Levels of *ple* transcripts are high during the 24 hour period spanning eclosion (Fig. 3A). The early appearance of *ple* transcripts is not surprising, because pigmentation of the pharate adult bristles and epidermis occurs between 84 and 96 hours APF. Both *ple* (and *Ddc*) transcription are normal in EH-KO, CCAP-KO, $burs^{Z1091}$ and rk^{4} flies (data not shown). The accumulation of *ple* transcripts before eclosion, the maintenance of high levels of TH transcription until 12 hours after eclosion and the fact that neuropeptide mutant and ablation knockout flies exhibit normal *ple* transcription, led us to conclude that the precise onset of tanning following eclosion is not due to regulation of *ple* transcription.

TH protein and activity levels are high before eclosion when pigmentation of the pharate adult bristles and epidermis occurs (Fig. 4). Levels fall rapidly just before eclosion and rise thereafter. During this entire time, *ple* transcripts are present, suggesting that protein levels are being regulated. The drop in TH protein levels may occur through repression of translation from *ple* transcripts and/or increased turnover of the protein. The complete failure of CCAP-KO flies to accumulate TH protein following eclosion (Fig. 4C), although they transcribe *ple* normally (data not shown), indicates a role for CCAP in this process. This could occur at the level of translation; alternatively, CCAP signalling may alter TH protein stabilisation. Since PKA signalling has been shown to regulate proteins involved in translational control (Denman et al., 2004), it is more likely that CCAP signalling activates PKA to cause translation, not stabilisation, of TH following eclosion.

EH-KO, $burs^{Z1091}$ and rk^{4} flies all appear to have relatively normal TH protein and activity profiles (compare Fig. 4C with 4A and 4D with 4B). Although all three exhibit a considerable range of activity in WPP, the pupal cases of these organisms tan normally. Despite the initial delay in TH accumulation in EH-KO flies following eclosion (Fig. 7A), these flies, and $burs^{Z1091}$ and rk^{4} mutants, maintain high levels of TH until 144 hours APF, a time when TH is undetectable in control flies (Fig. 4A). This persistence of TH indicates a delay in the execution of the neuropeptide hormone cascade. Interestingly, rk^{4} flies also show a delay in degradation of TH following pupariation (Fig. 4C). Perhaps there is a requirement for RK signalling to trigger TH degradation following tanning of the puparium.

Neck-ligation of flies at eclosion prevents tanning, whereas flies ligated 30 minutes after eclosion tan normally (Fraenkel and Hsiao, 1962). Furthermore, tanning of flies neck-ligated at eclosion is rescued by injection of 8-Br-cAMP (Baker and Truman, 2002). TH protein begins to accumulate 1 hour after eclosion in control flies (Fig. 5A). Phosphorylation of the protein by PKA at Ser32 leads to enzyme activity rising between 1.5 and 3 hours after eclosion (Fig. 5). We conclude that the translational and activational state of TH is responsible for controlling tanning following eclosion. We found that TH protein accumulates, but is not phosphorylated (Fig. 6A) in flies neck-ligated at eclosion resulting in reduced TH activity (Fig. 6B). Interrupting neuropeptide signalling after eclosion reveals that the element that controls TH translation is released before eclosion. The loss of TH accumulation in CCAP-KO flies (Fig. 4C and Fig. 8A) and the restoration of TH accumulation upon injection of CCAP (see Fig. S1 in the supplementary material) suggests that CCAP is responsible for inducing TH translation.

Flies neck-ligated 30 minutes after eclosion, translate and phosphorylate TH normally (Fig. 6A). By allowing neuropeptide signalling after eclosion, we have demonstrated that a factor is released within 30 minutes of eclosion that causes phosphorylation and therefore activation of TH. The reduced phosphorylation of Ser32 in $burs^{Z1091}$ flies, and complete loss of phosphorylation in rk^{4}, $burs^{Z1091}/burs^{Z5569}$ and rk^{1}/rk^{4} flies (Fig. 7A and see Fig. S2 in the supplementary material) suggests that bursicon signalling through RK controls this process. Activity levels of TH are significantly reduced in flies neck-ligated at eclosion compared with control flies (Fig. 6B). Flies ligated 30 minutes after eclosion show twofold higher levels than flies neck-ligated at eclosion and this difference probably accounts for the presence or absence of tanning. This suggests that a critical threshold of TH activity exists that is surpassed in the flies ligated at 30 minutes. Thus, although the activity present in these flies is significantly less than that in control flies, the organisms have sufficient TH activity to tan, whereas flies ligated at eclosion do not attain the threshold of activity required for tanning. Injection of 8-Br-cAMP into flies neck-ligated at eclosion rescues tanning by restoring phosphorylation and therefore activation of TH (Fig. 6). Although injection of 8-Br-cAMP does not restore TH activity

to control levels, it increases activity nearly sixfold (Fig. 6B), achieving the threshold of activity required for tanning following eclosion.

These results, taken together, suggest that at least two factors control the precise timing of tanning after eclosion. One, released before eclosion, causes translation of TH; the other, released after eclosion, causes phosphorylation and activation of TH. Both EH and CCAP are released before eclosion to control pre-ecdysis and ecdysis, respectively (Clark et al., 2004; Ewer and Truman, 1996; Gammie and Truman, 1999; Horodyski et al., 1993). EH-KO and CCAP-KO flies both exhibit extreme post-eclosion tanning defects. EH-KO flies take more than 9 hours to tan and CCAP-KO flies fail to tan (M.M.D., unpublished results). TH protein is undetectable in EH-KO flies immediately following eclosion (Fig. 7A), but these flies do eventually accumulate TH (Fig. 4C) and tan. The complete failure of CCAP-KO flies to tan, combined with the fact that CCAP-KO flies fail to accumulate TH (Fig. 5A and Fig. 8A) from the *ple* transcripts that are present at eclosion (data not shown), suggest that CCAP is responsible for inducing TH translation. The initial failure of EH-KO flies to accumulate TH (Fig. 7A) is probably caused by a failure to trigger the rapid release of CCAP (Fig. 1). Presumably, enough CCAP is eventually released in these flies to effect the translation of TH and eventually tanning, because the EH genetic ablation is leaky. Consistent with this prediction, EH-KO flies that expand their wings accumulate TH normally (data not shown), suggesting that CCAP is released normally in these flies. TH translation is restored in CCAP-KO flies injected with CCAP (see Fig. S1 in the supplementary material) and rescue of TH accumulation and phosphorylation occurs when EH-KO and CCAP-KO flies are injected with 8-Br-cAMP (Fig. 7). Rescue of both defects probably occurs because injection of 8-Br-cAMP activates PKA in CCAP target cells, thus circumventing the need for CCAP release (Fig. 1), and also activates PKA in TH-expressing cells, leading to phosphorylation and activation of TH.

Our data suggest that the post-eclosion factor causing the phosphorylation of Ser32 is the heterodimeric hormone bursicon. It is responsible for tanning and wing expansion (Dewey et al., 2004; Fraenkel et al., 1966; Luo et al., 2005; Mendive et al., 2005) and acts through its receptor RK (Baker and Truman, 2002). Consistent with the role of bursicon in the phosphorylation of TH, rk^4 flies fail to phosphorylate TH and have reduced activity (Fig. 7). These flies show a delay in tanning, taking up to 9 hours to tan (Baker and Truman, 2002). Injection of 8-Br-cAMP rescues tanning by restoring phosphorylation and therefore activation of TH (Fig. 7).

Two mutants in the α subunit of bursicon have been identified, of which one – $burs^{Z5569}$ – shows a delay in tanning in 40% of the progeny, whereas a delay is present in 82% of $burs^{Z1091}/burs^{Z5569}$ flies (Dewey et al., 2004). The $burs^{Z1091}$ mutant does not show a delay in tanning, although phosphorylation of TH is reduced in these flies (Fig. 7A). Phosphorylation of Ser32 is undetectable in $burs^{Z1091}/burs^{Z5569}$ flies (see Fig. S2 in the supplementary material), probably causing the more severe tanning defect seen in these flies. The reduced phosphorylation of TH in $burs^{Z1091}$ flies corresponds to a minor loss of TH activity (Fig. 7B). Thus, it seems that the threshold TH activity required for proper tanning is achieved in $burs^{Z1091}$ flies, although they do not have wild-type levels of TH phosphorylation or activity. Normal tanning in these flies cannot be attributed to residual activity of the β subunit of bursicon, CG15284, recently proposed to be encoded by *pu* (S. McNabb and J. Truman, personal communication), because neither subunit independently confers bursicon activity (Luo et al., 2005; Mendive et al., 2005). The $burs^{Z1091}$ allele is probably a hypomorph, and creation of a null allele would be useful. Additional studies on the activational state of TH in *pu* or *burs* null mutants will help to elucidate why tanning is not delayed in $burs^{Z1091}$ flies.

Our data indicate that CCAP is responsible for initiating TH translation following eclosion. In *Drosophila*, translational regulation often occurs through microRNA (miRNA)-dependent RNAi-mediated repression through binding sites in the 3′UTR of transcripts (Wilhelm and Smibert, 2005). Three miRNAs – let-7, mir-iab-4-3p and mir-iab-4-5p – have been predicted to regulate TH translation in *Drosophila* (Enright et al., 2003). It is conceivable that one or more of these miRNAs, in association with the RISC complex, could bind to *ple* transcripts to cause the repression of translation through a miRNA-dependent RNAi-mediated mechanism. It is also plausible that PKA, activated by CCAP signalling, might relieve repression of TH translation by phosphorylation of one of the subunits of the RISC complex or associated proteins. Future work will establish whether there is a role for these miRNAs in the repression of TH translation before eclosion.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/24/4395/DC1

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