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CREB-binding protein contributes to the regulation of endocrine and developmental pathways in insect hemimetabolan pre-metamorphosis

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ABSTRACT

Background: CREB-binding protein (CBP) is a promiscuous transcriptional co-regulator. In insects, CBP has been studied in the fly *Drosophila melanogaster*, where it is known as Nejire. Studies in *D. melanogaster* have revealed that Nejire is involved in the regulation of many pathways during embryo development, especially in anterior/posterior polarity, through Hedgehog and Wingless signaling, and in dorsal/ventral patterning, through TGF-ß signaling. Regarding post-embryonic development, Nejire influences histone acetyl transferase activity on the ecdysone signaling pathway.
Methods and results: Functional genomics studies using RNAi have shown that CBP contributes to the regulation of feeding and ecdysis during the pre-metamorphic nymphal instar of the cockroach *Blattella germanica* and is involved in TGF-ß, ecdysone, and MEKRE93 pathways, contributing to the activation of Kr-h1 and E93 expression. In *D. melanogaster*, Nejire's involvement in the ecdysone pathway in pre-metamorphic stages is conserved, whereas the TGF-ß pathway has only been described in the embryo. CBP role in ecdysis pathway and in the activation of Kr-h1 and E93 expression is described here for the first time.
Conclusions: Studies in *D. melanogaster* may have been suggestive that CBP functions in insects are concentrated in the embryo. Results obtained in *B. germanica* indicate, however, that CBP have diverse and important functions in post-embryonic development and metamorphosis, especially regarding endocrine signaling.
General significance: Further research into a higher diversity of models will probably reveal that the multiple post-embryonic roles of CBP observed in *B. germanica* are general in insects.

1. Introduction

One of the most promiscuous transcriptional co-regulators reported to date is CREB-binding protein (CBP), and its paralog in vertebrates p300, which in addition to other properties, possesses histone acetyltransferase (HAT) activity [1]. More than 400 interaction partners have been described, thus CBP and p300 proteins act as hubs in transcription networks [2]. This makes them important players in regulating cell differentiation during development, as well as decisive factors in human diseases [3–5].

In insects, the properties of these transcriptional co-regulators have only been studied in the fly *Drosophila melanogaster*, which have a single homolog of CBP, known as dCBP or Nejire. As occurs in CBP/p300 knockout mice, flies mutant for Nejire have highly pleiotropic phenotypes, indicating that it is required for multiple developmental processes [6,7]. Both oogenesis and embryogenesis require Nejire function, and RNAi depletion of Nejire expression in Drosophila kc cells leads to cell death [8].

The first roles of Nejire reported in *D. melanogaster* embryogenesis related to the regulation of the anterior/posterior polarity of embryonic segments through Hedgehog and Wingless signaling [5,6]. However, Nejire is more fundamentally involved in dorsal–ventral patterning in *D. melanogaster* embryogenesis, especially through the regulation of the TGF-ß signaling pathway. Early studies suggested that Nejire acts as a coactivator for Mad [9], a Smad protein that plays an important transducer role in the bone morphogenetic protein (BMP) branch of the TGF-ß signaling pathway [10]. Recent reports [11] point to widespread roles in dorsal–ventral patterning, targeting master regulators such as Medea (Med), which is a co-Smad common to the BMF and TGF-ß/activin branches of the TGF-ß signaling pathway [10,12].

With regard to post-embryonic development, contrasting with species where juvenile stages are similar to the adult, i.e., the hemimetabolan mode of metamorphosis, *D. melanogaster* follows the holometabolan mode, where juveniles can be extremely divergent with respect to the adults [13]. Available data into Nejire's roles in post-embryonic

Abbreviations: Bi, Bifid; Bmm, Brummer lipase; BMP, Bone Morphogenetic Protein; Burs, Bursicon; CBP, CREB-binding protein; 20E, 20-hydroxyecdysone; EcR, Ecdysone receptor; Eh, Eclosion hormone; JH, Juvenile hormone; Kr-h1, Krüppel homolog 1; LMS, Leucomyosuppresin; MEKRE93, Met-Kr-h1-E93; Med, Medea; Met, Methoprene-tolerant; PEPCK, Phosphoenol pyruvate carboxykinase; RNAi, RNA interference; Sk, Sulfakinin; T4-5, Tergites 4 and 5; T7-8, Tergites 7 and 8; TGF, Transforming Growth Factor; Tk, Tachykinin; vg, Vestigial.

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development is scarce and focused on the Nejire HAT activity and the ecdysone signaling pathway in *D. melanogaster*. In insects, ecdysone, or rather its main active form 20-hydroxyecdysone (20E), promotes the successive juvenile molts in the presence of juvenile hormone (JH), and triggers the metamorphic molt in its absence [14]. 20E exerts its action upon binding to its receptor EcR and then activating the expression of a cascade of early and late genes that codify other transcription factors in a hierarchical signaling pathway [15]. A study on *D. melanogaster* prepupae showed that Nejire catalyzes acetylation of histone H3 at lysine 23 of early genes of the 20E signaling pathway, which is required for the proper activation of them [16]. A second study carried out in Drosophila S2 cells revealed that Nejire is crucial for acetylating the histone H3 at lysine 27 of Sox14, which is a 20E-induced gene crucial for regulating dendrite pruning during metamorphosis [17].

We recently carried out transcriptome analysis on male nymph tergites 7 and 8 (T7–8) (the epidermal tissue where the adult tergal gland will be formed) of the hemimetabolan insect *Blattella germanica*, comparing metamorphic and non-metamorphic stages [18]. Results showed that CBP was significantly upregulated in metamorphic stages [18], which suggested to us that CBP may be important in the metamorphosis of this cockroach. The purpose of the present study is to test this conjecture.

As one of the most obvious results of CBP depletion was a reduction of feeding, we examined the levels of transcripts corresponding to peptides involved in feeding regulation in *B. germanica*, like tachykinin (Tk) and sulfakinin (Sk), which stimulate [19] and inhibit feeding [20], respectively. We also explored CBP's possible roles in the TGF-ß signaling pathway by examining the expression of Mad and Med, as well as that of bifid (bi, also known as optomotor blind, omb) and vestigial (vg) which are downstream targets in the BMP branch of the TGF-ß signaling pathway [21,22] and that were present in the T7–8 transcriptomes. Furthermore, we studied the effects of CBP depletion on the expression of E75A, E75B, and HR3A, early-mid response genes of the ecdysone signaling cascade in *B. germanica* [23,24], and on transcripts corresponding to peptides involved in ecdysis, such as the eclosion hormone (Eh) [25] and Bursicon (Burs), which regulate post-ecdysis cuticle tanning and wing extension [26]. In *D. melanogaster*, moreover, Burs is also expressed before the ecdysis, which points to a role of this factor in the ecdysis process itself [27]. Transcripts of Eh and Burs were also found in our T7–8 transcriptomes. Finally, we tested possible roles of CBP in the MEKRE93 pathway [28], which essentially switches metamorphosis on and off. In pre-last nymphal instars of *B. germanica*, this pathway starts with JH, whose signal is transduced by the transcription factor Methoprene-tolerant (Met) [29] and stimulates the expression of another transcription factor, Krüppel homolog 1 (Kr-h1), which represses metamorphosis [30]; thus, the molts produce a new nymphal instar. However, at the beginning of the last nymphal instar, JH production vanishes [31], transcription of Kr-h1 declines [30], and the expression of E93, a trigger of metamorphosis previously repressed by Kr-h1 [28,32], starts to increase, thus eliciting metamorphosis. During our work, we measured the expression of the two main players of metamorphosis regulation, Kr-h1 and E93, in CBP-depleted specimens.

2. Material and methods

2.1. Insects

All specimens of *B. germanica* used in the experiments were obtained from a colony reared in the dark at 30 ± 1 °C and 60–70% relative humidity. Freshly ecdysed male nymphs were selected and used at the chosen ages. Prior to injection treatments, dissections and tissue sampling, the specimens were anesthetized with carbon dioxide.

2.2. RNA extraction and retrotranscription to cDNA

Total RNA extraction was carried out from specific tissues using the miRNeasy extraction kit (QIAGEN). A sample of 100-ng from each RNA extraction was treated with DNase (Promega) and reverse transcribed with first Strand cDNA Synthesis Kit (Roche) and random hexamers primers (Roche). RNA quantity and quality were estimated by spectrophotometric absorption at 260 nm using a Nanodrop Spectrophotometer ND-1000® (NanoDrop Technologies).

2.3. Cloning and sequencing of CBP in B. germanica

To obtain the CBP cDNA (as well the cDNA of other factors studied during the present work), we combined BLAST search in *B. germanica* transcriptomes available in our laboratory (accession codes GSE63993, GSM1560373, GSM1560374, GSM1560375, SRX796238, SRX796239, SRX796244, and SRX790658), results of mapping these sequences in the *B. germanica* genome (https://www.hgsc.bcm.edu/arthropods/german-cockroach-genome-project) and RT-PCR strategies to validate the transcriptome and genome sequences. RT-PCR studies were performed using specific primers and cDNA from penultimate instar male nymphs of *B. germanica* as a template. All PCR products were subcloned into the pSTBlue-1 vector (Novagen) and sequenced.

2.4. Determination of mRNA levels by quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) reactions were carried out in triplicate in an iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories), using SYBR®Green (iTaq ™ Universal SYBR® Green Supermix; Applied Biosystems). A template-free control was included in all batches. The primers used to detect mRNA levels of all factors studied are detailed in Table S1. The efficiency of each set of primers was first validated by constructing a standard curve through four serial dilutions. Levels of mRNA were calculated relative to BgActin-5c (accession number AJ862721) expression, using the Bio-Rad iQ5 Standard Edition Optical System Software (version 2.0). Results are given as copies of mRNA per 1000 copies of BgActin-5c mRNA. Statistically significant differences between samples were assessed with the Relative Expression Software Tool (REST) [33]. This program makes no assumptions about the distributions, evaluating the significance of the derived results by Pair-wise Fixed Reallocation Randomization Test [33].

2.5. RNA interference

The procedures for RNAi experiments were as detailed previously [34]. The primers used to generate the dsRNA templates to target CBP, Tk, and Sk mRNAs are summarized in Table S1. The fragments were amplified by PCR and cloned into the pSTBlue-1 vector. A 307-bp sequence from *Autographa californica* nucleopoyhedrovirus was used as control dsRNA (dsMock). The dsRNAs were prepared as reported elsewhere [34]. A volume of 1 μL of dsRNA solution (1 or 2 μg/μL) was injected into the abdomen of specimens at chosen ages and stages with a 5-μL Hamilton microsyringe. Control specimens were treated with the same dose and volume of dsMock.

2.6. Treatment with juvenile hormone

In a series of experiments, dsRNA-treated specimens were further treated with JH III as follows. Male nymphs freshly emerged to N6 received a 1-μg dose of dsRNA (dsMock or dsCBP) and were topically treated with 10 μg of JH III (Sigma) in 2 μl of acetone. JH III is the native JH of *B. germanica* [35], and the commercial source used is a mixture of isomers containing ca. 50% of the biologically active (10R)-JH III, thus the active dose applied was around 5 μg per specimen, which is an effective dose to inhibit metamorphosis [30].

2.7. Quantification of food intake

Food intake was measured as reported previously [19] with some minor modifications. Quantifications were carried out at intervals of

24 h in groups of two sixth instar male nymphs, which were provided with a portion of food (dry dog food) of known mass; the remaining food was dried in an oven and its mass recorded. The water lost due to evaporation from a similar portion of food placed in a control box, containing only the water vial, was used as a correction factor. Results were expressed as food consumption (in mg) per nymph.

3. Results

3.1. Structure and expression of CBP in B. germanica

Based on sequences derived from the tergal gland transcriptomes of *B. germanica* [18] and using PCR strategies, we obtained a cDNA of 10,095 bp, including a complete ORF of 8409 bp whose conceptual translation rendered a 2803 amino acid protein with sequence similarity to *D. melanogaster* Nejire and CBP protein orthologs in other insect species. The N and C terminal sections of the *B. germanica* sequence contain the so-called transcriptional adaptor zinc finger (TAZ finger), and between them are found the KIX domain, the Bromodomain, the CBP/p300-type histone acetyltransferase domain, and the Zinc finger ZZ-type motif (Fig. 1A, Supplementary Fig. 1), all of which are typical of *D. melanogaster* Nejire and other CBP proteins [3,5]. Thus, we identified it as the CBP homolog of *B. germanica*.

We measured the expression of *B. germanica* CBP in tergites 7–8 of male nymphs in the sixth instar (last, N6), which are the tergites where the tergal gland is formed during metamorphosis [18] (Fig. 1B). mRNA levels fluctuated between 0.1 and 0.5 copies per 1000 copies of actin, peaking on day 2 (N6D2) (ca. 0.5 copies of CBP mRNA per 1000 copies of actin, as average). As a reference, we also measured CBP expression in tergites 4 and 5, in which no special structure is formed during metamorphosis, and observed that the pattern was relatively similar, also peaking on day 2 (ca. 1 copy of CBP mRNA per 1000 copies of actin, as average, although differences with respect to tergites 7–8 are not statistically significant) (Fig. 1B). We then measured CBP expression in different tissues, such as the brain, fat body, midgut, and wing pads, in male nymphs in N6D6. Results indicated that CBP was expressed in all these tissues at similarly low levels (Fig. 1C).

3.2. Depletion of CBP impairs growth and delays molting

In order to deplete CBP transcripts from the very beginning of N6, we treated male nymphs freshly ecdysed to the 5th instar (N5D0) with 1 μg of dsCBP (n = 10) or 1 μg of dsMock (n = 10). The dsCBP-treated insect survived until day 8–12 in the same instar, but died without molting to the 6th instar. This treatment reduced CBP expression ca. 40% in all tergites on N6D4 (Supplementary Fig. 2). Conversely, dsMock-treated specimens molted to the 6th instar after 6 days, as occurs in untreated

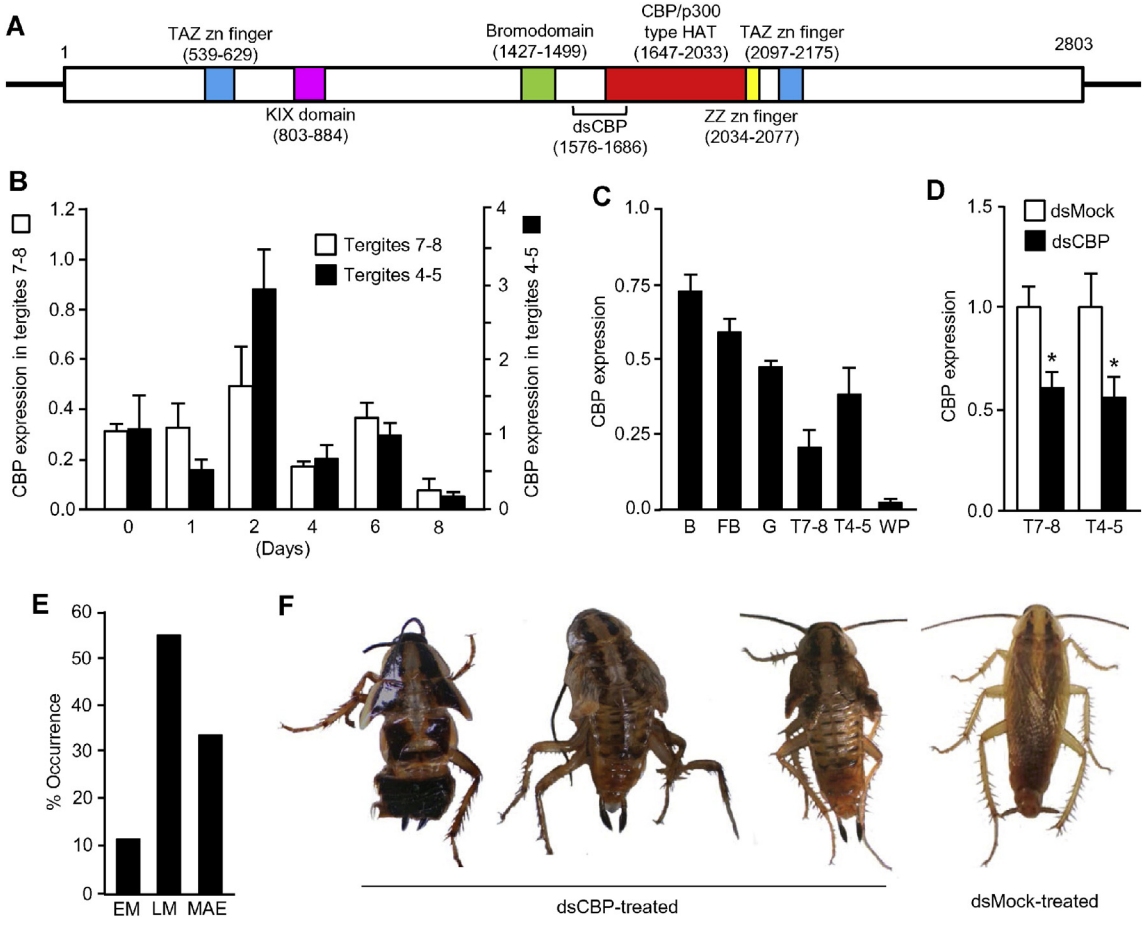

Fig. 1. *Expression and function of CBP in* Blattella germanica. (A) Scheme of the organization of CBP protein, with indication of the main functional motifs and the site where the dsRNA was designed; the lengths represented are proportional to the real lengths in amino acids. (B) mRNA levels of CBP in tergites 7–8 and 4–5 of male nymphs in last (N6) instar. (C) Expression of CBP in different tissues of male nymphs on day 6 of the last instar; B: brain, FB: fat body, G: midgut, T7–8: tergites 7–8, T4–5: tergites 4–5; WP: metathoracic wing pads. (D) Transcript decrease provoked by dsCBP treatment; male nymphs freshly emerged to N6 received a 1-μg dose of dsCBP, and transcript levels were measured on day 6 (N6D6); controls received an equivalent treatment with dsMock. (E) Percentage of specimens that died between days 6 and 8 after the treatment (early mortality, EM), between days 13 and 22 (late mortality, LM), and when molting to adults (mortality during adult ecdysis, MAE). (F) Dorsal view of adult specimens resulting from dsCBP and dsMock treatments. Each point of quantitative data in the histograms represents four biological replicates and results are expressed as the mean ± SEM; data in A, B, and C represent copies of CBP mRNA per 1000 copies of BgActin-5c mRNA; data in D are normalized against the dsMock-treated samples (reference value = 1), and the asterisk indicates statistically significant differences with respect to controls ($p < 0.05$), calculated on the basis of a Pair-wise Fixed Reallocation Randomization Test implemented in the REST [33].

specimens. In a second set of experiments, we treated male nymphs freshly ecdysed to the 6th instar (N6D0) with a dose of 1 μg of dsCBP (n = 36) or 1 μg of dsMock (n = 10). The treatment reduced CBP expression ca. 50% in all tergites on N6D4 (Supplementary Fig. 2). When transcript decrease was measured in specific tergites on N6D6, when the peak of ecdysone is produced [36], we observed that dsCBP treatment resulted in a similar decrease of CBP expression in tergites 7–8 (ca. 40%) and in tergites 4–5 (ca. 45%) (Fig. 1D). Treated specimens were observed to practically ignore food and produced fewer feces. In general, growth was deficient, and development, slower. Of the 36 dsCBP-treated animals, 4 did not molt and died between the 6th and 8th days after treatment (early mortality, EM, in Fig. 1E), 20 did not molt and died between the 13th and 22nd days after treatment (late mortality, LM, in Fig. 1E), and 12 were able to molt to adults between days 18 and 23, but died while molting (mortality during adult ecdysis, MAE, in Fig. 1E). Specimens in this third group were unable to escape from the exuvium and the wings' extension was very incomplete (Fig. 1F). All dsMock-treated specimens molted to perfect adults between 8 and 9 days after treatment.

3.3. Depletion of CBP impairs food intake

As the most apparent effect of CBP depletion was feeding impairment, we measured food intake in dsCBP-treated specimens. Daily measurements of food consumption revealed that the peak which occurred on day 3 in controls did not occur in dsCBP-treated insects; rather, food consumption remained stable at around 1 mg/specimen during the first 5 days and then decreased much more abruptly compared to the controls during days 6, 7, and 8 (Fig. 2A). Expression of enzymes involved in gluconeogenesis, e.g., phosphoenolpyruvate carboxykinase (PEPCK), and in lipidogenesis, e.g., Brummer lipase (Bmm), have been shown to increase when *B. germanica* is under fasting [37]. We thus measured the mRNA levels corresponding to these enzymes in fat body on day 5, 48 h after day 3, when differences of food consumption between controls and CBP-depleted specimens were significant. Both transcripts increased in dsCBP-treated animals (Fig. 2B), which indicates that the reduced food intake observed in CBP-depleted specimens was enough to provoke metabolic consequences derived from poor feeding. Expression of insulin receptor (InR) in the present study, which has been observed to increase in starved specimens of *B. germanica* [38], tended to increase in dsCBP-treated specimens, but differences with respect to controls were not statistically significant (Fig. 2B).

We then examined whether CBP depletion had affected the expression of tachykinin (Tk) and/or sulfakinin (Sk), which stimulate [19] and inhibit feeding [20], respectively. We also measured the expression of leucomyosuppresin (LMS), a typical myotropic peptide which has also been associated to antifeeding activities in *B. germanica* [39]. Measurement of mRNA levels of the precursors for the three peptides in brain tissues in N6D2 and N6D6 showed that those of Tk were lower and those of Sk were higher in dsCBP-treated specimens, whereas mRNA levels of LMS were unaffected by the treatment (Fig. 2C). The same brain tissue was used to measure mRNA levels of CBP, which were significantly lower (22% as average) in N6D2 and remained lower in N6D6 (23% as average), although in this case, differences with respect to controls did not result statistically significant (Fig. 2C).

The above results suggested that the low food intake phenotype observed after CBP depletion could be due, at least partially, to Tk downregulation and Sk upregulation. Pharmacological treatments with synthetic

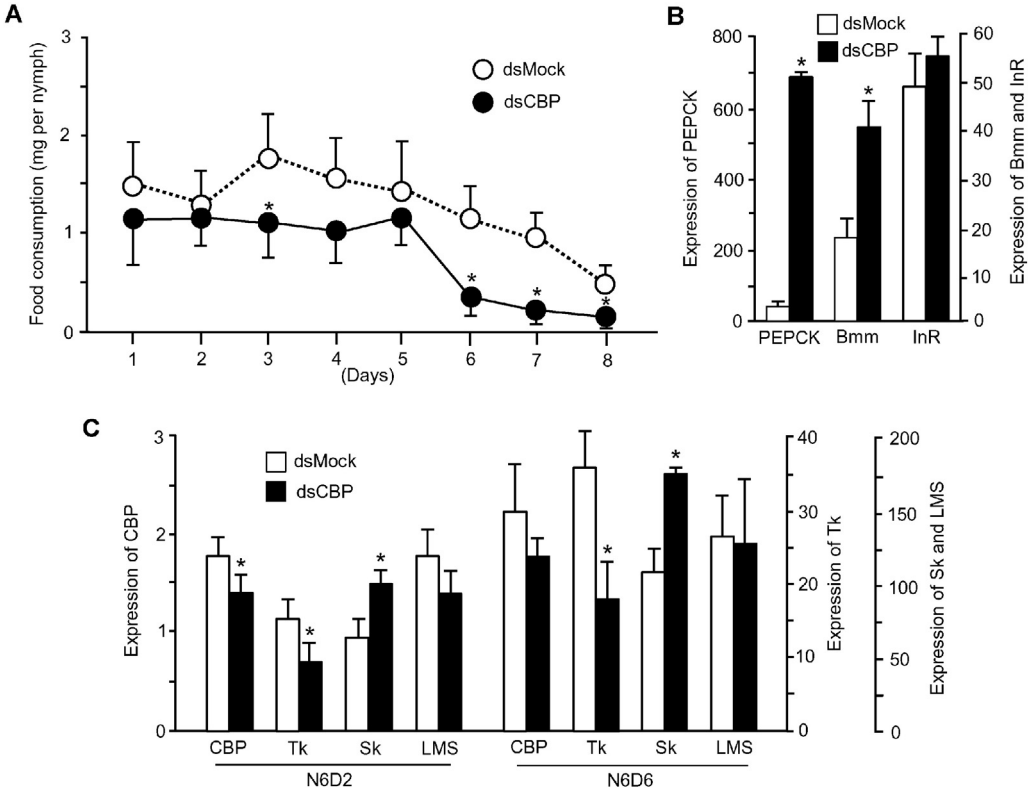

Fig. 2. *Effects of CBP depletion on food intake in* Blattella germanica (A) Daily food consumption by male nymphs during the first 8 days of the 6th nymphal instar, treated with dsCBP or with dsMock as described in Fig. 1. (B) mRNA levels of PEPCK, Bmm, and InR in male nymphs that had been treated with a 1-μg dose of dsCBP when freshly emerged to N6 and suffered from impaired food consumption; transcript levels were measured in fat body tissues on day 5 (N6D5), 48 h after N5D3, when differences of food consumption between controls and CBP-depleted specimens were significant. (D) Effects of CBP depletion on mRNA levels of Tk, Sk, and LMS; male nymphs freshly emerged to N6 received a 1-μg dose of dsCBP and transcript levels were measured in brain tissue on days 2 (N6D2) and 6 (N6D6); CBP mRNA levels are also shown. Each point of panel A represents six groups of two male nymphs each and results are expressed (mean ± SEM) as mg of food consumed per nymph. Each mRNA data point (panels B and C) represents four biological replicates and is expressed as copies of the given transcript per 1000 copies of BgActin-5c mRNA (mean ± SEM). The asterisk indicates statistically significant differences with respect to controls ($p < 0.05$), calculated on the basis of a Pair-wise Fixed Reallocation Randomization Test implemented in the REST [33].

Tk [19] and Sk [20] have shown the respective food intake stimulation and inhibition properties of these peptides. However, we wanted to assess the extent to which they are physiologically relevant in terms of feeding regulation. Firstly, we studied the expression pattern of both peptide precursors in brain tissue from N6 specimen. Results showed that both Tk and Sk have an irregular pattern presenting no clear correlation with either feeding fluctuations or the CBP expression pattern (Supplementary Fig. 3A). Using RNAi, we efficiently depleted the mRNA levels of the Tk precursor (Supplementary Fig. 3B), but food intake during N6 in Tk-depleted specimens (Supplementary Fig. 3C) only significantly decreased on N6D3, when controls were peaking. RNAi depletion of Sk precursor expression was also efficient (Supplementary Fig. 3D), but food intake remained practically unchanged (Supplementary Fig. 3E).

3.4. Depletion of CBP delays molting and modifies the expression of ecdysis factors

The considerable delay of the imaginal molt in CBP-depleted specimens suggested that ecdysone signaling was affected by the treatment. Thus, at N6D6, the stage when ecdysone levels peak in the hemolymph [36], we measured the expression of HR3A, E75A and E75B, early-mid response genes of the ecdysone signaling cascade [23,24], in all abdominal tergites. Results (Fig. 3A) showed that mRNA levels in the three factors were much lower in dsCBP-treated specimens compared to controls. This suggests that N6D6 CBP-depleted specimens were not producing the ecdysteroid peak on time, which is in agreement with the delay observed in the subsequent molt.

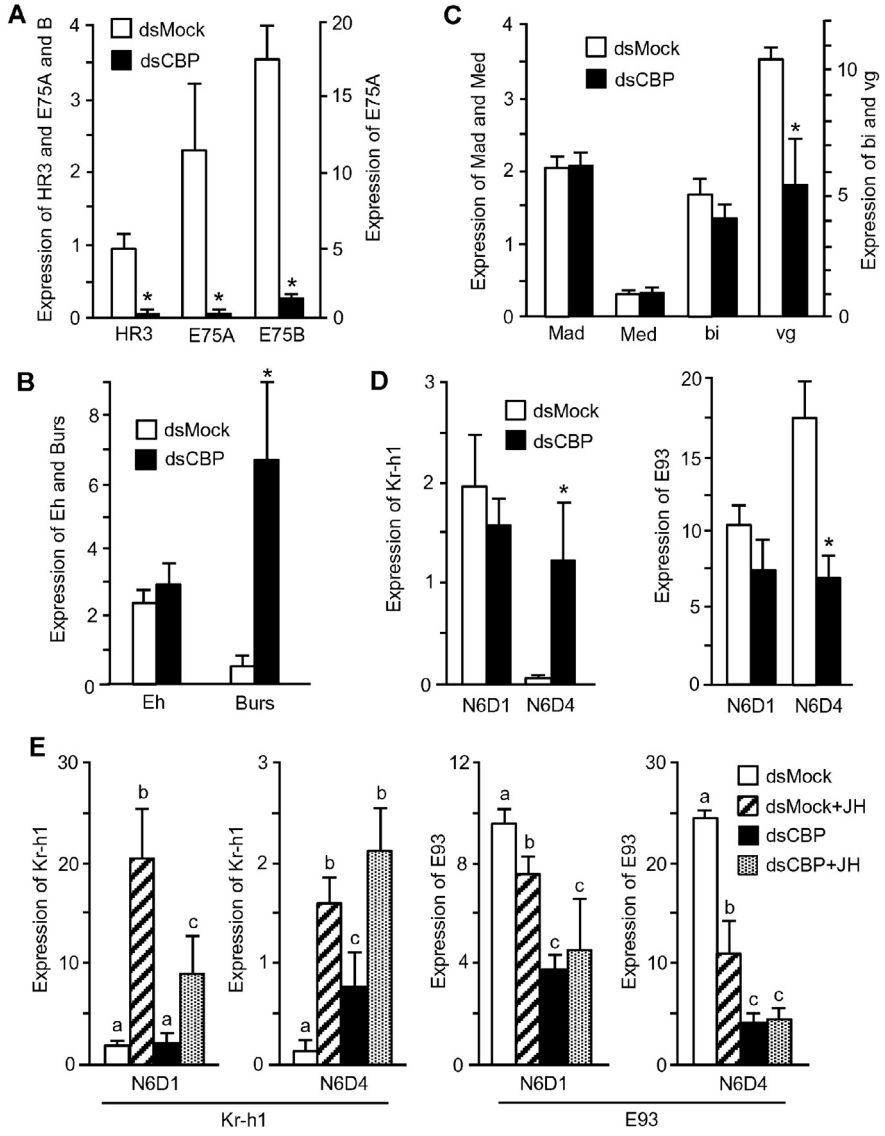

Fig. 3. *Effects of CBP depletion on ecdysone, TGF-ß, and MEKRE93 signaling pathways and in ecdysis factors in* Blattella germanica (A) Effects on the mRNA levels of HR3, E75A, and E75B ecdysone signaling factors in male nymphs that had been treated with a 1-μg dose of dsCBP when freshly emerged to N6; transcript levels were measured on day 6 (N6D6), when the ecdysteroid peak occurred in control specimens. (B) Effects of CBP depletion on Eh and Burs mRNA levels, factors involved in ecdysis; male nymphs freshly emerged to N6 received a 1-μg dose of dsCBP, transcript levels were measured during the ecdysis into adults in both control and dsCBP-treated specimens. (C) Effects of CBP depletion on mRNA levels of Mad, Med, bi, and vg, factors of the BMP branch of the TGF-ß signaling pathway; male nymphs freshly emerged to N6 received a 1-μg dose of dsCBP and transcript levels were measured on day 4 (N6D4). (D) Effects of CBP depletion on mRNA levels of Kr-h1 and E93, factors of the MEKRE93 pathway; male nymphs freshly emerged to N6 received a 1-μg dose of dsCBP and transcript levels were measured on day 1 (N6D1), when Kr-h1 starts to decline and E93 to increase in controls, and on day 4 (N6D4), when Kr-h1 expression practically vanished and E93 approaches maximum expression levels in controls. (F) Effects of JH treatment on Kr-h1 and E93 expression in CBP-depleted specimens; male nymphs freshly emerged to N6 received a 1-μg dose of dsCBP and were immediately treated with 10 μg of JHIII; controls for this experiment were equivalently treated with dsMock and JHIII; transcript levels were measured on N6D1 and N6D4. mRNA levels were measured in all abdominal tergites. Each point represents four biological replicates and is expressed as copies of the given transcript per 1000 copies of BgActin-5c mRNA (mean ± SEM). The asterisk (or different letters in panel E) indicates statistically significant differences with respect to controls ($p < 0.05$), calculated on the basis of a Pair-wise Fixed Reallocation Randomization Test implemented in the REST [33].

Moreover, the deficiencies observed in the imaginal ecdysis and wing extension of dsCBP-treated specimens led us to examine factors specifically involved in these processes, i.e., Eh and Burs. We measured the expression of both factors during the ecdysis to adults (while the insects were escaping from the exuvium), which occurred on N6D9 in controls or between N6D10 and N6D14 in dsCBP-treated specimens in these experiments. Results (Fig. 3B) revealed that while Eh mRNA levels were practically unaffected by the dsCBP treatment, those of Burs were remarkably upregulated.

3.5. Depletion of CBP impairs signaling in the TGF-β pathway and disrupts the MEKRE93 pathway

To explore whether the TGF-β signaling pathway had been affected by CBP depletion, we measured the expression of Mad and Med, as well as those of bi and vg, which are downstream targets in the pathway. Results showed that Mad, Med, and bi were unaffected, but vg expression was significantly lower in comparison with controls (Fig. 3C).

Finally, we studied the effects of CBP depletion on the two main players in the MEKRE93 pathway, the transcription factors Kr-h1 and E93, which repress and trigger metamorphosis, respectively. In *B. germanica*, during the first 24 h of the last nymphal instar, JH titer decreases, and consequently, Kr-h1 expression vanishes and that of E93 increases, which triggers adult morphogenesis (see [28]). We examined the effects of CBP depletion on Kr-h1 and E93 mRNA levels on day 1 (N6D1), when Kr-h1 starts to diminish in controls and E93 to increase, and on day 4 (N6D4), when Kr-h1 declined practically to zero in controls and E93 approaches maximum expression levels. Results demonstrated that levels of both transcripts were practically unaffected at N6D1, whereas at N6D4, levels of Kr-1 mRNA in dsCBP-treated were higher than in controls, whereas those of E93 were lower (Fig. 3D). This suggested to us that CBP depletion directly or indirectly affected the decrease of Kr-h1 and/or the increase of E93 in N6. In order to clarify this issue, we treated CBP-depleted specimens with JH on N6D0, which should stimulate Kr-h1 expression [30], in order to examine in a more direct way the effect of CBP on Kr-h1. Measurements carried out on N6D1 showed that control specimens treated with JH increased ca. 4-fold the expression of Kr-h1 and that CBP depletion reduced ca. 50% the JH-driven increase of Kr-h1 expression (Fig. 3E). On N6D4, levels of Kr-h1 mRNA were very low in controls and significantly higher in all other treatments; specimens treated with JH showed the highest levels, with no significant differences between those treated with JH and those treated with dsCBP and JH (Fig. 3E). The effect of JH treatment on E93 expression is also shown in Fig. 3E. In controls, either in N6D1 as in N6D3, JH treatment reduced E93 expression. In dsCBP-treated specimens (further treated with JH or not), the expression of E93 resulted more dramatically reduced (Fig. 3E).

4. Discussion

Our studies have shown that *B. germanica* possesses a structurally well conserved homolog of CBP. We initially detected CBP transcripts in the abdominal tergites 7 and 8 (T7-8) of male *B. germanica* nymphs, where the tergal gland is formed in the adult [18]. However, our expression studies have shown that CBP is expressed not only in other tergites (T4–5) but also in a diversity of tissues. We conclude that CBP is ubiquitously expressed in *B. germanica*.

Treatment with a 1-μg dose of dsCBP in N5 was lethal and treatment with 1 μg of dsCBP in N6 yielded ca. 30% survival. High mortality elicited by dsCBP treatments already accounts for the vital functions of CBP in *B. germanica*. Survivors of N6 molted to adult but showed severe ecdysis deficiencies, such as incomplete exuviation and unextended wings. Conversely, the morphology of the tergal glands was that CBP was initially detected [18], was normal, thus suggesting that either CBP is not directly related with morphogenesis of these glands or that the levels of depletion reached by RNAi were not enough to impair this process. Interestingly, CBP depletion elicited a dramatic upregulation of Burs during ecdysis, which suggests that CBP directly or indirectly represses Burs expression. As Burs plays important roles in ecdysis itself in *D. melanogaster* [27], their upregulation in CBP-depleted specimens may have disrupted the process and impaired the ecdysis. Moreover, the essential roles played by Burs in post-ecdysis cuticle tanning and wing extension [26] also suggest that incomplete exuviation and wing extension might have resulted by precocious tanning of the cuticle and hardening of the wings in animals undergoing ecdysis.

The most striking phenotype, however, was the reduced food intake observed in dsCBP-treated specimens. This triggered the increase in expression of enzymes involved in gluconeogenesis (PEPCK) and lipidogenesis (Bmm) to levels similar to those observed in starving *B. germanica* [37]. This indicates that dsCBP-treated specimens were suffering of poor feeding conditions. One of the consequences of reduced food intake was that the duration of N6 was considerably lengthened. Indeed, early–mid genes of the ecdysone signaling cascade, such as E75A, E75B, and HR3, were practically unexpressed at N6D6, when the ecdysone peak that triggers the adult molt is produced in control animals. This, and the actual molting time, suggests that the ecdysone peak was considerably delayed in dsCBP-treated specimens.

Two peptides associated with food intake regulation, Tk and Sk, which stimulate and inhibit the process, were shown to be downregulated and upregulated, respectively, in dsCBP-treated specimens. However, RNAi depletion of Tk or Sk expression showed that their influence in regulating food intake is rather modest in *B. germanica*. In the case of Sk, this contrasts with data described for the beetle *Tribolium castaneum*, where RNAi of Sk resulted in a consistent increase of food intake [40]. It appears that food intake regulation in *B. germanica* does not heavily rely on Sk or Tk and that stimulatory or inhibitory effects on food intake, provoked respectively by treatments with Tk [19] or Sk [20], were pharmacological rather than physiological. In any case, our data indicate that CBP may directly or indirectly influence the expression of the precursors of these two peptides, but full food intake regulation mainly appears to rely on other factors, the regulation of which might also involve CBP.

Our studies in the last nymphal instar of *B. germanica* also revealed that CBP depletion reduces the expression of vg in epidermal tissues, a gene that is regulated by the BMP branch of the TGF-β signaling pathway [22]. This is consistent with data obtained from *D. melanogaster* embryos, which indicate that CBP is a coactivator for Mad and Med [9,11,41], which are important transducers in the TGF- β pathway. The effect of CBP depletion on TGF-β signaling pathways (especially the TGF-β/activating branch through Med) may have also contributed to the delay in molting, as it has been shown that this pathway is involved in regulating the timing of the metamorphic molt in *D. melanogaster* by influencing ecdysone production [42,43]. Moreover, CBP depletion may have directly impaired the expression of genes of the 20E signaling pathway, as demonstrated in *D. melanogaster* for E74 and E75 [16], or affected the transducer activity of EcR, as shown also in *D. melanogaster* [17]. The latter activity is reminiscent of the modulatory action of CBP upon the activity of steroid receptors described in mammals [44]. Another possibility to explain our results is that CBP affects the ecdysone biosynthesis, as occurs in mammals, where CBP in cooperation with other partners regulates the first step of steroid synthesis by modulating the expression of cytochrome P450 enzymes [45,46]. This possibility has been unexplored in insects and would deserve specific research.

Finally, we examined whether CBP plays a role in the MEKRE93 signaling pathway [28]. Our results demonstrated that CBP depletion in N6 of *B. germanica* impaired the decrease of Kr-h1 expression and the increase of E93 expression that normally occurs during the first 24 h of N6. Although lower than normal, the E93 expression levels were apparently high enough to trigger adult morphogenesis as the insects molted into an adult phenotype (Fig. 1F). The experiments treating dsCBP-treated specimens with JH showed that CBP depletion reduces the JH-driven increase of Kr-h1 (Fig. 3E), which suggests that CBP contributes

to Kr-h1 expression, possibly acting as a coactivator. The same experiments showed that JH treatment impaired the expression of E93, which can derive from the stimulation of Kr-h1 in this experiment, as Kr-h1 represses the expression of E93 [28]. In CBP-depleted specimens, however, either treated with JH or not, E93 expression resulted notably reduced (Fig. 3E), which suggests that CBP might act as an activator also of E93.

In insects, CBP functions have been studied only in *D. melanogaster*, where data available suggested that the main functions of CBP are concentrated in the embryo, especially in anterior/posterior polarity, through Hedgehog and Wingless signaling, and also in dorsal/ventral patterning, through TGF-ß signaling [5–7,9,11,41]. In *D. melanogaster* post-embryonic development, known data on CBP are limited to two contributions that describe the influence of CBP HAT activity on the ecdysone signaling pathway [16,17]. In the present work, we have demonstrated that CBP influences a significant number of regulatory pathways in the pre-metamorphic nymphal instar of the cockroach *B. germanica*. This includes those involved in regulating feeding, of which we have incomplete knowledge, as well as in the ecdysis, TGF-ß, ecdysone, and MEKRE93 pathways, in the latter case contributing to the activation of Kr-h1 and E93 gene expression. CBP's participation in the ecdysone pathway is conserved in *D. melanogaster* pre-metamorphic stages. Its participation in the TGF-ß pathway has been described in *D. melanogaster* embryo but not in post-embryonic development. CBP's influence over ecdysis and MEKRE93 pathways is described here for the first time.

5. Conclusions

Studies in the fly *D. melanogaster* may have suggested that CBP functions in insects are concentrated in the embryo. Results obtained in the cockroach *B. germanica* indicate, however, that CBP have diverse and important functions in post-embryonic development and metamorphosis, especially in endocrine signaling. We predict that further research into a higher diversity of models, including *D. melanogaster*, will reveal that the multiple post-embryonic roles of CBP observed in *B. germanica* are general in insects.

Transparency document

The Transparency document associated with this article can be found, in the online version.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2015.12.008.

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SUPPLEMENTARY DATA

CREB-binding protein contributes to the regulation of endocrine and developmental pathways during insect hemimetabolan pre-metamorphosis

Ana Fernandez-Nicolas and Xavier Belles

CONTENTS

TGDMADHLVDGPPSKRQKMTDPFQGTSDSSVPMLMHFNLAPTLGNNGGGDKQQQQLLHLQQQWNPQKRNQFTNMDMFD
LENDLPDELMTSGSWGSSVESTASSKPPATGPGPGPGPGQQQQAQQQQQTGAGGPGLQNGAVDQQQQPQSTDPQRQQQ
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KGLKRKEN**KFNAKRLPVTKLGAYIEARVNNFLKKKEAGAGEVAIRVVA**SSDKIVEVKPGMRNRFVEPGDLPDQFPYRA
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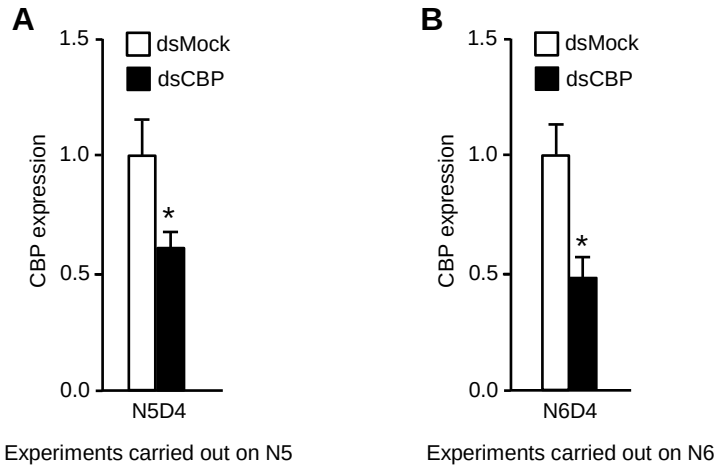
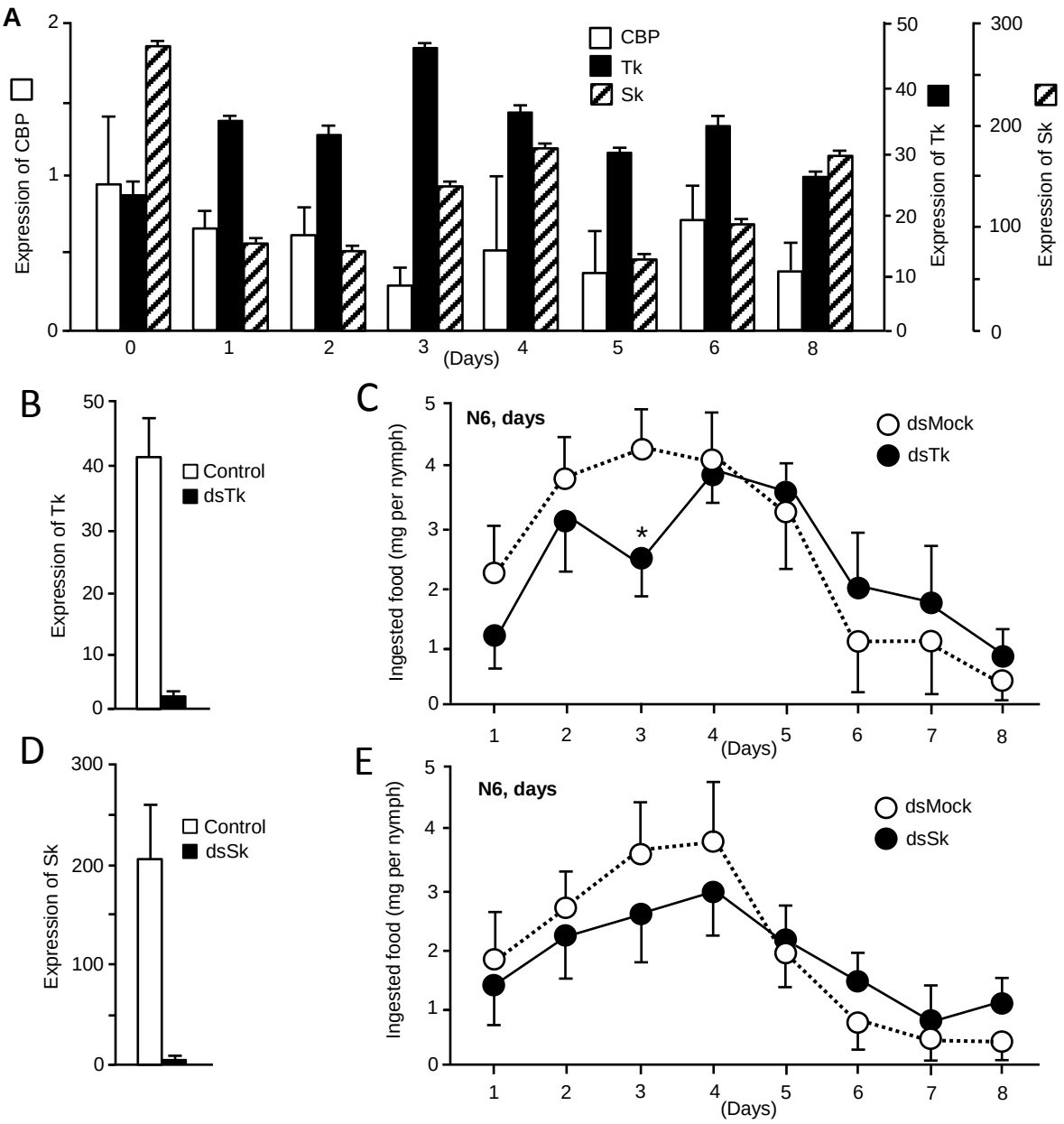
GGSSEMMLSQLSGGAAGQQHGAGTGMPHHPSPAGAPPGSAGSSGQGPTAGGSDPNEVPPMTPQDKLTKFVEQL

Supplementary Figure 1. Sequence of *Blattella germanica* CBP with the main functional motifs highlighted. TAZ zn finger: blue, KIX domain: fucsia, Bromodomain: green, CBP/p300-type histone acetyltransferase domain: red, ZZ zn finger: yellow. The region where the dsRNA was designed is in bold italics and underlined.



Supplementary Figure 2. Transcript decrease elicited by dsCBP treatment in N5 and N6. (A) In the experiments carried out on N5 male nymphs freshly emerged to N5 received a 1-µg dose of dsCBP, and transcript levels were measured in all abdominal tergites on day 4 (N6D4). (B) In the experiments carried out on N6 male nymphs freshly emerged to N6 received a 1-µg dose of dsCBP, and transcript levels were measured in all abdominal tergites on day 6 (N6D4). In both cases, controls received an equivalent treatment with dsMock. Each value represents 5 biological replicates and is expressed as copies of CBP per 1000 copies of BgActin-5c mRNA (mean ± SEM). Data are normalized against the dsMock-treated samples (reference value = 1), and the asterisk indicates statistically significant differences with respect to controls ($p < 0.05$), calculated on the basis of a Pair Wise Fixed Reallocation Randomisation Test implemented in the REST [34].



Supplementary Figure 3. Contribution of tachykinin and sulfakinin to feeding regulation in *Blattella germanica*. (A) mRNA levels of Tk and Sk in brain tissues of male nymphs during the last (N6) nymphal instar; CBP expression is also shown as a reference. (B) Transcript decrease provoked by dsTk treatment; male nymphs freshly emerged to N6 received a 1-µg dose of dsTk, and transcript levels were measured on day 4 (N6D4) in brain tissues; controls received an equivalent treatment with dsMock. (C) Daily food consumption by male nymphs during the 8 first days of the 6th nymphal instar, treated with dsTk or with dsMock. (D) Transcript decrease provoked by dsSk treatment; procedure was as described in panel B. (E) Daily food consumption by male nymphs during the 8 first days of the 6th nymphal instar, treated with dsSk or with dsMock. Each point of mRNA measurement (panels A, B and D) represent 4 biological replicates and is expressed as copies of the given transcript per 1000 copies of BgActin-5c mRNA (mean ± SEM). Each point of panels C and E represents 6 groups of two male nymphs each, and results are expressed (mean ± SEM) as mg of consumed food per nymph. In all cases, the asterisk indicates statistically significant differences with respect to controls ($p < 0.05$), calculated on the basis of a Pair Wise Fixed Reallocation Randomisation Test implemented in the REST [34].

Supplementary Table 1. Primers used to detect the transcripts studied, and to prepare the dsRNAs to target CBP, Sk and Tk.

Primer set	bp	Forward primer (5'-3')	Reverse primer (5'-3')	GenBank accession number
Bmm	90	GACATCTGTCCCCGTGATTTG	CCTGTAGATATTGTGGCGAGACAA	HG005308.1
Burs	158	AATGGACGAGTGTCAGGTGACA	AACACATGCATGAACGTTCCAT	LN901328
CBP	131	AAAATGTCCCGTGCCATTCT	ATGGCACTGTTGATCTCGTG	LN901329
E75A	101	GTGCTATTGAGTGTGCGACATGAT	TCATGATCCCTGGAGTGGTAGAT	AM238653.1
E75B	168	CGCAATCCAGTTCAACTCAG	CCGGAAGAATCCCTGTGAAT	AM238654.1
E93	143	TCCAATGTTTGATCCTGCAA	TTTGGGATGCAAAGAAATCC	CCM97102.1
Eh	180	GTTGTACCTGGTCATTCTTATC	GGATTGTTGCAATCAGGCAAA	LN901330
HR3	101	GATGAGCTGCTCTTAAAGGCGAT	AGGTGACCGAACTCCACATCTC	CAJ90622.1
InR	109	CACAGGGCCTAATTCCACAGA	ACAGCGCCGGTTCAGATACTT	HG518668.1
Kr-h1	77	GCGAGTATTGCAGCAAATCA	GGGACGTTCTTTCGTATGGA	CCC55948.1
LMS	185	AATGAAGTACGTCAGCGTAGTT	AACTTTCAAATGCGATGGAAG	CAF04070.1
Mad	148	TGGATGCTGATGAAGCGGAA	TCAACAGCCTTTTCTGCCCA	LN901331
Med	71	AGGTGTGGTGGGAGGTACTG	TTGTTTGAGGAACCGTGTGA	LN901332
bi	154	GATCCTCAACTCGATGCACA	TGCGTGATCTTTTCGTTCTG	LN901333
PEPCK	151	ATCTTGCCATGATGTGCCCT	TTATTTGATGTGCCTGGTGCA	HG005312.1
Sk	120	TCAACGATTTTGTTATTGACGA	CAAATCTCATGTGCCCATAAT	P85555.1
Tk	186	CAGATGAATTAAACGATGTACT	GACCTCTCTTATCCAAATATTC	LN901334
vg	250	AACTGTGTGGTGTTCACTCACT	AAGGAGGGAAGTTGCGAGC	LN901335
dsCBP	330	GATGATCCTACTCAGCCGCA	GCAACAACACGAATTGCTACCT	LN901329
dsSk	258	AGCTACACTATTAGTGACCTTA	TTACTGAAGTCCACAACATCA	P85555.1
dsTk	454	AAAGAAGACTTTGAAGCAGAAG	TTTCAGGTTCAGTTCCCGTTT	LN901334