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Full Length Article

BmNPV-induced hormone metabolic disorder in silkworm leads to enhanced locomotory behavior

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ABSTRACT

Many parasites alter the host locomotory behaviors in a way that increases their fitness and progeny transmission. Baculoviruses can manipulate host physiology and alter the locomotory behavior by inducing 'hyperactivity' (increased locomotion) or 'tree-top disease' (climbing high up to the top before dying). However, the detailed molecular mechanism underlying virus-induced this hyperactive behavior remains elusive. In the present study, we showed that BmNPV invaded into silkworm brain tissue, resulting in severe brain damage. Moreover, BmNPV infection disturbed the insect hormone balance. The content of 20-hydroxyecdysone (20E) in hemolymph was much lower during the hyperactive stage, while the dopamine (DA) titer was higher than mock infection. Exogenous hormone treatment assays demonstrated that 20E inhibits virus-induced ELA (enhanced locomotory activity), while dopamine stimulates this behavior. More specificity, injection of dopamine or its agonist promote this hyperactive behavior in BmNPV-infected larvae. Taking together, our findings revealed the important role of hormone metabolism in BmNPV-induced ELA.

1. Introduction

Many parasites adaptively control host behavior in ways that enhance the transmission of their genes into the next generation. Behavior manipulation following infection with parasites has been defined as a phenotypic change in the host organism (Poulin, 1995). There are a growing number of overall diversity parasites known to manipulate host behavior. One example is that parasitoid wasps, *Cotesia congregate*, secrete ecdysteroids into their hosts to hijack host development (Gelman et al., 1998). Some rodents infected with *Toxoplasma gondii*, which parasites within host central nervous system, are manipulated to become more attracted to cat urine (Vyas et al., 2007). Dengue virus infection displays an increase of up to ~50% in locomotor activity of *Aedes aegypti* mosquitoes (Lima-Camara et al., 2011). Given the broad taxonomic diversity of parasites that can manipulate insects behavior from a variety of organisms including viruses, macroscopic worms, fungi, and parasitic insects, the proximate mechanism underlying these parasite-induced behavioral manipulations remain poorly understood (Moore, 2002).

Baculoviruses are the arthropod-specific pathogens, which infect insect larvae through the consumption of contaminated plant. One classical example of parasite-manipulated host behavior is tree-top disease (*Wipfelkrankheit*) that infected caterpillars climb high up to the upper foliage of the tree before death (Goulson, 1997). At this elevated position, the host dies and liquefies, leading to better transmission of virus particles on leaves that are then consumed by new caterpillar hosts.

The baculovirus encodes an enzyme known as ecdysteroid UDP-glucosyltransferase (EGT), which catalyzes the transfer of glucose from UDP-glucose to ecdysteroids. The encoded EGT enzyme inactivates insect molting hormone 20-hydroxyecdysone (20E), thereby prolonging feeding time and increase the yield of viral progeny (O'Reilly and Miller, 1989). However, *egt* does not have a conserved role among baculoviruses in inducing tree-top disease (Ros et al., 2015). Another interesting study identified a baculovirus-encoded gene, protein tyrosine phosphatase (*ptp*), which induces the enhanced wandering-like behavior in *Bombyx mori* larvae (Kamita et al., 2005). Further report revealed that BmNPV PTP functions as a virion structural protein rather than an

Abbreviations: B.mori, *Bombyx mori*; BmNPV, *Bombyx mori* nucleopolyhedrovirus; ELA, Enhanced locomotory activity; qRT-PCR, quantitative real-time RT-PCR; 20E, 20-hydroxyecdysone; DA, dopamine; ELISA, enzyme-linked immunosorbent assay; Flu, Flupentixol Dihydrochloride; SKF, SKF389393 HCl; Hsps, heat shock proteins; HE, hematoxylin & eosin; DR, dopamine receptor; EcR, ecdysone receptor; USP, ultraspiracle protein; DopEcR, Dopamine/Ecdysteroid receptor.

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enzyme. Interestingly, virus propagation was markedly reduced in brain tissues when *ptp* was deleted from the BmNPV genome (Katsuma et al., 2012). These results were very impressive and made significant contributions to the field, and they also suggested that baculovirus may play a crucial role in the induction of ELA by infecting the brain tissue.

Although the above-mentioned studies have provided valuable information about mechanisms behind baculovirus-induced insect behavior, the impact of baculovirus infection on host development and physiological processes was still not completely understood. Considering that the central nervous system (CNS) plays an important role in regulating the insect behaviors, it is necessary to investigate the effect of baculovirus infection on CNS, particularly in brain. The brain is a behavior regulation center for coordinating body function. A signal generated in the brain is conveyed by a population of descending neurons (DNs) (Namiki and Kanzaki, 2018), which is also related to the activity of growth, development, reproduction and diapause. Thus, it is necessary to clarify how baculovirus controls the host brain and its role in regulating locomotion and synaptic transmission. It remains to be elucidated how the locomotor signal is generated and processed in brain to trigger BmNPV-induced ELA in silkworm larvae.

In the present study, we examined the effects of heat shock response and hormone metabolism in silkworm larvae. Our results demonstrated that BmNPV infection leads to abnormal expression in heat shock proteins and hormone corresponding receptors in brain during the ELA stage. Elisa assay showed that BmNPV interferes with host endogenous hormone homeostasis (20E and dopamine), resulting in host hormone metabolism disorder. Furthermore, exogenous hormone treatment assays found that dopamine triggered this hyperactive behavior, while it was inhibited by 20E. This study explains the mechanisms of ELA behavior in silkworm, and provides a new insight of baculovirus-induced host behavioral manipulation.

2. Materials and methods

2.1. Insect, cell culture and virus preparation

The silkworm larvae (commercial strain: Qiufeng × Baiyu) were reared on fresh mulberry leaves at 25 °C as described previously (Wang et al., 2015a). BmN cells were cultured at 27 °C in TC-100 insect medium supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, and 30 μg/ml streptomycin. The T3 strain of BmNPV was maintained in our laboratory. The virus titers were determined by $TCID_{50}$ method. OBs (occlusion bodies) were amplified from infected larvae and stored at 4 °C (Rahman and Gopinathan, 2004).

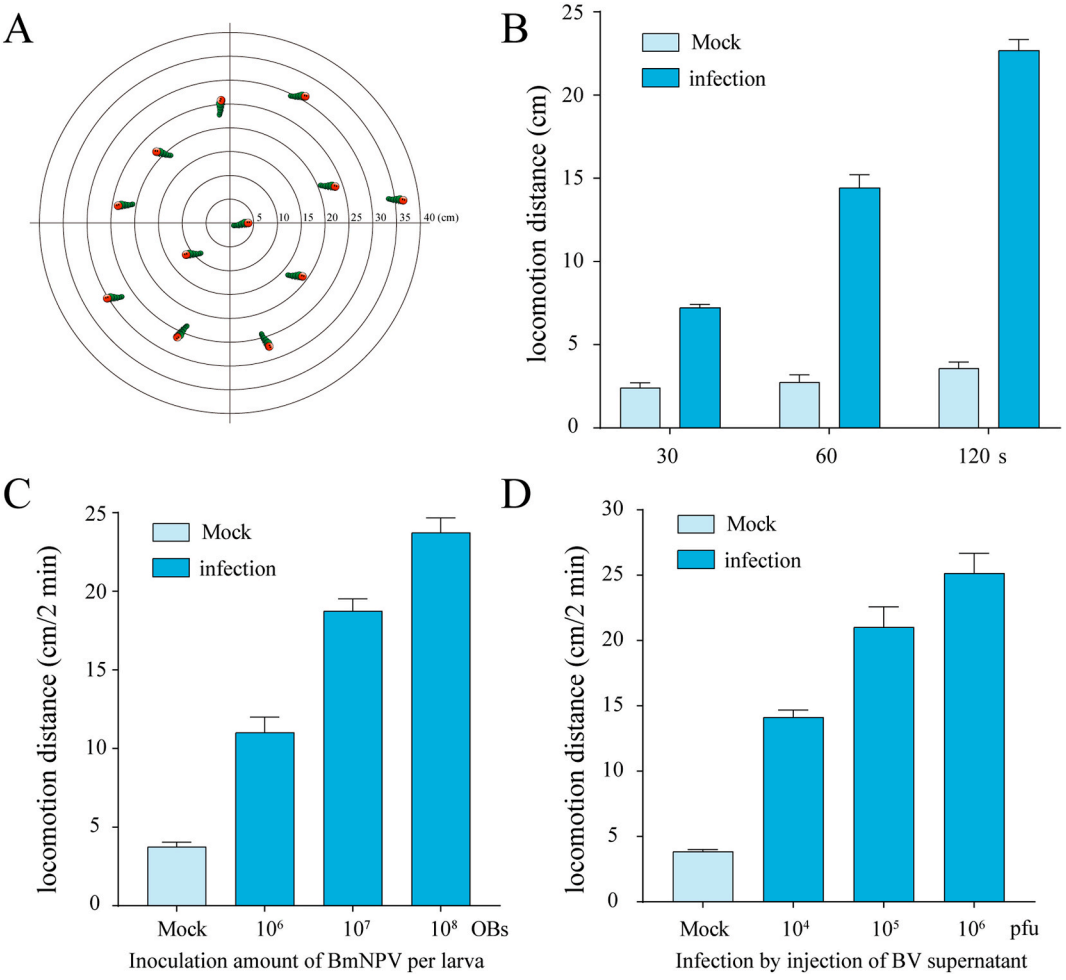

Fig. 1. Locomotion assays system for ELA larvae.
(A) Schematic drawing of behavioral assay. Each group of larvae was placed in the center of A0 paper marked with concentric circles (the radius of each circle was 5 cm greater than the previous circle, with a maximum radius of 40 cm in A0 paper). (B) Locomotion distances of larvae at each time point. The data are presented as mean locomotion of 10–20 larvae tested at 30s, 1 min, 2 min. (C) BmNPV at three different concentrations induced ELA behavior in silkworm. Newly molted larvae were inoculated with OBs (10^6, 10^7 and 10^8 OBs per larva). (D) Induction of ELA in silkworm larvae injected with BV supernatant at three different PFU (10^4, 10^5 and 10^6 pfu per larva). Each data point was determined by three independent assays. All data were performed in triplicate. Values above the bars indicate means and error bars represent standard deviations.

2.2. Behavioral assay for locomotion activity

To quantify ELA locomotor behavior, locomotion assays were performed as described previously with some modification (Katsuma et al., 2012). Briefly, newly molted fifth instar larvae were starved for one night and fed with mulberry leaves contaminated with OBs (10^6, 10^7 and 10^8 OBs per larva) or injected with BV (10^4, 10^5 and 10^6 pfu per larva). Each group of larvae (10–20) was placed in the center of a piece of paper marked with concentric circles (the radius of each circle was 5 cm greater than the previous circle, with a maximum radius of 40 cm in A0 paper) in Fig. 1A. Photographs were taken by a digital camera at 30 s, 1 min and 2 min after release. The coordinates of each larvae at the fifth and sixth body segments were determined at each time point.

2.3. Hematoxylin & eosin staining and immunohistochemistry

The brains were dissected, washed in PBS and immediately fixed in 4% formalin at 25 °C for 1 h. Then, the brain tissues were embedded in paraffin and cut into 4-μm-thin sections. Hematoxylin & eosin (HE) staining was applied to analyze tissue viability and morphology. The slides were viewed under the light microscope equipped with a digital camera. The sections were analyzed for structural changes, degenerative alterations, necrosis, and signs of inflammation.

For immunohistochemistry staining, paraffin slides were deparaffinized and subjected to antigen retrieval by using citrate sodium solution (pH 6.0). To reduce non-specific staining and permeabilize the sample, slides were incubated in TBS solution containing 1% BSA and 0.5% Triton for 1 h at room temperature. Slides were then washed 3 times with PBS and incubated with primary anti-GP64 antibody (viral envelope fusion proteins, Sigma) at 1:350 dilution overnight at 4 °C. After incubation, the samples were subsequently stained with FITC-labeled Rabbit anti-mouse secondary antibodies. Nuclei were stained using 4', 6-diamidino-2-phenylindole (DAPI, Beyotime). Images were acquired using ZEISS LSM 780 confocal laser scanning microscopy.

2.4. RNA extraction and qRT-PCR assay for quantification of viral and host gene expression

Total RNA was extracted from brain using RNAiso Plus (TaKaRa) according to the manufacturer's instructions. To remove potential genomic contaminants, the RNA samples were first treated with DNase I (Invitrogen). First-strand cDNAs were synthesized from 2 μg of total RNA by EasyScript® One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech). Quantitative Real-time PCR was performed using Hieff® qPCR SYBR® Green Master Mix (Yeasen) with primer pairs shown in supplementary Table S1. Melting curve analysis was performed for each PCR to confirm the specificity of the amplifications. For normalization of gene expression, the *B. mori* 28S ribosomal transcript was used as an internal reference to calculate the expression levels of the target genes. Each amplification was carried out in 20 μl reaction mixture for 95 °C for 5min and 40 cycles of 95 °C for 10s and 60 °C for 30s. The melting-curve was obtained during the last step from 58 to 95 °C. Relative expression levels of the target genes were calculated and determined using the $2^{-\Delta\Delta Ct}$ method. All data were obtained from three independent biological replicates.

2.5. Western blot analysis

Larval brains were lysed in lysis buffer [20 mM Tris PH7.5, 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM EDTA, 1% Na_3VO_4, 0.5 μg/ml leupeptin, 1 mM phenylmethanes DNA synthesi-sulfonyl fluoride (PMSF)] for 30 min on ice. Protein concentrations in lysates were determined using BCA Protein Assay Kit (TaKaRa). A 20 μg amount of protein extract was separated by a 15% SDS-PAGE gel and transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked in 5% non-fat milk in TBST for 1 h and incubated overnight at 4 °C following antibodies: viral proteins (anti-POLH, anti-GP64) and insect heat shock proteins (anti-Hsp90, anti-Hsc70-4). After incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies, membranes were developed by enhanced chemiluminescence. Alpha-tubulin was served as an internal reference.

2.6. Measurement of 20E and DA titer by Elisa kit

Silkworm larvae were anesthetized on ice cooling before dissection. The hemolymph extracted from both mock and infected larvae was diluted in TBS at the same multiple. After centrifugation, the supernatant was subjected to enzyme-linked immunosorbent assay (ELISA).

20E and dopamine levels were determined using an enzyme immunoassay kit (MEIMIAN, Jiangsu) according to the manufacturer's instructions. The accession number of each Elisa kit: MM-0681O1 (20E kit), MM-91073O1 (Dopamine kit). The assay range is 0.8–32 ng/L and 1.5–60 pg/ml, respectively. The hemolymph supernatant was diluted and determined from a standard curve of diluted 20E or dopamine. The absorbance value was measured at a wavelength of 450 nm to calculate the sample content. The test was repeated three times.

2.7. Hormone treatment

To determine the hormone homeostasis on ELA behavior, exogenous hormone treatment was applied. Briefly, 20E and dopamine (DA) were dissolved in dimethyl sulfoxide (DMSO) to prepare a 10 mg/ml stock solution. The work solution of 20E and DA was diluted to 100 μg/ml with PBS (pH 7.4, 10 mM sodium phosphate, 140 mM NaCl).

To examine the potential effect of hormone treatment on BmNPV-induced aberrant hyperactivity, newly molted fifth-instar silkworm larvae were inoculated with 10^8 OBs of BmNPV per larva. At 48 h post-inoculation, 20E (1 μg/larva; NPV+20E) or dopamine (1 μg/larva; NPV + DA) was injected into larvae hemocoel. For the control group, larvae received no treatment (NPV) or were injected with an equal volume of DMSO (NPV + DMSO). The brain tissues were collected at 48 h post-treatment to extract total mRNA and protein. The methods described above for qRT-PCR and Western blot were used to measure the expression of polyhedrin/GP64 (virus proteins) and Hsp90/Hsc70-4 (heat shock proteins) after hormone treatments. An additional cohort of virus-infected larvae were placed in the center of A0 paper marked with concentric circles for behavioral assay as described above. Each treatment was replicated with 10–20 larvae and the experiment was repeated three times.

2.8. DA receptor agonist and antagonist treatment

To determine the role of DA in modulating ELA behavior, larvae with 48 h post-infection were injected with dopamine receptor agonist (SKF389393 HCl) or antagonist (Flupentixol Dihydrochloride). As mentioned above, agonist (1 μg/larva; NPV + SKF) or antagonist (1 μg/larva; NPV + Flu) was injected into larvae hemocoel. An equal amount of DMSO was injected into larvae as the control.

At 48 h post-treatment, the brain tissues were collected for qRT-PCR and Western blot analysis. An additional cohort of virus-infected larvae were selected for behavioral assay. Each treatment consisted of 10–20 larvae and the experiment was repeated three times.

2.9. Statistical analysis

The data for qRT-PCR analysis and behavioral assay (travel distance) are represented as the means ± standard error (SE) with three independent repetitions. The differences in means between multiple sets of data were compared by one-way ANOVA. The Student's t-test was conducted to compare the data by using the GraphPad Prism6. $p < 0.05$ was considered statistical significance (denoted by * $p < 0.05$, and ** $p < 0.01$).

3. Result

3.1. The establishment of behavioral assay system

To quantify this enhanced locomotory activity induced by BmNPV, behavioral assay was examined under three doses of OBs (10^6, 10^7 and 10^8 OBs per larva). The horizontal climbing distance of infected larvae on a circle grid, and the results are shown in Fig. 1. At 30s after release, the locomotion distances of infected larvae (7.22 ± 0.22 cm) significantly exceeded those mock-infected larvae (1.82 ± 0.44 cm). As time lapsed, the locomotion distances of larvae reached 10.82 ± 0.79 cm (1 min after release) and 22.67 ± 0.67 cm (2 min after release), but those of mock-infected larvae were only 2.31 ± 0.30 cm (1 min) and 3.60 ± 0.37 (2 min). Behavioral assays were recorded on time scale, indicating that the BmNPV infection is required for the induction of ELA. And the different infection dose indicates the difference of infection speed. Larvae infected with a higher dose of BmNPV exhibited longer crawling paths comparing to larvae infected with a lower dose and mock-infected larvae.

3.2. BmNPV infection results in severe brain pathology

It is of great interest to decipher how baculovirus has evolved the ability to manipulate behavior and how brain neurotransmission of the manipulated host can occur. To address this question, brain tissues were dissected for pathological examination. The collected brain tissues were treated using HE staining and further stained by immunofluorescence using GP64 antibody (Fig. 2A and B). Histological assessment of the brain tissue revealed the presence of tissue and cellular damage in the infected group. After baculovirus infection, viral DNA replication triggers host DNA damage responses, resulting in the depletion of host inhibitors of apoptosis (IAP) proteins and the initiation of apoptosis (Mitchell and Friesen, 2012; Vandergaast et al., 2011). The altered structure of brain tissue likely results from pathological damage and apoptosis.

At several designed time points, the expression levels of viral

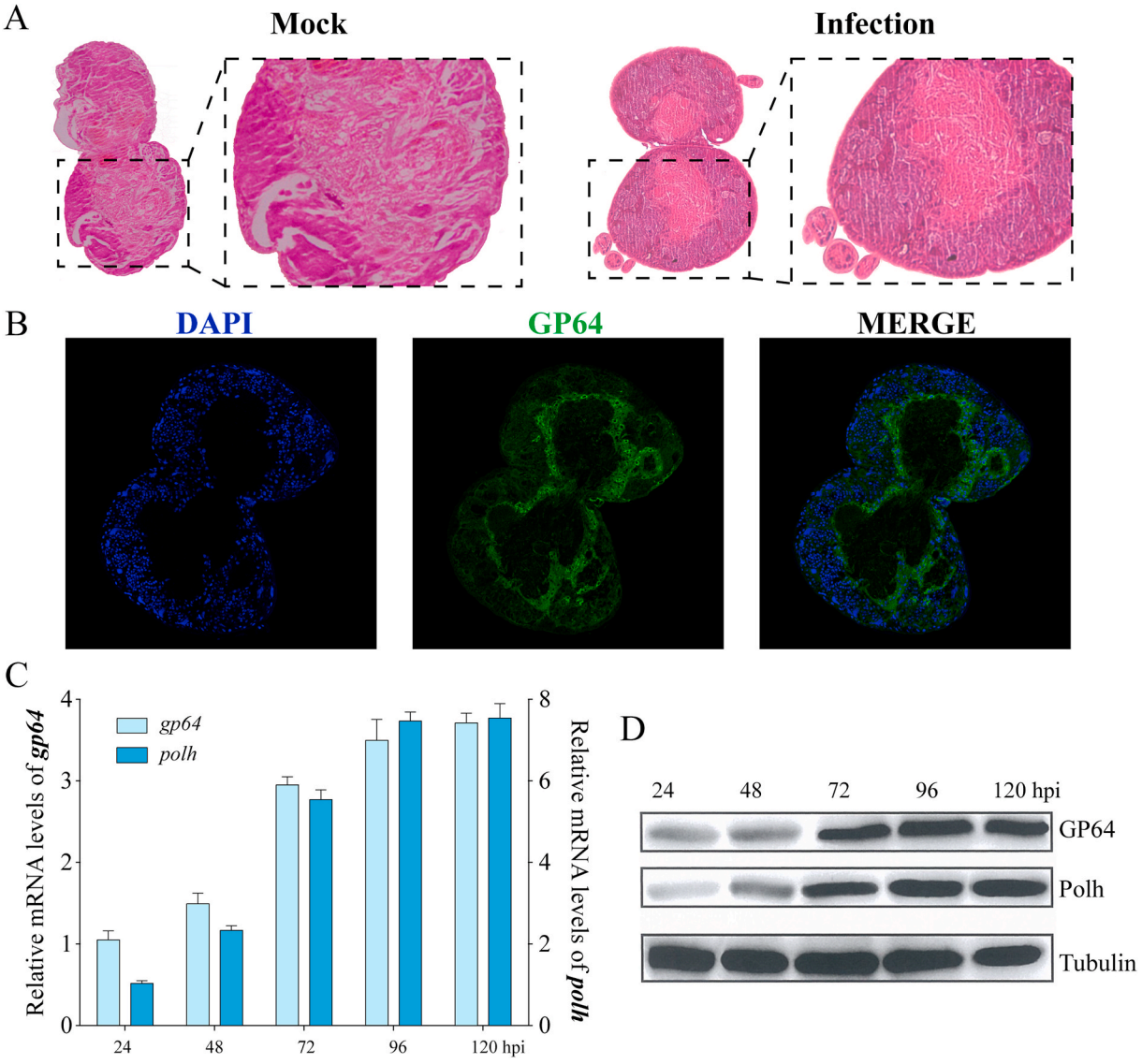

Fig. 2. Morphology and histopathological changes of brain tissue after BmNPV infection.
(A) Hematoxylin and eosin staining of brain from mock (left) and infection (right). Magnified view of the boxed area showed the detail of infected brain tissue, indicating the pathological change after BmNPV infection. (B) The brain was examined by immunofluorescence staining with anti-GP64 (green) and DAPI counterstaining for DNA (blue). The green fluorescent spots indicate the abundant spread of virus in the infected brain at 72 h post infection. (C, D) Time course of BmNPV infection in brain tissue. At the indicated time points, the virus invasion progress was determined in mRNA and protein level. Abbreviations: GP64, viral major envelope fusion proteins; Polh, polyhedrin. Tubulin served as the internal control.

genomic RNA and viral proteins in the infected brain were determined by qRT-PCR and Western blot, respectively (Fig. 2C and D). The above results showed that the expression of viral protein was gradually increased during the infection, which could be indicative of viral presence and its ongoing replicative process in the brain tissue.

3.3. BmNPV infection triggers heat shock response in brain tissue

It was reported that heat-shock proteins exhibited significantly change after baculovirus challenge (Katsuma, 2021; Lyupina et al., 2010). Interestingly, heat shock proteins are involved in protein folding and facilitating numerous cellular processes, which are conducive for virus replication such as ubiquitin-proteasome pathway (Mayer, 2005; Nguyen et al., 2013). Then, the heat shock proteins (Hsp90 and Hsc70-4) were selected for qRT-PCR and Western blot analysis. In a continuous infection process, both Hsp90 and Hsc70-4 were expressed at high level (Fig. 3).

In addition, Hsp90 was reported to function in the regulation of signal transduction, which interacts with USP (ultraspiracle protein) to induce gene expression in the 20E and juvenile hormone signaling pathway in insects (Liu et al., 2013). Another heat-shock protein (Hsc70-4) was involved in BmNPV propagation and was regarded as a novel structural protein of BmNPV (Iwanaga et al., 2014). The above-referenced studies indicated the strong association between host heat shock response and virus infection. The pathological changes of infected brain may result from the complicated heat shock responses.

3.4. BmNPV infection changed the host endogenous hormone homeostasis

Previous studies revealed the potential involvement of viral *egt* gene in this hyperactive behavior. The *egt* gene, a conserved gene among baculovirus, encodes ecdysteroid UDP-glucosyl transferase (EGT). As a consequence, the molting hormone 20E become inactivated in the infected larvae (O'Reilly and Miller, 1989; Ros et al., 2015). To investigate the role of hormones in the process of ELA, the concentrations of 20E and DA were measured in larval hemolymph. The content of 20E in hemolymph was at 14.50 ± 0.77 ng/L (mean ± SD) in the infected group, which is much lower than that in mock groups (23.07 ± 1.04 ng/L) (Fig. 4A). The effects of BmNPV infection on 20E-related genes in silkworm larvae at the transcript level were analyzed by real-time PCR. The results showed that the transcript levels of 20E receptors (*EcR*, *USP*) were significantly inhibited at 48, 72 and 120 h post infection (Fig. 4C and D), which were consistent with the reports in *Helicoverpa armigera* (Zhang et al., 2015).

As the ELA occurred at 72h post-infection, the concentration of DA in hemolymph was significantly decreased to 27.88 ± 1.82 pg/L (mean ± SD), which is higher than that in mock infection (19.17 ± 1.25 pg/L) (Fig. 4B). The transcript levels of the corresponding dopamine receptor (*DR1* and *DR2*) were up-regulated after infection at 48, 72 and 120h, respectively (Fig. 4E and F). These results indicated that BmNPV altered the levels of hormones metabolism in hemolymph, and disrupted the balance of endogenous hormone in insects.

3.5. 20E and DA counteractively regulate ELA behavior induced by BmNPV

Correspondingly, several studies have found that biogenic amine and amine biosynthesis process play an important role in parasitic manipulation of host behavior (Gasque et al., 2019; Helluy and Holmes, 1990; Xing et al., 2017). To investigate the roles of hormone and biogenic amine in virus fitness and host ELA behavior, exogenous 20E and dopamine were injected into BmNPV-infected larvae to evaluate the effect of these treatments on BmNPV-induced ELA behavior. The expression of viral envelope fusion proteins (GP64) and major occlusion body protein (POLH) in brain tissue were measured after hormone treatment. qRT-PCR and Western blot analysis showed that, the expression level of BmNPV replication (*gp64* and *polh*) were significantly decreased after 20E treatment, while treatment with DA facilitated BmNPV replication, compared to mock-infected larvae without injection or injection with DMSO (Fig. 5A and B).

Behavioral analysis demonstrated that 20E treatment decreased the movement distance (in 2 min after release), while DA treatment significantly increased the distance of BmNPV-infected larvae compared with the single infection (Fig. 5F). Previous studies indicated that DA promoted this hyperactive behavior while 20E has the opposite effect (Kang et al., 2019). There is one possible explanation that the higher 20E titer may trigger molting, repressing host growth and viral proliferation. Therefore, we propose that the infected larvae did not progress to the next molt or to the spinning and prepupal stages, so ecdysone synthesis was probably not stimulated. Nevertheless, dopamine, that could promote dopaminergic signal systems hijacked by BmNPV, to some extent, was helpful to trigger this aberrant hyperactive behavior.

3.6. Effects of DA receptor agonist and antagonist treatments on infected larvae

To determine the effect of DA on ELA behavior, the dopamine receptors were stimulated or inhibited by pharmacological intervention

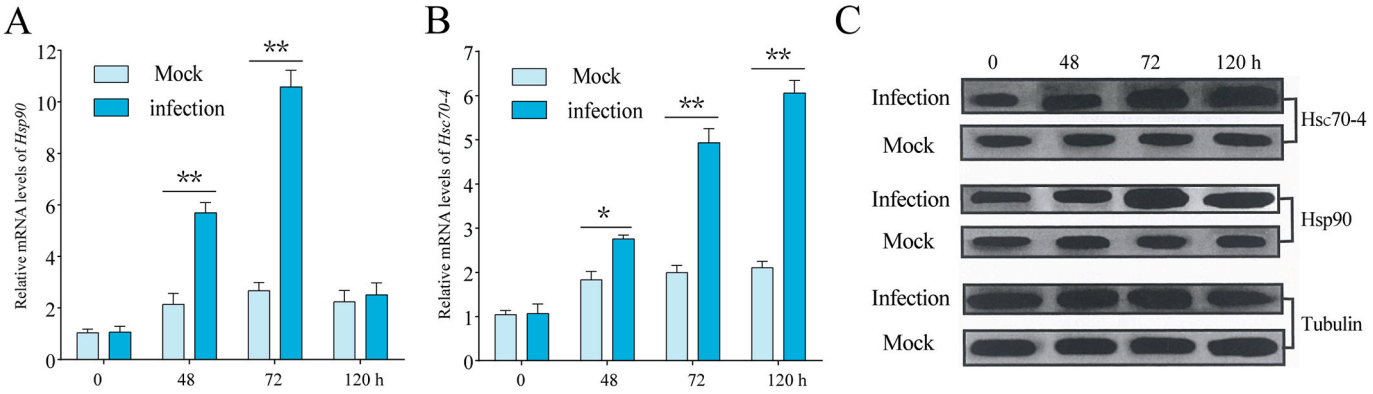

Fig. 3. BmNPV infection triggers a heat shock response in brain.
The brain total RNAs were extracted at the indicated time points. The transcript levels of *Hsp90* (A) and *Hsc70-4* (B) were quantified by qRT-PCR. Relative gene transcript levels (28sRNA as internal controls) were analyzed using the $2^{-\Delta\Delta CT}$ method. (C) Western blot analysis of heat shock proteins (HSPs) expression. At designated time points, protein extracted from whole brain tissue were analyzed by Western blot using the indicated antibodies (anti-Hsp90 and anti-Hsc70-4). Anti-tubulin was used as the loading control. Our result showed that BmNPV infection induced heat shock response, resulting in high expression of Hsp90 and Hsc70-4. The pathological change in brain may result from heat shock responses.

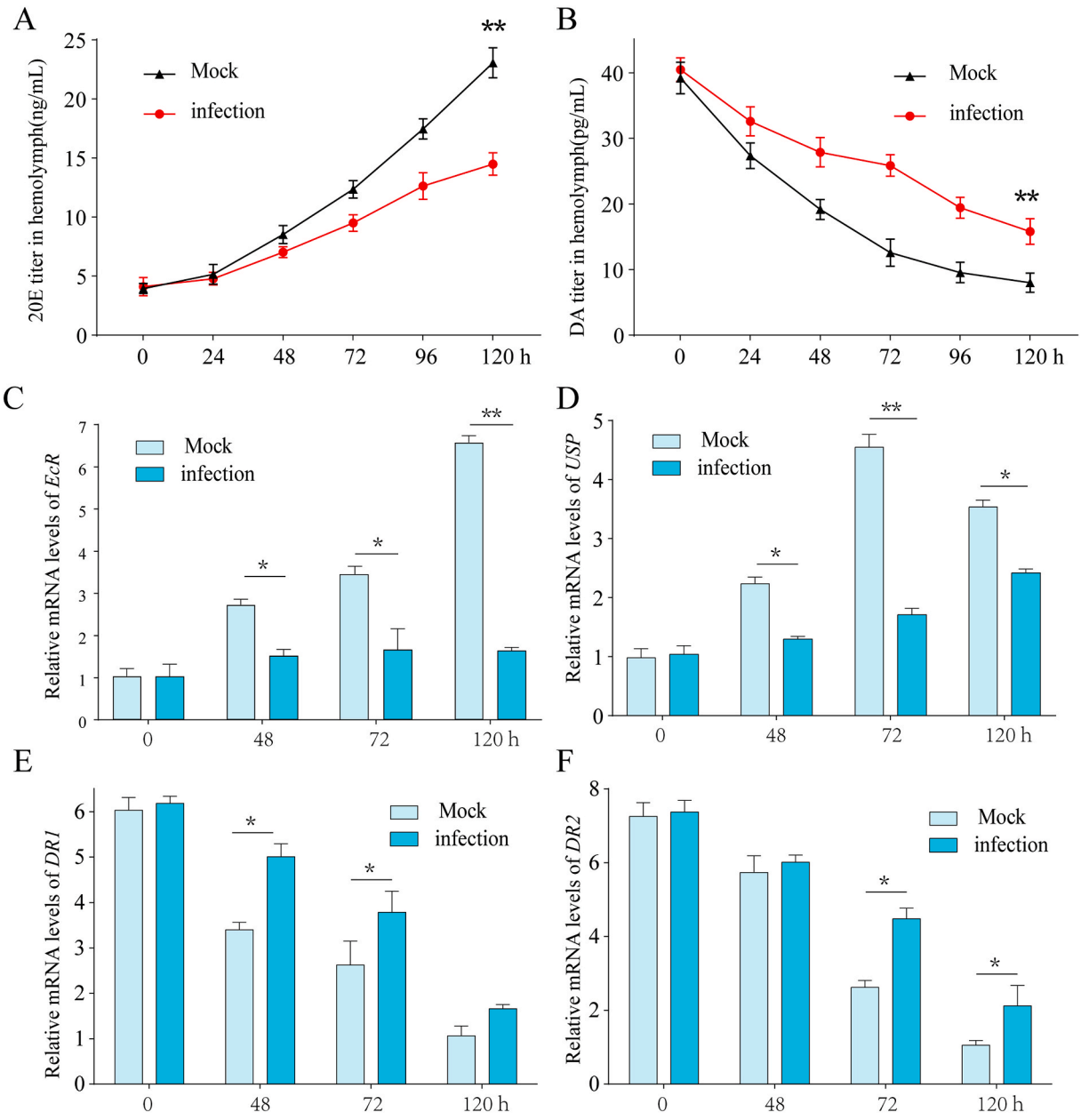

Fig. 4. The effect of BmNPV infection on endogenous hormone and its metabolism-related genes.
The content of 20E (A) and dopamine (B) in hemolymph were measured at 0, 24, 48, 72, 96 and 120 h post-infection. The collected hemolymph supernatant was subjected to ELISA to determine the titers of 20E and dopamine. (C, D) The transcriptional levels of 20E-response genes (*EcR*, *USP*) after BmNPV infection. The transcript levels of *EcR* and *USP* were significantly decreased after virus infection. (E, F) The mRNA expression levels of dopamine receptor (*DR1*, *DR2*) after BmNPV challenge. The transcript levels of *DR1* and *DR2* were significantly up-regulated following virus infection, implying that virus infection might induce this hyperactive behavior by activating dopamine receptors. Bars indicate means, and error bars represent standard deviations. The statistical significance was calculated using Student's t-test (*$p < 0.05$; **$p < 0.01$). Abbreviations: EcR, ecdysone receptor; USP, ultraspiracle protein; DR, dopamine receptor. The above results revealed that BmNPV infection disturbed the hormone balance in silkworm.

(agonist or antagonist). After injection of the antagonist (Flupentixol Dihydrochloride), *polh* and *gp64* expression were significantly decreased in both transcript and protein levels (Fig. 6A and B). By contrast, activation of DA receptor could stimulate ELA behavior through injecting agonist (SKF389393 HCl). Control larvae were injected with an equal volume of DMSO.

The inhibition of DA receptor could suppress this hyperactive behavior, whereas dopamine receptor agonists stimulate ELA behavior (Fig. 6F), indicating that the ELA behavior is induced and maintained by activating dopaminergic signaling pathway after BmNPV infection. The BmNPV could manipulate larvae behavior through molecular targets in the host brain, leading to extensive alteration of synaptic efficacy and behavioral changes that promote successful proliferation of BmNPV progeny.

4. Discussion

Baculoviruses have evolved versatile strategies to benefit optimal from their insect hosts (Gasque et al., 2019; Wang and Hu, 2019). Infected silkworm larvae show increased locomotion behavior, which is thought to enhance viral replication and transmission. This process may involve four physiological systems (neural, endocrine, neuromodulatory

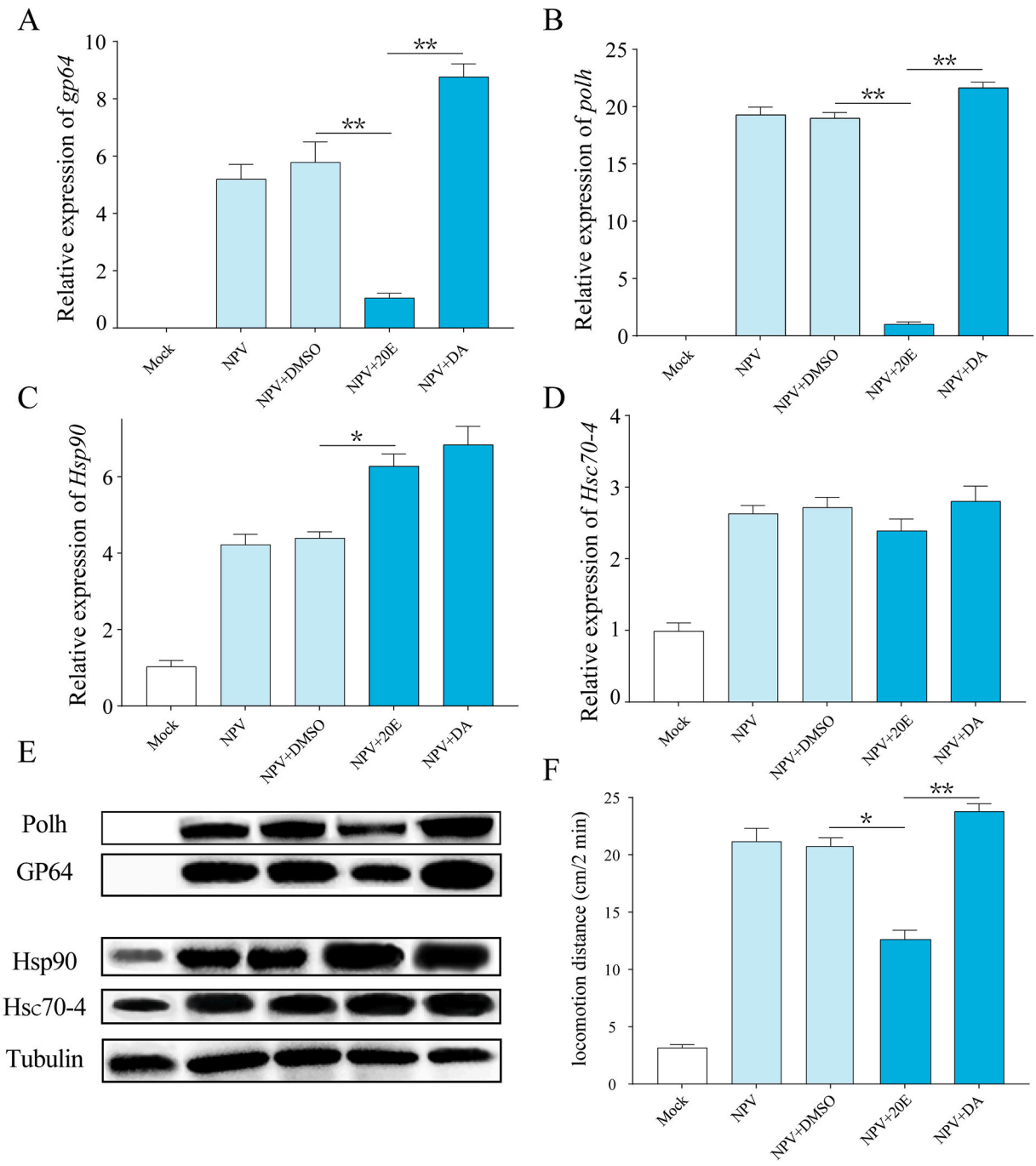

Fig. 5. The effect of exogenous hormones on BmNPV-induced ELA behavior.
(A, B) Effect of exogenous hormone treatments on BmNPV replication (*polyhedrin* and *gp64* gene expression). The mRNA expression levels of virus replication in brain were quantified by qRT-PCR. (C, D) Effect of exogenous hormone treatments on host gene expression (*Hsp90*, *Hsc70-4*). The mRNA expression levels of heat shock protein in brain were quantified by qRT-PCR. 20E or DA was injected into larvae at 48 h post-inoculation; For control group, larvae received no infection (Mock) or no treatment (NPV); DMSO was used as the solvent control. (E) Western blot analysis of virus proteins and heat shock proteins (HSPs) expression. After exogenous hormone treatments, protein extracted from whole brain tissue were analyzed by Western blot (GP64, Polh, Hsp90 and Hsc70-4). Tubulin serves as a loading control. (F) Locomotion distance of infected larvae after exogenous hormone treatments. The statistical significance was calculated using Student's t-test (*$p < 0.05$; **$p < 0.01$). Abbreviations: 20E, 20-hydroxyecdysone; DA, dopamine. The results show that 20E and DA counteractively regulate ELA behavior induced by BmNPV.

and immunomodulatory), making it difficult to elucidate the specific mechanisms underlying host behavioral manipulation (Lafferty and Shaw, 2013).

With the rapid development of multi-omics technology, several pathways hijacked by baculovirus and other parasites have been identified (Lin et al., 2018; Nguyen et al., 2013; Xing et al., 2017). A recent study showed that several signal transduction pathways are enriched during this hyperactive stage (Bhattarai et al., 2018a, 2018b). However, they did not focus on the brain which plays a decisive role in regulating host behavior. To gain a deep understanding on BmNPV-induced ELA in silkworm, we shall focus our attention on the brain tissue between virus-infected and control hosts. In our previous studies, some behavior-related proteins in the silkworm brain were identified by shotgun proteomic (Wang et al., 2016). More specifically, several

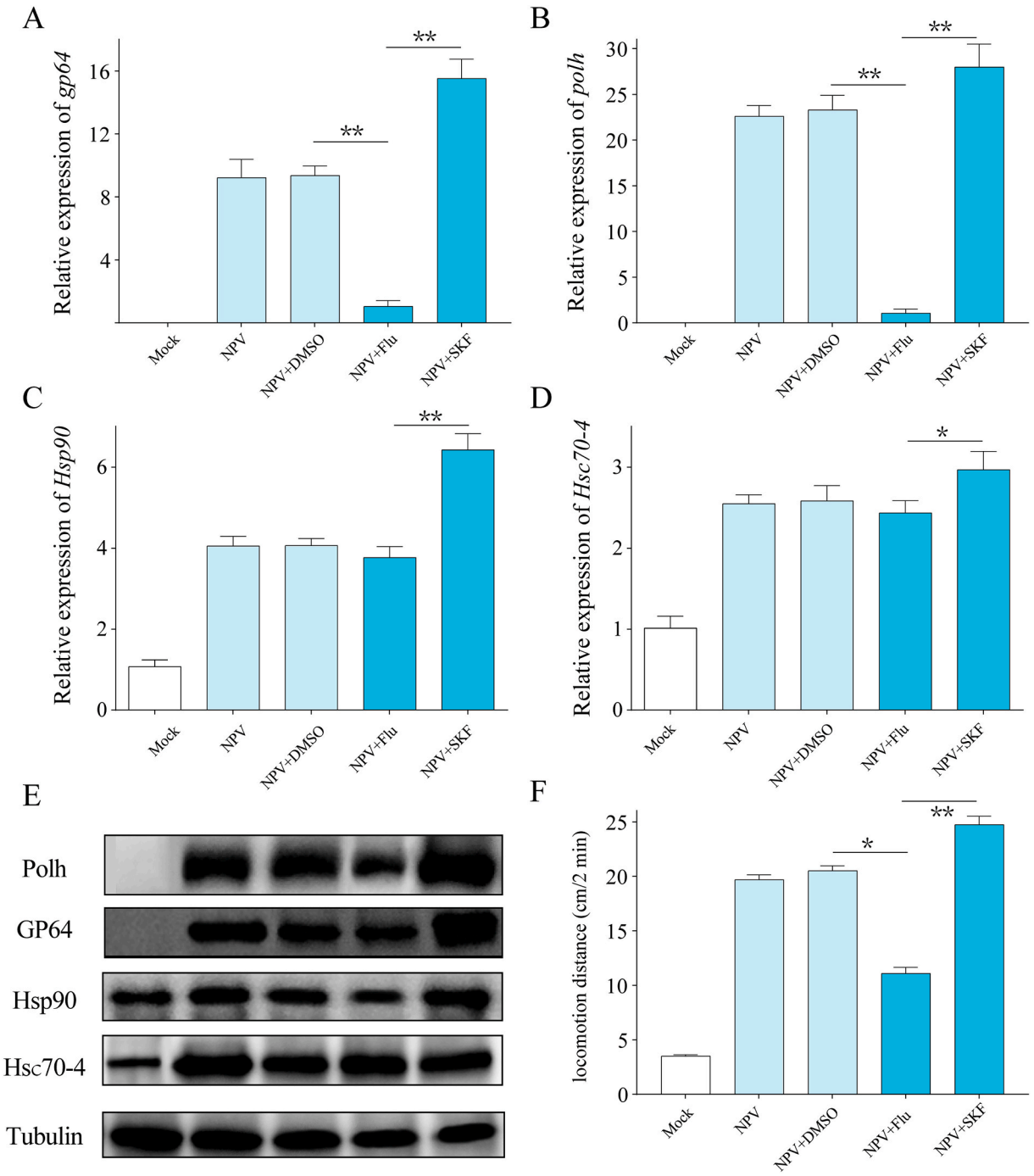

Fig. 6. The effect of dopamine signaling pathways on BmNPV-induced ELA behavior.
(A, B) Effects of DA receptor agonist and antagonist treatments on BmNPV replication (*polyhedrin* and *gp64* gene expression). The mRNA expression levels of virus replication in brain were quantified by qRT-PCR. (C, D) Effect of DA receptor agonist and antagonist treatments on host gene expression (*Hsp90*, *Hsc70-4*). The mRNA expression levels of heat shock protein in brain were quantified by qRT-PCR. Flu (antagonist) or SKF (agonist) was injected into larvae at 48 h post-inoculation; For control group, larvae received no infection (Mock) or no treatment (NPV); DMSO was used as the solvent control. (E) Western blot analysis of virus proteins and heat shock proteins (HSPs) expression. After injecting the agonist or antagonist of dopamine receptor, protein extracted from whole brain tissue were analyzed by Western blot (GP64, Polh, Hsp90 and Hsc70-4). (F) Effect of DA receptor agonist or antagonist treatment on locomotion distance. The statistical significance was calculated using Student's t-test (*$p < 0.05$; **$p < 0.01$). Abbreviations: Flu, Flupentixol Dihydrochloride (antagonist); SKF, SKF389393 HCl (agonist). The results show that injecting dopamine and its receptor agonists could promote ELA behavior in infected larvae.

differentially expressed genes were identified in BmNPV-infected brain, which are involved in circadian rhythms, synaptic transmission and the serotonin receptor signaling pathway (Wang et al., 2016), implying the importance of genetic and epigenetic factors in triggering this hyperactive-like behavioral alterations.

Accordingly, viruses trigger diverse cellular responses, including the activation of apoptosis, DNA damage, and heat shock response, aimed at fighting the infection by preventing virus replication and dissemination. HE staining and immunohistochemistry results showed that BmNPV invades host brain, resulting in severe pathological change. It is worth noting that heat-shock proteins (Hsps) were detected in an abnormally high expression during ELA stage. Several studies reported that

baculoviruses hijacked host cells and utilized host Hsp90/70 (heat shock protein) to facilitate its own replication and proliferation (Li et al., 2019; Wu et al., 2019). Hsc70-4 was characterized as a novel component protein of ODV and BV, which may play an important role in BmNPV infection (Iwanaga et al., 2014). In *Helicoverpa armigera*, Hsp90 was reported to interact with steroid hormone receptors, signaling kinases, and various transcription factors by differential phosphorylation and protein interactions (Liu et al., 2013). These studies suggested a potential connection between BmNPV and hormone metabolism.

In addition, it was also discovered that a single gene of baculovirus (*egt* [known as ecdysteroid UDP-glycosyl-transferase]) that inactivates insect host ecdysteroid hormones and prevents insect molting. Therefore, EGT might suppress the growth and development by disturbing endogenous hormone balance. A more recent study showed that two host miRNAs are found to target the BrZ2 gene and are involved in the cross-talk between the 20E and JH signaling pathways (Zhang et al., 2018). Theese results indicated the close relationship between host hormone metabolism and virus infection. For that, we further measured 20E level in larvae hemolymph by enzyme-linked immunosorbent assay (ELISA). The results showed that the hemolymph 20E content (both infected and non-infected) was increased. However, the transcription level of 20E response genes (*EcR* and *USP*) were greatly up-regulated following virus infection, compared with the mock group. And the content of 20E in hemocoel was lower in infected larvae than the control group. The decreased 20E content might result from EGT expression in silkworm, which was verified by over-expressing EGT in Gal4/UAS system (Shen et al., 2018).

Moreover, dopamine is also an important neuromodulator to regulate larval development and behavior, especially in neuromodulation (Kang et al., 2019; Kume, 2005). Several studies have suggested the significance of dopamine in locomotor activity and behavioral manipulation in various animal species. In migratory locust, dopamine receptor pathway is responsible for maintaining gregarious behavior (Ma et al., 2011). Juvenile hormone was reported to be involved in the regulation of suppressing aggregation behavior by influencing antennal gene expression in locusts (Guo et al., 2020). Indeed, several reports suggest that parasites (like *Toxoplasma*) have evolved an ingenious strategy to manipulate host metabolism (such as dopamine or other hormones) (McConkey et al., 2015; Wang et al., 2015b). Therefore, we set out to investigate the function of dopamine in infected larvae during hyperactive stage. Our results found that hormone-sustained homeostasis in larvae might be completely disturbed by BmNPV, indicating the importance of hormone in the regulation of ELA.

To understand how hormone acts on insect ELA behavior, exogenous hormone was injected into larvae hemolymph. The result showed that treatment of BmNPV-infected larvae with 20E interfered with BmNPV replication. In contrast, injecting DA promoted BmNPV replication and larvae moved longer crawling paths. It has been confirmed that 20E antagonizes DA's functions (Kang et al., 2019). Thus, clarification of how DA functions to promote ELA behavior is required. We further injected DA receptor agonist or antagonist treatments into larvae at 48 h post infection, respectively. It is striking that the activation of DA receptors (agonist, SKF38393) could amplify the effects of ELA behavior, which is contrary to the result of injecting its antagonist. Our results indicate that dopamine has a stimulatory role in locomotor activity in baculovirus-infected insect. A series of biogenic amines (such as dopamine, octopamine, serotonin, tyramine and gamma-aminobutyric acid) has been revealed to play important roles in host behavioural manipulation. Dopamine in silkworm larvae may act as a coordinator of ELA behavior in response to BmNPV infection by modulating peripheral and central nervous system signaling transmission during hyperactive stage.

It has been reported that injecting dopamine and its agonists promoted the gregarious behavior in the migratory *Locust* (Ma et al., 2011), and *Toxoplasma* parasite could increase dopamine secretions in the brain of infected mouse (Prandovszky et al., 2011). However, injection of dopamine (or dopamine receptor agonist) into normal larvae could not trigger similar hyperactive behavior, indicating that BmNPV-induced ELA is not the result of a single factor determining. More importantly, the underlying processes are likely to be more complicated within the host CNS. Taking together, BmNPV infection in silkworm larvae is a highly dynamics process (Nguyen et al., 2013; van Houte et al., 2013), relying on the complex turnover and activation of signaling cascades. Correspondingly, dopamine is a biogenic amine, found at elevated levels during the hyperactive stage. The injection of dopamine and dopamine receptor agonists stimulate ELA behavior in infected larvae, indicating that this hyperactive behavior is induced and maintained by dopaminergic modulation. Further research is still required to disclose the formative mechanism of hormone disorder in infected larvae. Meanwhile, it is also an interesting scientific question to deeply investigate about dopaminergic signal pathway, which could be conducive to understanding baculovirus-induced hyperactive behavior.

Author contributions

X.F.W conceived and designed the experiments; Y.L performed the experiments; Y.L processed the data and wrote the manuscript. J.J.Z and S.D.Z reviewed the manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2021.104036.

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