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DPP-mediated TGFβ signaling regulates juvenile hormone biosynthesis by activating the expression of juvenile hormone acid methyltransferase

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SUMMARY

Juvenile hormone (JH) biosynthesis in the corpus allatum (CA) is regulated by neuropeptides and neurotransmitters produced in the brain. However, little is known about how these neural signals induce changes in JH biosynthesis. Here, we report a novel function of TGFβ signaling in transferring brain signals into transcriptional changes of *JH acid methyltransferase* (*jhamt*), a key regulatory enzyme of JH biosynthesis. A *Drosophila* genetic screen identified that Tkv and Mad are required for JH-mediated suppression of *broad* (*br*) expression in young larvae. Further investigation demonstrated that TGFβ signaling stimulates JH biosynthesis by upregulating *jhamt* expression. Moreover, *dpp* hypomorphic mutants also induced precocious *br* expression. The pupal lethality of these *dpp* mutants was partially rescued by an exogenous JH agonist. Finally, *dpp* was specifically expressed in the CA cells of ring glands, and its expression profile in the CA correlated with that of *jhamt* and matched JH levels in the hemolymph. Reduced *dpp* expression was detected in larvae mutant for *Nmdar1*, a CA-expressed glutamate receptor. Taken together, we conclude that the neurotransmitter glutamate promotes *dpp* expression in the CA, which stimulates JH biosynthesis through Tkv and Mad by upregulating *jhamt* transcription at the early larval stages to prevent premature metamorphosis.

KEY WORDS: *Drosophila melanogaster*, *Broad*, TGFβ, JH acid methyltransferase, JH biosynthesis

INTRODUCTION

Juvenile hormone (JH) coordinates with 20-hydroxyecdysone (20E) in regulating insect molting and metamorphosis. The molting process is orchestrated by 20E, whereas the nature of the molt is determined by JH during critical JH-sensitive periods. In the presence of JH, 20E induces larva-larva molt, whereas in the absence of JH, 20E promotes larva-pupa or pupa-adult metamorphosis (Gilbert et al., 2000; Riddiford et al., 2003). The recent progress in our understanding of JH molecular action clarifies the function of Methoprene-tolerant (Met) and Krüppel-homolog 1 (Kr-h1) in transducing JH signaling. The *Drosophila Met* gene encodes a bHLH-PAS protein family member, which is proposed to be a component of the elusive JH receptor (Wilson and Ashok, 1998). Kr-h1 is considered to be a JH signaling component that works at downstream of Met (Minakuchi et al., 2008; Minakuchi et al., 2009).

JH biosynthesis is regulated at three closely linked steps. In the first step, developmental, environmental and physiological cues are received by the central nervous system, which determines the appropriate rate of JH synthesis (Riddiford, 1993). In the second step, the brain transfers these signals to mediate JH biosynthesis in an endocrine gland, the corpus allatum (CA). It has long been thought that JH biosynthesis is regulated primarily by two neuropeptides secreted by brain neurosecretory cells: allatotropin (AT) and allatostatin (AST), which stimulate and inhibit JH synthesis, respectively (Stay, 2000; Weaver and Audsley, 2009). Nevertheless, no AT-like neuropeptides or AT receptor genes have been found in the *Drosophila* genome thus far (Nassel, 2002; Hauser et al., 2006; Liu et al., 2006; Yamanaka et al., 2008). Although AST-like neuropeptides exist in *Drosophila* (Lenz et al., 2000), their function of inhibiting JH biosynthesis has not been demonstrated. Alternatively, the brain may directly control JH biosynthesis through neurotransmitters. For example, studies in cockroaches and *Drosophila* revealed that glutamatergic nerves innervate CA cells and that the N-methyl-D-aspartate subtype of glutamate receptors (NMDAR) are expressed in both the brain and CA. Additionally, glutamate and NMDA were shown to stimulate JH synthesis in vitro (Chiang et al., 2002). In the final step of JH biosynthesis regulation, the brain signals received in the CA should be translated into changes in the expression and/or activity of key regulatory JH biosynthetic enzymes, which directly determine the rate of JH biosynthesis. However, there are major voids in our current understanding of the pathways that lead from brain signals to the activities of JH biosynthetic enzymes.

The evolutionarily conserved TGFβ signaling pathway modulates a wide range of cellular processes, including proliferation, differentiation, migration, apoptosis and cell fate speciation (Kingsley, 1994; Massagué et al., 2000). In addition, studies in *C. elegans* reveal that the TGFβ signaling pathway controls dauer formation through modulation of dafachronic acid synthesis (for a review, see Hu, 2007). Here, we present a novel, gradient-independent function of Dpp, a TGFβ ligand, in controlling JH biosynthesis. Dpp-Tkv-Mad-mediated TGFβ signaling in the CA serves as a bridge to connect brain-derived neurotransmitter signals to the transcriptional changes of JH acid methyltransferase (JHAMT), a key regulatory enzyme of JH biosynthesis (Shinoda and Itoyama, 2003; Sheng et al., 2008).

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MATERIALS AND METHODS

Fly strains and genetics

All fly strains were grown on standard cornmeal/molasses/agar medium at 25°C. The *GAL4-PG12* line is a gift from H.-M. Bourbon (Bourbon et al., 2002). All lethal mutant lines used in the genetic screen were obtained from the Bloomington *Drosophila* Stock Center. The alleles for *dpp*, *tkv*, *Mad*, *Met*, *Kr-h1* and *Nmdar1* used in this study are dpp^{s11}, dpp^{d5}, tkv^{7}, tkv^{8}, tkv^{k16713}, Mad^{1-2}, Mad^{8-2}, Mad^{k00237}, $Mad^{kg00581}$, Met^{27} (a gift from T. Wilson, Ohio State University, Columbus, OH, USA), $Kr\text{-}h1^{k04411}$, $Kr\text{-}h1^{10642}$, $Nmdar1^{05616}$ and $Nmdar1^{DG23512}$. RNAi lines of these genes, including *UAS-dpp RNAi*, *UAS-tkv RNAi*, *UAS-Mad RNAi*, *UAS-jhamt RNAi* and *UAS-Nmdar1 RNAi*, were also obtained from the Bloomington *Drosophila* Stock Center. Other fly lines used in this study include *hs-GAL4*; *Dscam-GAL4* (Wang et al., 2004); *GAL4-Aug21* (Mirth et al., 2005); *UAS-Mad* (a gift from S. J. Newfeld, Arizona State University, Tempe, AZ, USA); and *UAS-dpp* (Bloomington *Drosophila* Stock Center).

Fly stocks used for the creation of fat body MARCM clones include *FRT40*; *FRT40, tkv*^{8}*/Cyo* (a gift from K. Moses, Emory University, Atlanta, GA, USA); *FRT40,* Mad^{8-2}*/Cyo*; *FRT40,* $Kr\text{-}h1^{10642}$*/Cyo*; *hs-Flp, UAS-mCD8GFP*; and *FRT40, tub-GAL80*. MARCM clones in the fat body were induced by *hs-Flp* through heat shock-independent induction as previously described (Britton et al., 2002). To generate *jhamt-GAL4* transgenic flies, a 2 kb *jhamt* promoter was isolated by genomic DNA PCR, fused with GAL4 cDNA and inserted into *pCaSpeR4*. Transgenic fly lines were generated by *P* element-mediated germline transformation at Rainbow Transgenic Flies (Camarillo, CA, USA).

Immunohistochemistry and microscopy

Larval fat bodies were dissected from the 2nd or 3rd instar larvae. Immunohistochemistry was performed as described previously (Wang et al., 2002). Florescence signals were captured with a Zeiss LSM510 confocal microscope (Carl Zeiss) and processed using Adobe Photoshop.

JHA treatment and JHAMT activity assay

The JHA pyriproxifen (Sigma) was dissolved in 95% ethanol to give a 300 ppm stock solution. JHA-containing fly food was prepared by adding JHA stock solution to the standard cornmeal-molasses-yeast food at 50-55°C to a final concentration of 0.1 ppm or as indicated. JHAMT activity in the brain-ring gland complex was measured as previously described (Liu et al., 2009).

Western blotting and quantitative real-time PCR

Protein extracts isolated from 2nd instar larvae were analyzed by standard SDS-PAGE and western blot. The expression of β-tubulin was used as a loading control. Br mouse monoclonal antibody Br-core (25E9.D7) (Emery et al., 1994) and β-tubulin mouse monoclonal antibody (AA12.1) were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa.

Total RNA samples were prepared from the whole body for 2nd instar larvae or ring glands for 3rd instar larvae and pupae. Primers used are listed in Table S1 in the supplementary material. Quantitative real-time PCR was performed using the LightCycler 480 SYBR Green I Master Kit (Roche). The mRNA levels of different genes were normalized to *actin* mRNAs, with three replicates for each sample.

RESULTS

A genetic screen identifies that Tkv and Mad regulate *br* expression

The *broad* (*br*) gene has been identified as a key regulator in mediating the crosstalk between 20E and JH signaling pathways. Studies in *Manduca* indicated that the expression of *br* is directly induced by 20E, but this induction can be prevented by the presence of JH (Zhou et al., 1998; Zhou and Riddiford, 2002). Therefore, in many tissues, *br* is predominantly expressed during the larval-pupal transition when 20E is high and JH is absent (Huet et al., 1993). To identify genes involved in JH biosynthesis or JH signaling, we developed a *Drosophila* genetic screen to isolate mutations that derepress *br* expression in the 2nd instar larvae. We reasoned that mutations that block JH biosynthesis or disrupt JH action should reduce JH activity and cause precocious *br* expression.

To monitor *br* expression in live organisms, we examined the expression patterns of GAL4 enhancer-trap lines inserted near the *br* gene. One of these lines, *GAL4-PG12*, closely resembles the temporal and spatial expression pattern of the endogenous *br* gene in tissues other than the salivary gland. In the genetic screen, *GAL4-PG12>UAS-mCD8GFP* on the X chromosome was used as a reporter of *br* expression, and lethal mutations or *P*-insertions on the 2nd or 3rd chromosome were made homozygous and screened for precocious *br* expression. Because most of the lethal lines allowed organisms to develop to early larval stages, we were able to examine GFP expression in the 2nd instar under a fluorescent microscope.

From 4400 lethal lines, 55 mutations were isolated based on GFP expression in the 2nd instar larvae. Genes associated with these mutations encode proteins with various molecular functions, including enzymes, signal transduction molecules, transcriptional factors and others. Some of them are known to be involved in JH biosynthesis, such as farnesyl diphosphate synthase (FPPS) (Sen et al., 2007) and NMDAR1 (Chiang et al., 2002).

Among these 55 genes were two main components of TGFβ signaling, *thick vein* (*tkv*) and *Mothers against Dpp* (*Mad*) (Raftery and Sutherland, 1999). As shown in Fig. S1 in the supplementary material, the expression of *GAL4-PG12>UAS-mCD8GFP* was restricted to salivary glands in the wild-type 2nd instar larvae, but ubiquitous expression of *GAL4-PG12>UAS-mCD8GFP* was detected at the same stage of the *tkv*, *Mad* and *Nmdar1* mutant larvae. Consistently, when assessed with Br-core antibody staining, endogenous Br proteins were not detectable in the fat body (FB) of wild-type 2nd instar larvae but were observed in the FB nuclei of both tkv^{k16713} and Mad^{k00237} mutant larvae (Fig. 1A). To further test this finding, we examined other *tkv* and *Mad* alleles, including tkv^{7}, tkv^{8}, Mad^{1-2}, Mad^{8-2} and $Mad^{kg00581}$. Precocious *br* expression was detected in all cases. These results suggest that Tkv- and Mad-mediated TGFβ signaling is required to repress *br* expression at the early larval stages, possibly through regulating JH titer or signaling.

The same genetic screen also isolated genes that are involved in JH signaling, such as *Kr-h1* (see Fig. S1 in the supplementary material; Fig. 1A). Another known JH signaling component, Met, was not analyzed by this screen because the *Met* gene is located on the X chromosome. A reverse genetic study showed that precocious *br* expression was also detectable in *Met* mutant larvae (Fig. 1A).

Exogenous JH agonist prevents precocious *br* expression in *tkv* and *Mad* mutants

We next asked whether the effects of *tkv* and *Mad* mutations on *br* expression were caused by the decrease in JH titer. Based on this hypothesis, we expected that precocious *br* expression in the *tkv* and *Mad* mutant larvae would be blocked by exogenous JH agonist (JHA). Wild-type, *tkv* and *Mad* larvae were reared on a diet containing 0.1 ppm pyriproxifen, an efficient JHA (Riddiford and Ashburner, 1991). The Br-core antibody was used to detect Br proteins in the 2nd instar larvae. Immunohistochemical results revealed that the precocious *br* expression was suppressed by exogenous JHA in the FB of *tkv* and *Mad* mutants (Fig. 1A). However, the precocious *br* expression in *Kr-h1* and *Met* mutants was not suppressed by exogenous JHA, which demonstrates that Met and Kr-h1 function as the JH signaling components in mediating *br* expression (Wilson and Ashok, 1998; Minakuchi et al., 2008; Minakuchi et al., 2009) (Fig. 1A).

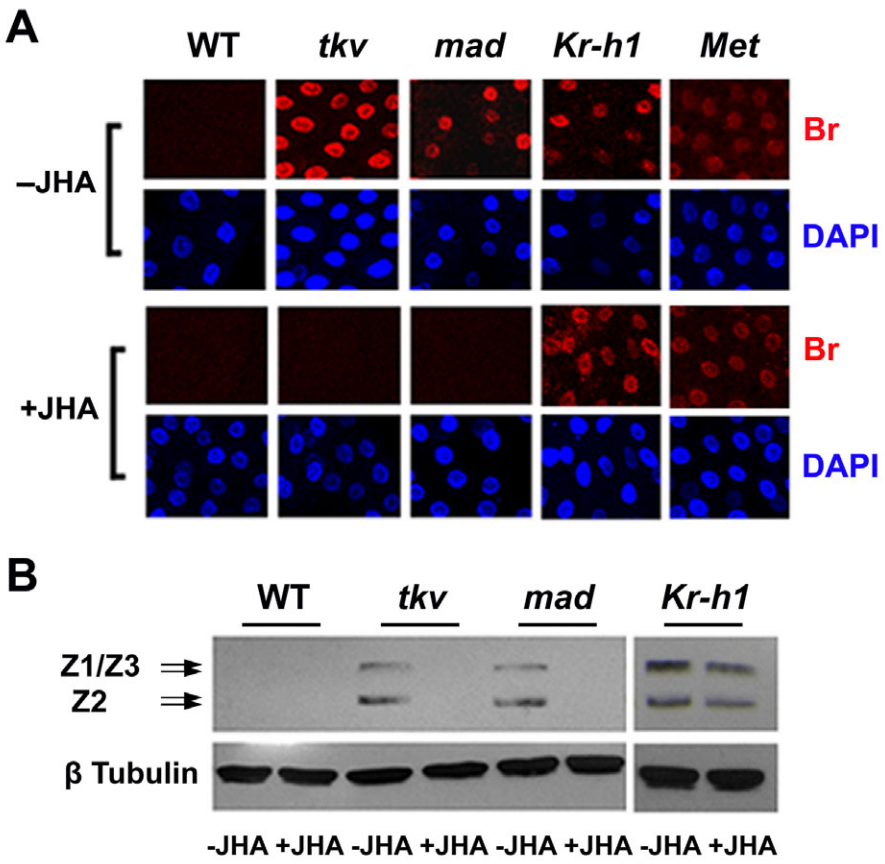

Fig. 1. Tkv and Mad repress *br* expression in the FB by maintaining JH levels. (**A**) Wild-type, tkv^{k16713}, Mad^{k00237}, $Kr\text{-}h1^{10642}$ and Met^{27} flies were reared on normal (–JHA) or 0.1 ppm pyriproxifen-containing (+JHA) food. Fat bodies of the 2nd instar larvae were stained with Br-core antibody (red). Nuclei were labeled with DAPI (blue). (**B**) Br proteins extracted from wild-type, tkv^{k16713}, Mad^{k00237} and $Kr\text{-}h1^{10642}$ 2nd instar larvae that were reared on normal (–JHA) or 0.1 ppm pyriproxifen-containing (+JHA) food were assessed by western blotting with Br-core antibody. Tubulin β was used as a loading control. Z1, Z2 and Z3 represent different isoforms of Br protein.

These observations were further confirmed by western blot analysis (Fig. 1B). Thus, we assume that Tkv/Mad-mediated TGFβ signaling maintains JH titers to inhibit *br* expression at the early larval stages, thereby blocking precocious metamorphosis.

Tkv and Mad non-cell-autonomously regulate *br* expression in the FB

Proteins that transduce JH signals should cell-autonomously regulate *br* expression in the JH-affected organs, whereas proteins that affect JH titer should non-cell-autonomously affect *br* expression. We asked whether the Tkv and Mad proteins were required for the regulation of *br* expression in the FB, a target organ of JH (Liu et al., 2009). A MARCM analysis of FB cells was conducted for tkv^{8}, $Mad^{8\text{-}2}$ and $Kr\text{-}h1^{10642}$ mutants. Br protein was assessed with Br-core antibody staining and compared between the homozygous mutant cells (GFP-positive) and the surrounding heterozygous or wild-type cells (GFP negative). As expected, *br* was not expressed in FB of the wild-type 2nd instar larvae (Fig. 2A,A′), but was expressed in the mutant clones homozygous for *Kr-h1* (Fig. 2D,D′). However, *br* expression was not detected in the *tkv* and *Mad* mutant FB clones at the same stage (Fig. 2B,B′,C,C′). This result is different from those shown in Fig. 1A, in which *br* was found to be highly expressed in FB cells of the *tkv* and *Mad* mutant larvae at the 2nd instar. Therefore, at the early larval stages, the presence of the Tkv and Mad proteins in the FB cells is not required for JH-mediated *br* suppression in these cells. Rather, these proteins function in other tissues to control *br* expression. These results further support the hypothesis that Tkv/Mad-mediated TGFβ signaling inhibits *br* expression at the early larval stages by maintaining JH titers.

Mad functions in the CA to suppress *br* expression in the FB

Because JH titer is mainly determined by JH biosynthesis in the CA, which is controlled by the brain, we tested whether the precocious *br* expression phenotype in the *Mad* mutant larvae

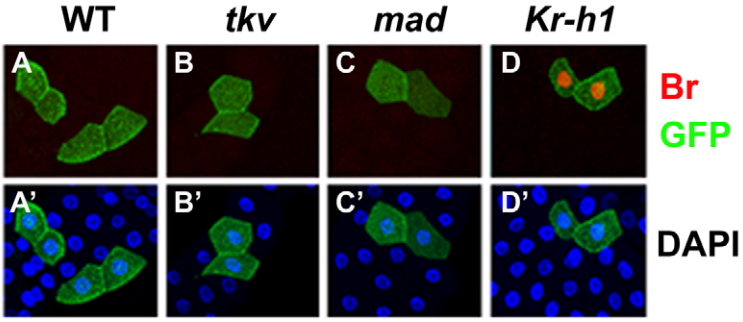

Fig. 2. Tkv and Mad non-cell-autonomously repress *br* expression in the FB. MARCM analyses were carried out in the FB of wild-type (**A**,**A′**), *tkv* (**B**,**B′**), *Mad* (**C**,**C′**), and *Kr-h1* (**D**,**D′**) 2nd instar larvae. Cells homozygous for wild type, tkv^{8}, $Mad^{8\text{-}2}$ or $Kr\text{-}h1^{10642}$ were marked by GFP (green). Br proteins were assessed with Br-core antibody (red). DAPI was used to label nuclei (blue).

could be suppressed by expressing *Mad* cDNA specifically in the CA or brain. When *hs-GAL4* was used to drive *UAS-Mad* ubiquitously in the *Mad*^{k00237} larvae, precocious *br* expression was fully suppressed as expected. By contrast, when we used *Dscam-GAL4*, a pan-neuronal expression driver (Wang et al., 2004), the precocious *br* expression phenotype was not affected. However, when we used *GAL4-Aug21*, a CA-specific GAL4 line (Mirth et al., 2005), precocious *br* expression was completely suppressed (Fig. 3A). As the expression of *Mad* in the CA was sufficient to suppress *br* expression in FB of the *Mad* mutant larvae, we infer that Tkv/Mad-mediated TGFβ signaling promotes JH biosynthesis in the CA at the early larval stages.

Tkv and Mad upregulate *jhamt* transcription and its enzymatic activity

Next, we asked whether TGFβ signaling regulates the expression of genes encoding crucial enzymes of JH biosynthesis. We first compared mRNA levels for these genes between wild-type and *Mad* mutant larvae at the 2nd instar using quantitative real-time PCR. Six enzymes, including JHAMT, farnesyl diphosphate synthase (FPPS), farnesol oxidase (FARox), farnesol dehydrogenase (FARD), cytochrome P450 6g2 (Cyp 6g2) and farnesoic acid O-methyltransferase (Famet), were chosen because they catalyze the key steps of JH biosynthesis and are predominantly expressed in the CA (Belles et al., 2005; Noriega et al., 2006). We found that in the *Mad*^{k00237} larvae, the mRNA levels of *jhamt* were decreased to less than half of that in wild type. However, no changes were detected for the mRNA levels of the other five enzymes (Fig. 3B). We also examined the mRNA levels for genes encoding major JH degradative enzymes, including juvenile hormone esterase (JHE), juvenile hormone epoxide hydrolase 1-3 (JHEh1-3) and juvenile hormone esterase duplication (JHEdup) (Goodman and Granger, 2005). Expression of these JH degradative enzymes was not affected in the *Mad* mutant larvae (see Fig. S2 in the supplementary material).

To test whether the reduced *jhamt* mRNA expression in *Mad* mutants resulted in a correspondingly reduced enzymatic activity, we further measured JHAMT activity in the brain-ring gland complex of Tkv- or Mad-deficient larvae. Because *tkv* and *Mad* mutants die at early larval stages, we carried out this experiment in the *tkv* and *Mad* RNAi larvae. *hs-GAL4*/*UAS-tkv RNAi*, *hs-GAL4*/*UAS-Mad RNAi* and *hs-GAL4*/+ control flies were reared under the normal conditions with or without heat-shock treatment. In the *hs-GAL4*/+ larvae, heat-shock treatment did not affect JHAMT activity. However, JHAMT activity in heat-shocked *hs-GAL4*/*UAS-tkv RNAi* and *hs-GAL4*/*UAS-Mad RNAi* larvae was reduced to 40-50% of that in the control (Fig. 3C). Additionally, JHAMT activity in non-heat-shocked *hs-GAL4*/*UAS-tkvRNAi* and *hs-GAL4*/*UAS-Mad RNAi* larvae was also mildly decreased (~80% of control), probably owing to leaky expression of *hs-GAL4*. The lower *jhamt* mRNA levels and JHAMT activity in Tkv- and Mad-deficit larvae indicate that Tkv/Mad-mediated TGFβ signaling in the CA promotes JH biosynthesis by upregulating *jhamt* expression.

Dpp is the TGFβ ligand in the regulation of JH biosynthesis

The *Drosophila* genome encodes seven TGFβ superfamily members. Three of them, Decapentaplegic (Dpp), Glass bottom boat (Gbb) and Screw (Scw), belong to the BMP subgroup and are suggested to signal through Tkv as the type I receptor (Raftery and Sutherland, 1999). In an attempt to identify TGFβ ligand(s) participating in the regulation of JH synthesis, we found that, like *tkv* and *Mad* mutations, hypomorphic *dpp* mutants, *dpp*^{s11} and *dpp*^{d5}, also caused precocious *br* expression in the FB (Fig. 4A), suggesting that Dpp may be the TGFβ ligand regulating JH biosynthesis in the CA. Noticeably, although both are hypomorphic alleles, *dpp*^{s11} and *dpp*^{d5} have rearrangements of the *disk* and *shv* regulatory regions, respectively (Johnston et al., 1990). It is likely that both regulatory regions are required for the normal *dpp* expression in the CA.

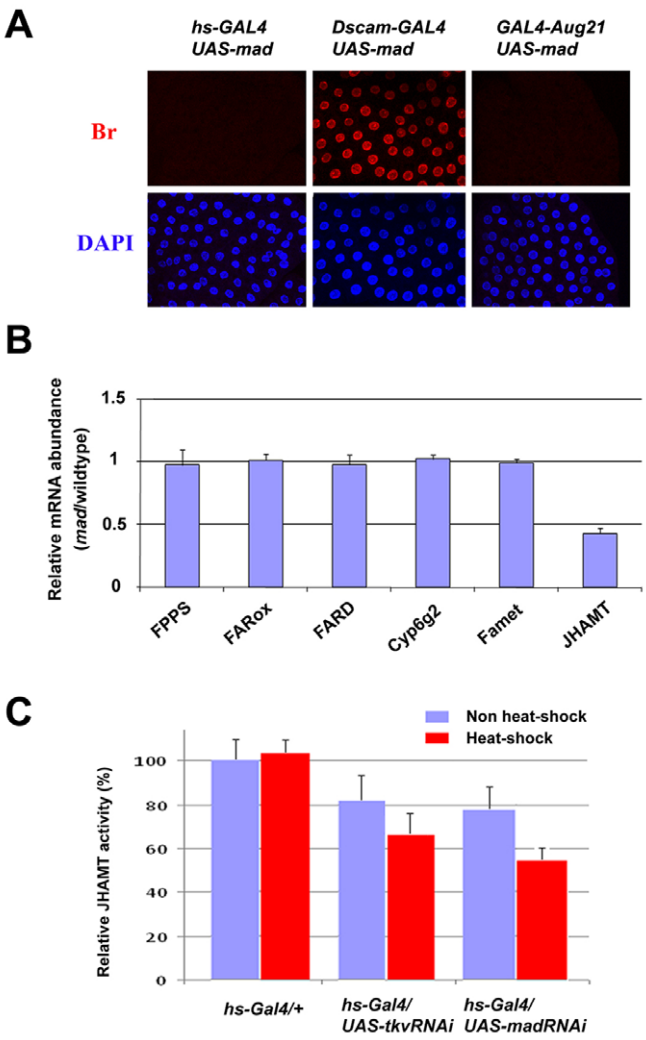

Fig. 3. Tkv and Mad are required in the CA to promote *jhamt* transcription and then repress FB *br* expression. (**A**) *UAS-Mad* was expressed in different tissues of *Mad*^{k00237} mutants using the GAL4 lines that are expressed ubiquitously (*hs-GAL4*), specifically in neurons (*Dscam-GAL4*) or specifically in the CA (*Aug21-GAL4*). FBs of 2nd instar larvae were stained with Br-core antibody (red) and DAPI (blue). (**B**) mRNA levels of JH biosynthetic enzymes in the wild-type and *Mad*^{k00237} 2nd instar larvae were analyzed by quantitative real-time PCR. The ratios of mRNA levels between *Mad*^{k00237} and wild-type larvae are presented as the mean of three independent experiments ± s.d. The accession numbers of genes and sequences of the primers are listed in Table S1 in the supplementary material. (**C**) JHAMT activity in the brain-ring gland complexes of wild-type, *tkv* RNAi and *Mad* RNAi organisms were measured at the wandering larval stage. Data are mean±s.d.

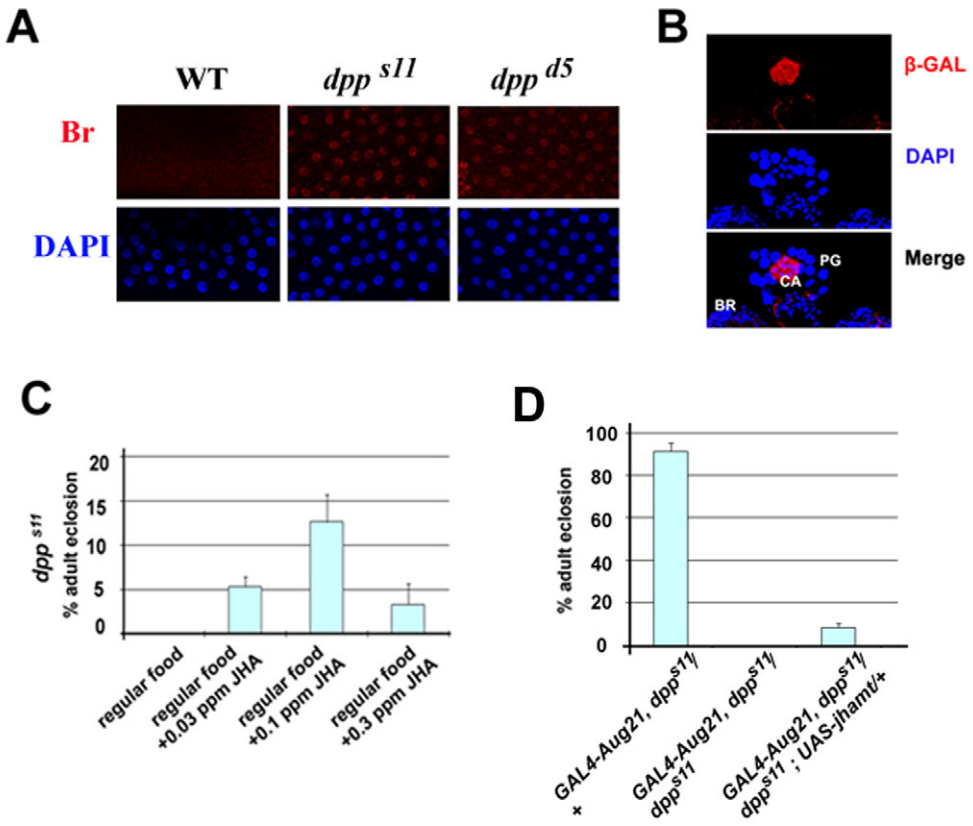

Fig. 4. Dpp is the ligand of Tkv that mediates JH biosynthesis in the CA. (**A**) FBs of wild-type, *dpp*^{s11} and *dpp*^{d5} 2nd instar larvae were stained with Br-core antibody (red) and DAPI (blue). (**B**) Brain-ring gland complexes of the *dpp-lacZ* transgene at the wandering larval stage were assessed with β-galactosidase antibody staining (red). Nuclei were labeled with DAPI (blue). CA, corpus allatum; PG, prothoracic gland; BR, brain. (**C**) One hundred 1st instar larvae of *dpp*^{s11} were reared on normal food or food containing different concentrations of pyriproxifen. The percentages of individuals that develop into adults are shown as the means of 10 replicates±s.d. (**D**) *GAL4-Aug21*, *dpp*^{s11}*/Cyo, GFP* flies were crossed with (1) *+/Cyo, GFP*, (2) *dpp*^{s11}*/Cyo, GFP* and (3) *dpp*^{s11}*/Cyo, GFP; UAS-jhamt*. One hundred GFP-negative 1st instar larvae of their progeny were reared on normal fly food at 25°C. The percentages of individuals that develop into adults are shown as the means of 10 replicates±s.d.

Next, we asked where the Dpp protein in the CA originates. Dpp is transported via intracellular trafficking initiated by receptor-mediated endocytosis (Entchev et al., 2000). Therefore, we first assessed *dpp* expression in the ring gland and found that *dpp-lacZ* was highly expressed in the CA but not in any other parts of the ring gland (Fig. 4B).

Null alleles of *dpp* mutations are embryonic lethal, whereas hypomorphic alleles, such as *dpp*^{s11}, were completely pupal lethal. Interestingly, this pupal lethality could be partially rescued by exogenous JHA. When reared on JHA-containing diet, more than 10% of *dpp*^{s11} larvae developed into adults (Fig. 4C). This finding was further supported by a CA-specific *dpp* RNAi assay (see Fig. S3 in the supplementary material). These data not only reinforce the importance of Dpp in regulating JH biosynthesis, but also imply that the pupal lethality of *dpp*^{s11} are partially caused by the reduced *jhamt* expression and JH levels. When *jhamt* was ectopically expressed in the CA, ~8% of *dpp*^{s11} mutants developed to adulthood (Fig. 4D). In light of all this evidence, we conclude that Dpp expressed in the CA is the TGFβ ligand of Tkv in stimulating JH biosynthesis.

CA-specific downregulation of TGFβ signaling induces precocious *br* expression

As mutations in *dpp*, *tkv* and *Mad* induce precocious *br* expression, we asked whether CA-specific knockdown of *dpp*, *tkv*, *Mad* or *jhamt* affects *br* expression. When the expression of *dpp*, *tkv*, *Mad* or *jhamt* was knocked down by CA-specific RNAi, precocious *br* expression was detected in the FBs of 2nd instar larvae in all cases (Fig. 5). These results further support that Dpp-Tkv-Mad-mediated TGFβ signaling in the CA is required to regulate JH biosynthesis.

Expression of *dpp* in the CA correlates with that of *jhamt*

We have demonstrated that Dpp and its downstream signaling molecules Tkv and Mad are required for normal *jhamt* expression and JH biosynthesis in the CA. It is crucial to determine whether TGFβ signaling is an efficient regulation mechanism for *jhamt* transcription. To address this, we first measured *jhamt* mRNA levels in the ring glands of Dpp-deficient flies at the wandering larval stage. We found that *jhamt* mRNA levels in the ring glands of *dpp*^{s11}, *dpp*^{d5} and CA-specific *dpp* RNAi larvae were only 10-40% of that in wild type. By contrast, when *dpp* was ectopically expressed in the CA, the mRNA level of *jhamt* increased fourfold (Fig. 6A).

We next compared the developmental profiles of *dpp* and *jhamt* expression in the CA of wild-type animals. From the late 3rd instar larva to early pupa, only a single high peak of *jhamt* mRNA levels was detected in the wandering larval stage, which is consistent both with a previous report (Niwa et al., 2008) and with JH titers in the hemolymph (Riddiford, 1993). As shown in Fig. 6B, *jhamt* mRNA level in the ring glands of wandering larvae was ~11-fold higher than that of the larvae 10 hours before the wandering stage. The expression pattern of *dpp* in the CA was similar to that of *jhamt*, but the increase of *dpp* mRNA prior to the wandering larval stage occurred hours earlier than that of *jhamt* mRNA (Fig. 6B). Therefore, despite differences in developmental stages of wild-type animals or in the Dpp-deficient larvae with distinct genetic backgrounds, *dpp* expression in the CA always correlates with *jhamt* expression in vivo. These findings further demonstrate that Dpp-mediated TGFβ signaling plays a crucial role in controlling JH biosynthesis through upregulating *jhamt* expression.

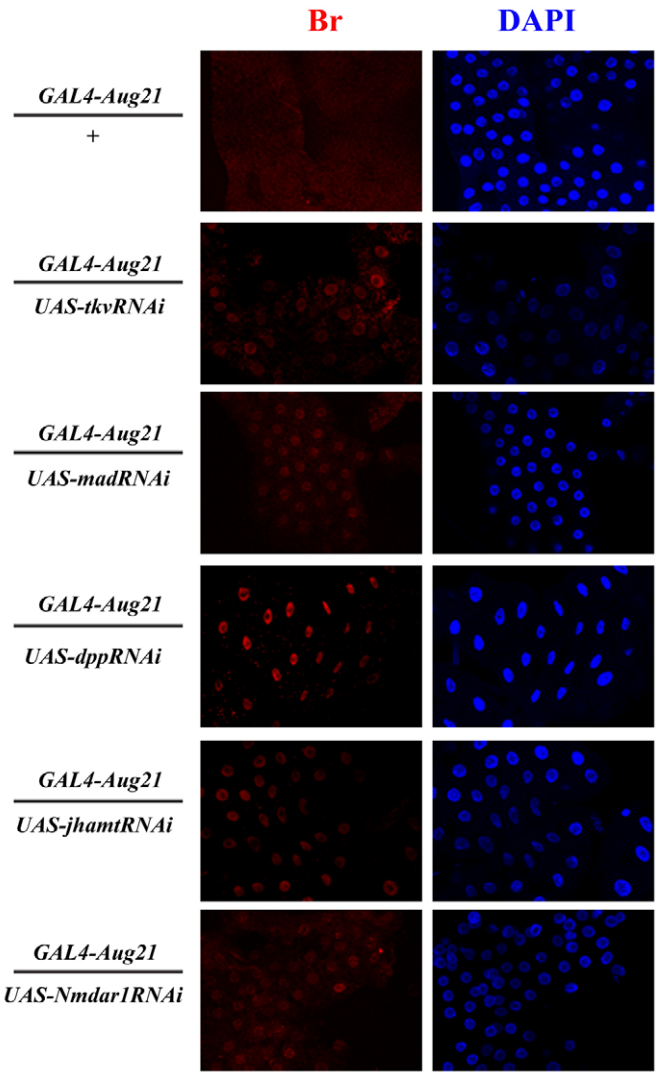

Fig. 5. CA-specific knockdown of *dpp*, *tkv*, *Mad*, *Nmdar1* or *jhamt* induces precocious *br* expression. *GAL4-Aug21* flies were crossed with *UAS-dpp RNAi*, *UAS-tkv RNAi*, *UAS-Mad RNAi*, *UAS-Nmdar1 RNAi* and *UAS-jhamt RNAi*. The FBs of their progeny were dissected at the 2nd instar larval stage and stained with Br-core antibody (red) and DAPI (blue).

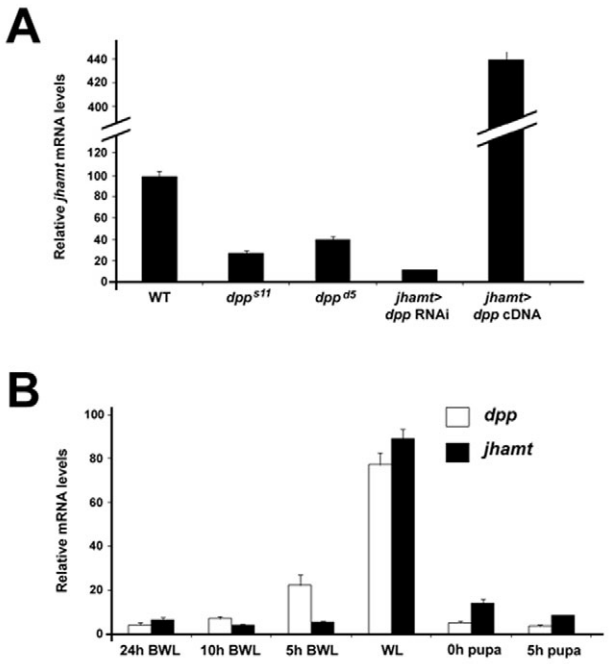

Fig. 6. Expression of *dpp* in the CA correlates with that of *jhamt*. (**A**) Relative *jhamt* mRNA levels at the wandering larval stage were compared among flies with different genetic backgrounds, including wild-type, dpp^{s11}, dpp^{d5}, *jhamt-GAL4>UAS-dpp RNAi* and *jhamt-GAL4>UAS-dpp* flies. Total RNA was extracted from the ring gland. The mRNA levels of *jhamt* were assessed by quantitative real-time PCR and normalized to *actin* mRNA. Values shown are the means of three independent experiments±s.d. (**B**) Relative mRNA levels of *dpp* and *jhamt* in the ring gland were compared among different developmental stages of wild-type organisms (Oregon R). Tissue and total RNA preparation, as well as quantitative real-time PCR, are the same as in A. Values shown are the means of three independent experiments±s.d.

Expression of *dpp* in the CA is controlled by neurotransmitter signals

Finally, we asked whether *dpp* expression in the CA was regulated by brain signals. *Drosophila Nmdar1*, a glutamate receptor, is expressed in the CA and plays a role in regulating JH biosynthesis (Chiang et al., 2002). Our genetic screen also identified that mutations in *Nmdar1* caused precocious *br* expression (see Fig. S1 in the supplementary material). As shown in Fig. 7, at the wandering larval stage, mRNA levels of both *dpp* and *jhamt* in $Nmdar1^{DG23512}$ and $Nmdar1^{05616}$ mutants were reduced to below 30% of those in wild type. In addition, CA-specific knock-down of *Nmdar1* expression also induced precocious *br* expression in the FBs of 2nd instar larvae. These data suggest that *dpp* expression in the CA is directly controlled by neurotransmitter signals from the brain.

DISCUSSION

Roles of TGFβ signaling in insect metamorphosis

The functions of the TGFβ superfamily and other morphogens in regulating insect metamorphosis are rarely reported. In two independent genetic screens, we discovered that *Drosophila* TGFβ signaling controls two different aspects of insect metamorphosis. In a previous study, we found that Baboon (Babo) and dSmad2-mediated TGFβ signaling regulates larval neuron remodeling, which is part of the insect central nervous system metamorphosis induced by 20E during the pupal stage. Further investigation revealed that Babo/dSmad2-mediated TGFβ signaling controls larval neuron remodeling through regulating the expression of *EcR-B1*, a specific isoform of the 20E receptor (Zheng et al., 2003).

In this paper, we report several findings. First, *br* is precociously expressed in 2nd instar *tkv* and *Mad* mutant larvae (see Fig. S1 in the supplementary material). Second, the precocious *br* expression phenotype in *tkv* and *Mad* mutant larvae can be suppressed by exogenous JHA (Fig. 1). Third, Tkv and Mad repressed *br* expression in a non-cell-autonomous manner (Fig. 2). Fourth, the presence of Mad in the CA is sufficient to repress *br* expression in the FB (Fig. 3A). Fifth, *jhamt* mRNA levels and JHAMT activity were significantly reduced in the Mad-deficient larvae (Fig. 3B,C). These results demonstrate that Tkv-

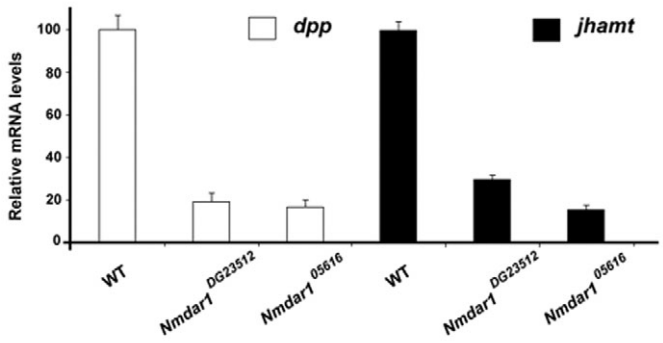

Fig. 7. The expression of *dpp* and *jhamt* in the CA is reduced in *Nmdar1* mutants. The relative mRNA levels of *dpp* and *jhamt* at the wandering larval stage were compared between wild type and two *Nmdar1* mutant alleles, $Nmdar1^{05616}$ and $Nmdar1^{DG23512}$. Tissue and total RNA preparation, as well as quantitative real-time PCR, were performed as described in Fig. 6. Values shown are the means of three independent experiments±s.d.

and Mad-mediated signaling is required in the CA to activate *jhamt* expression and thus JH biosynthesis, which in turn controls insect metamorphosis.

The *Drosophila* genome encodes two TGFβ type II receptors, Punt (Put) and Wishful thinking (Wit) (Raftery and Sutherland, 1999). Our genetic screen failed to identify a role for either of these receptors in the regulation of JH biosynthesis. Put and Wit are most probably functionally redundant in this biological event, as in the case of TGFβ-mediated mushroom body neuron remodeling (Zheng et al., 2003).

Dpp converts brain signals into JH biosynthesis in the CA

Dpp is a key morphogen that controls dorsal/ventral polarity, segmental compartment determination and imaginal disc patterning. Dpp function usually depends on its gradient distribution (Affolter and Basler, 2007). In an attempt to identify the ligand for Tkv/Mad-mediated TGFβ signaling in the CA, we have discovered a novel, gradient-independent role for Dpp that controls JH biosynthesis. We demonstrate that Dpp is the ligand of Tkv, which regulates *jhamt* transcription. Loss of Dpp, even RNAi reduction of Dpp in the CA specifically, causes precocious *br* expression at the early larval stages, which phenocopies *tkv* and *Mad* mutants (Fig. 1A, Fig. 3A and Fig. 5). Phenotypes of *dpp*, including precocious *br* expression and lethality, are at least partially rescued by JHA treatment (Fig. 4C) or ectopic *jhamt* expression in the CA (Fig. 4D). Notably, *dpp-lacZ* is strictly expressed in the CA cells, but not in the other two types of endocrine cells in the ring gland: the prothoracic gland and corpus cardiacum cells (Fig. 4B). The developmental expression profile of *dpp* in the CA is always consistent with that of *jhamt* (Fig. 6). Finally, *dpp* expression in the CA may be directly controlled by neurotransmitter signals in the brain, which is supported by reduced *dpp* and *jhamt* transcription levels in the *Nmdar1* mutant wandering larvae (Fig. 7).

Role of Met/Gce and Kr-h1 in JH action

Several lines of evidence suggest that Met is a crucial regulator at or near the top of a JH signaling hierarchy, possibly acting as a JH receptor (Wilson and Ashok, 1998). However, null *Met* mutants of *Drosophila* are completely viable, which is unexpected if Met is a JH receptor. A recent investigation indicated that another *Drosophila* bHLH-PAS protein, Germ cell-expressed (Gce), which has more than 50% homology to Met (Godlewski et al., 2006), may function redundantly to Met in transducing JH signaling (Baumann et al., 2010). Because *Met* is on the X chromosome in the fly genome, it was not covered by our genetic screen. We tested the *br* protein in the FBs of a *Met* null allele, Met^{27}, at the 2nd instar larval stage and observed precocious *br* expression. Importantly, this precocious *br* expression phenotype could not be suppressed by exogenous JHA (Fig. 1A). This result not only supports the previous reports regarding the function of Met in transducing JH signaling but also suggests that the precocious *br* expression is a

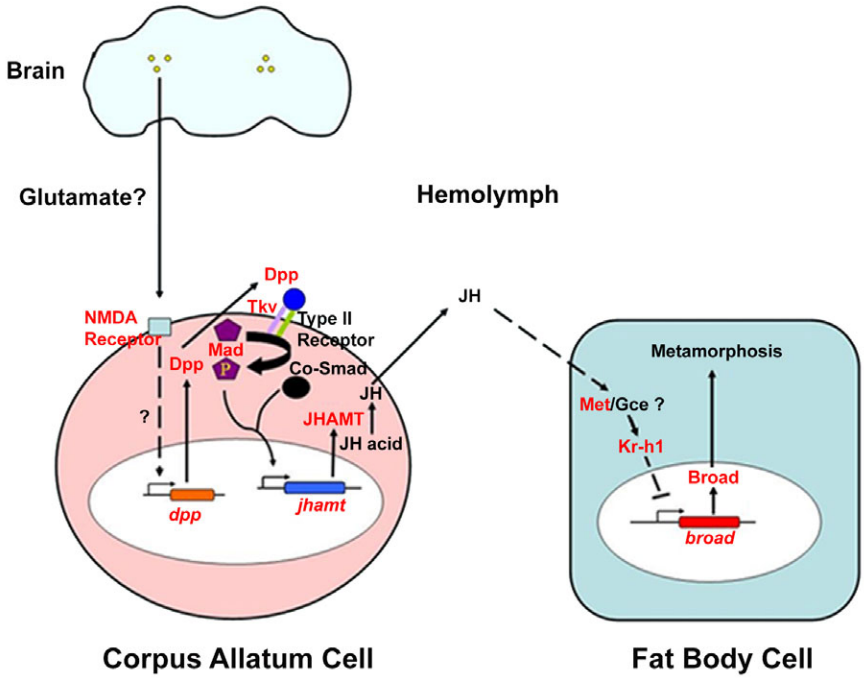

Fig. 8. A model for the function of TGFβ signaling in controlling JH biosynthesis and insect metamorphosis. Proposed model as described in the text illustrating the function of TGFβ signaling in controlling JH biosynthesis and insect metamorphosis. The genes and proteins involved in this study are highlighted in red.

more sensitive indicator for the reduced JH activity in *Drosophila* compared with precocious metamorphosis, lethality and other phenotypes.

Kr-h1 was reported to act downstream of Met in mediating JH action. Studies in both *Drosophila* and *Tribolium* reveal that, at the pupal stages, exogenous JHA induces *Kr-h1* expression, which in turn upregulates *br* expression (Minakuchi et al., 2008; Minakuchi et al., 2009). Our genetic screen successfully identified that Kr-h1 is cell-autonomously required for the suppression of *br* expression at young larval stages (see Fig. S1 in the supplementary material; Figs 1 and 2). Precocious *br* expression occurred in the FBs of *Kr-h1* mutants and was not suppressed by JHA treatment (Fig. 1). Therefore, our studies further suggest that Kr-h1 functions as a JH signaling component in mediating insect metamorphosis. However, our finding shows that, at the larval stages of *Drosophila*, the JH-induced Kr-h1 suppresses, rather than stimulates, *br* expression. This result is consistent with the facts that Kr-h1 functions to prevent *Tribolium* metamorphosis (Minakuchi et al., 2009) and Br is a crucial factor in promoting pupa formation (Zhou and Riddiford, 2002).

A working model for function of Dpp-mediated TGFβ signaling in controlling insect metamorphosis

Taken together, we find a novel function of Dpp, Tkv and Mad-mediated TGFβ signaling in controlling insect metamorphosis. As summarized in our model (Fig. 8), the brain sends neurotransmitters, such as glutamate, to the CA through neuronal axons. Glutamate interacts with its receptor (NMDAR) on the surface of CA cells to induce *dpp* expression. Dpp protein produced and secreted by CA cells forms a complex with TGFβ type I receptor (Tkv) and type II receptor on the membrane of CA cells, followed by phosphorylation and activation of Tkv. Activated Tkv in turn phosphorylates Mad, which is imported into the nucleus together with co-Smad and stimulates *jhamt* expression. JHAMT in CA cells transforms JH acid into JH, which is released into hemolymph. The presence of JH in young larvae prevents premature metamorphosis through Met/Gce and Kr-h1 by suppressing the expression of *br*, a crucial gene in initiating insect metamorphosis.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at
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