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# Transcriptome analysis of the brain of the silkworm *Bombyx mori* infected with *Bombyx mori* nucleopolyhedrovirus: A new insight into the molecular mechanism of enhanced locomotor activity induced by viral infection

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ABSTRACT

Baculoviruses have been known to induce hyperactive behavior in their lepidopteran hosts for over a century. As a typical lepidopteran insect, the silkworm *Bombyx mori* displays enhanced locomotor activity (ELA) following infection with *B. mori* nucleopolyhedrovirus (BmNPV). Some investigations have focused on the molecular mechanisms underlying this abnormal hyperactive wandering behavior due to the virus; however, there are currently no reports about *B. mori*. Based on previous studies that have revealed that behavior is controlled by the central nervous system, the transcriptome profiles of the brains of

the changes in the BmNPV-infected brain on the transcriptional level and to provide new clues regarding the molecular mechanisms that underlies BmNPV-induced ELA. Compared with the controls, a total of 742 differentially expressed genes (DEGs), including 218 up-regulated and 524 down-regulated candidates, were identified, of which 499, 117 and 144 DEGs could be classified into GO categories, KEGG pathways and COG annotations by GO, KEGG and COG analyses, respectively. We focused our attention on the DEGs that are involved in circadian rhythms, synaptic transmission and the serotonin receptor signaling pathway of *B. mori*. Our analyses suggested that these genes were related to the locomotor activity of *B. mori* via their essential roles in the regulations of a variety of behaviors and the down-regulation of their expressions following BmNPV infection. These results provide new insight into the molecular mechanisms of BmNPV-induced ELA.

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An intriguing phenomenon in which parasites manipulate the behavior of their hosts to increase parasite transmission rates has been observed in a variety of organisms (Beckage, 1997; Moore, 2002; Lefèvre et al., 2009). Parasites are capable of imposing dramatic behavioral influences on their hosts that include alterations in foraging, location and locomotor activity (Moore, 2002). Some parasitoid wasps coerce spiders into spinning 'sleeping bags' that are suspended from branches and provide a safe pupating site for the wasp (Eberhard, 2000). *Aedes aegypti* mosquitoes carrying Dengue virus (*Flavivirus iridae*) display a 50% increase in locomotor activity compared with uninfected mosquitoes (Lima-Camara et al., 2011) and exhibit longer probing and feeding times (Platt et al., 1997). Many of these behavioral changes help to increase parasite transmission rates and are thought to be conversed strategies of the parasite for manipulating their hosts' behaviors (Moore, 1995). Although numerous examples of such manipulations are known, the molecular mechanisms underlying these phenomena are still largely enigmatic, particularly regarding the changes that occur in the host.

Research on the parasitic manipulation of behavior has focused on the effects of parasites on host neural function. This emphasis is reasonable given that behavior is controlled by the central nervous system (Moore, 2002; Klein, 2003). Because the brain is a behavior-regulating center, parasites control their hosts' behavior by manipulating brain activity to a certain extent (Adamo, 2002; Thomas et al., 2005). For example, the phenomenon in which the gammarid host *Gammarus lacustris* is infected by the acanthocephalan

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*Polymorphus paradoxus* and subsequently displays abnormal escape behavior has been proven to result from the adverse effect exert by the parasite on the host's serotonergic system in the brain (Maynard et al., 1996). Several lines of research have applied proteomic technology to explore the molecular mechanisms underlying the phenomena of parasite manipulation of the host brain at the protein level (Biron et al., 2005, 2006; Ponton et al., 2006; Lefèvre et al., 2007a,b). However, a detailed report about the molecular mechanisms in the host brain at the gene expression level is lacking.

Baculoviruses have been known to induce hyperactive behavior in their lepidopteran hosts for over a century (Goulson, 1997; Hoover et al., 2011; Houte et al., 2012). The diseased larvae often display enhanced locomotor activity (ELA), which leads them to migrate to the upper plant foliage and subsequently die. The decaying corpse will contaminate a larger surface area of the host plant resulting in increased dispersal and transmission of the virus. Similar to baculovirus hosts, the silkworm (*Bombyx mori*), which is a typical lepidopteran insect that has been domesticated for over 5000 years, displays enhanced locomotor activity after infection by the *B. mori* nucleopolyhedrovirus (BmNPV). Although Kamita et al. (2005) and Katsuma et al. (2012) found that the protein tyrosine phosphatase (*ptp*) gene of BmNPV plays an essential role in this ELA phenomenon, there are no further investigations of the molecular mechanisms of this ELA phenomenon in *B. mori*.

*B. mori* is not only an important economic insect but is also a rising model organism. *B. mori* possesses a variety of advantages, such as ease of rearing and experimental manipulation as compared with other Lepidoptera insects. Moreover, the whole-genome sequencing of *B. mori* has been completed (Xia et al., 2004). Recently, RNA-sequencing (RNA-Seq), which is a powerful tool for transcriptome analyses and is based on deep sequencing technology, has been used to explore the molecular mechanisms of multiple physiological events in the silkworm (Li et al., 2012; Nie et al., 2014).

In the present study, RNA-Seq was applied to the polyadenylate-enriched mRNAs from the silkworm brains to better understand the complexity of the molecular mechanisms underlying BmNPV-induced ELA in *B. mori*. We obtained both up- and down-regulated genes by comparing the brain transcriptome profiles between BmNPV-infected and non-infected silkworm groups. We also screened the ELA-related genes from the DEGs via bioinformatic analyses. We believe that our data will provide useful information that will expand our current knowledge about the molecular mechanisms underlying BmNPV-induced ELA in *B. mori*.

## 2.1. Viruses and silkworm strain

BmNPV was stored in our laboratory. The Dazao silkworm strain was used in the study. The larvae were reared on fresh mulberry leaves in an environment with a 12-h light/12-h dark at 25 ± 1 °C and 70–85% relative humidity. Newly molted larvae of the fifth instar were used for the experiments.

## 2.2. Oral infection of the silkworms with B. mori nucleopolyhedrovirus (BmNPV) for transcriptome analyses

Approximately 15 newly molted silkworm larvae of the 5th-instar from each of a total of 10 groups were placed in 10 petri dishes and deprived of food prior to infection. For the oral infections, the silkworms were fed mulberry leaves with added BmNPV polyhedra. The infection dose was calculated to be $2 \times 10^4$ polyhedra per larva. By 3 h, most of the viral meal was ingested. The larvae were then transferred to a large petri dish with normal mulberry leaves. For the non-infected controls, the same volume of 0.9% physiological saline was mixed in the feed for the silkworms, and the rearing conditions were identical to those of the BmNPV-infected groups. All experiments were performed in three independent biological replications.

## 2.3. RNA extraction

The larval brains were dissected from the BmNPV-infected and non-infected groups (Fig. 1) at 96 h post-infection when the BmNPV-induced silkworms began to display abnormal hyperactive wandering behaviors and were then immediately snap9-frozen in liquid nitrogen. Total RNA was extracted from each sample using Trigol reagent (DingGuo Biotechnology, Beingjing, China) following the manufacturer's instructions. The RNA was quantified by measuring the absorbance at 260 nm using a NanoVue UV–Vis spectrophotometer (Bio-Science). The purities of all RNA samples were assessed at absorbance ratios of $OD_{260/280}$ and $OD_{260/230}$. The RNA integrity number (RIN) values (>8.0) of the samples were assessed using an Agilent 2100 Bioanalyzer (Santa Clara, CA, USA).

## 2.4. cDNA library construction and Illumina RNA-Seq

Libraries construction and RNA-Seq were performed by the Biomarker Biotechnology Corporation (Beijing, China). Briefly, the mRNAs were enriched with a NEBNext Poly (A) mRNA Magnetic Isolation Module (NEB, E7490). The enriched mRNAs were used as templates, and cDNA libraries were constructed using the NEBNext mRNA Library Prep Master Mix Set for the Illumina (NEB, E6110) and NEBNext Multiplex Oligos for the Illumina (NEB, E7500) following the manufacturer's instructions. The inserts sizes were detected with 1.8% agarose gel electrophoresis. cDNA library quantification was performed with quantitative RT-PCR using the Library Quantification Kit-Illumina GA Universal (Kapa, KK4824) according to the protocol. Clusters of the cDNA libraries were generated on an Illumina cBot. Finally, the cDNA libraries were sequenced on an Illumina HiSeq™ 2000.

## 2.5. RNA-Seq data analysis

The clean reads were filtered from the raw reads by removing the reads with only adaptors and unknown nucleotides >5% and those that were of low quality. The clean reads were then mapped to the silkworm genome sequence obtained from the SilkDB

Fig. 1. BmNPV-infected and non-infected silkworms. (A) A BmNPV-infected silkworm. (B) A non-infected silkworm. Bar = 1 cm.

database (http://silkworm.swu.edu.cn/silkdb/) using the TopHat software (Trapnell et al., 2009). The gene expression levels were calculated using the fragments per kilobase of exon per million fragments mapped (FPKM) method (Mortazavi et al., 2008). A FPKM >0.1 threshold was applied to ensure that the genes were expressed in two groups. The criteria of a fold change (FC) ⩾ 2 and a false discovery rate (FDR) < 0.01 were used to isolate the differentially expressed genes. The ratios of gene expression between the two groups (i.e. the BmNPV and control groups) with values greater than 2.0 were taken to indicate up-regulation, and those below 0.5 were taken to indicate down-regulation. The sequencing data generated in this study were deposited in the NCBI Sequence Read Archive (SRA, http://www.ncbi.nlm.nih.gov/sra) with accession number SRR1928169.

### 2.6. Bioinformatics analysis

The sequences of the differentially expressed transcripts were subjected to BLAST queries against the Ami gene ontology (GO) database (http://amigo.geneontology.org/cgi-bin/amigo/blast.cgi). The corresponding GO terms were extracted from the most homologous genes using a Perl program. The GO annotation results were plotted with the Web Gene Ontology Annotation Plot (WEGO) (http://wego.genomics.org.cn/cgi-bin/wego/index.pl) tool by uploading compiled WEGO-native format files containing the obtained GO terms. The FASTA gene sequences of the differentially expressed transcripts were searched against the online Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/tools/blast/). The corresponding KEGG pathways were extracted. The pathways were classified according to the definitions of the KEGG (http://www.genome.ad.jp/kegg/pathway.html). Additionally, the gene sequences of the differentially regulated transcripts were subjected to the Clusters of Orthologous Groups (COG) database (http://www.ncbi.nlm.nih.gov/COG/), and the corresponding COG annotation results were extracted.

### 2.7. Validation of RNA-Seq by qPCR quantitative RT-PCR

To validate the transcriptome data, 10 genes (7 down-regulated and 3 up-regulated) were selected for qPCR. The primer sequences and related information are shown in Table S1.

The total RNAs isolated from both the BmNPV-infected and non-infected groups were reverse transcribed using a GoScript™ Reverse Transcription System (A5001, Promega) following the manufacturer's instructions. According to the SYBR Premix Ex Taq™ Kit (TaKaRa) protocol, the reactions were run on an ABI7300 real-time PCR system using a 20-μL reaction system with reaction procedures of 40 cycles of 94 °C for 3 s and 60 °C for 30 s followed by a dissociation for the quality control of the amplified products. All samples were examined in triplicate. The expression level of each gene was calculated with the $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen, 2001) and normalized to the silkworm housekeeping gene *actinA3*. Following normalization, the ratios were expressed as fold changes compared with the samples from the control group.

## 3. Results

### 3.1. General transcriptional patterns

Whole genome mRNA sequencing was used to monitor the global changes in gene expression in the silkworm brain. After filtering, 64.05% and 87.85% clean reads from the two groups were matched to the silkworm genome using Tophat software; the proportions of the unique mapped reads accounted for the mapped reads were 95.92% and 96.48%, respectively (Table 1). Alignment against the silkworm genomic sequence revealed 742 differentially expressed genes (DEGs) that included 218 up-regulated and 524 down-regulated genes in the BmNPV-infected group (Table S2).

### 3.2. Gene ontology (GO) analysis of the differentially expressed genes

The DEGs were assigned to various GO categories to determine their functional classifications (Fig. 2). A total of 499 genes (123 up-regulated and 376 down-regulated, Table S3) with GO annotations were classified into the following 3 functional categories: cellular component, molecular function and biological process. Overall, 17, 12 and 22 catalogs of cellular component, molecular function and biological process were clustered, respectively. The majority of the annotated DEGs were assigned to the cell, membrane and organelle catalogs in the cellular component category. The widest distribution of the DEGs was associated with binding and catalytic activity in the molecular function category. In the category of biological process, the majority of the DEGs were involved in metabolic process, cellular process, multicellular organismal process, developmental process, response to stimulus and biological regulation.

### 3.3. KEGG pathway analysis of the differentially expressed genes

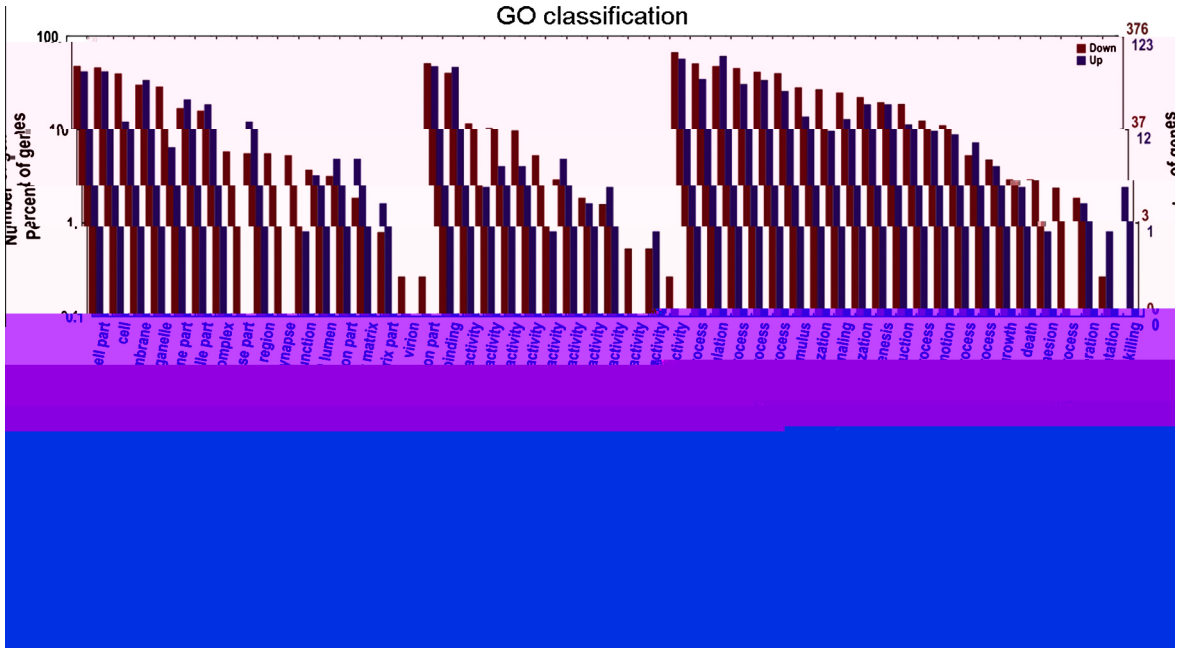
A total of 117 DEGs were assigned to 87 different pathways in this study (Table S4). As shown in Fig. 3, the pathways were classified into the following six categories: metabolism, genetic information processing, environmental information processing, cellular processes, organismal systems and human diseases. In the mapped pathways of the up-regulated genes, the abundant genes mapped onto carbohydrate, nucleotide and amino acid metabolisms. Similarly, the down-regulated genes were also primarily related to carbohydrate metabolism. However, it is noteworthy that there were numerous down-regulated genes that were associated with signal transduction and interactions between signaling molecules, suggesting that abnormal signal transmission occurred in the brains of the silkworms following infection with BmNPV.

### 3.4. COG analysis of the differentially expressed genes

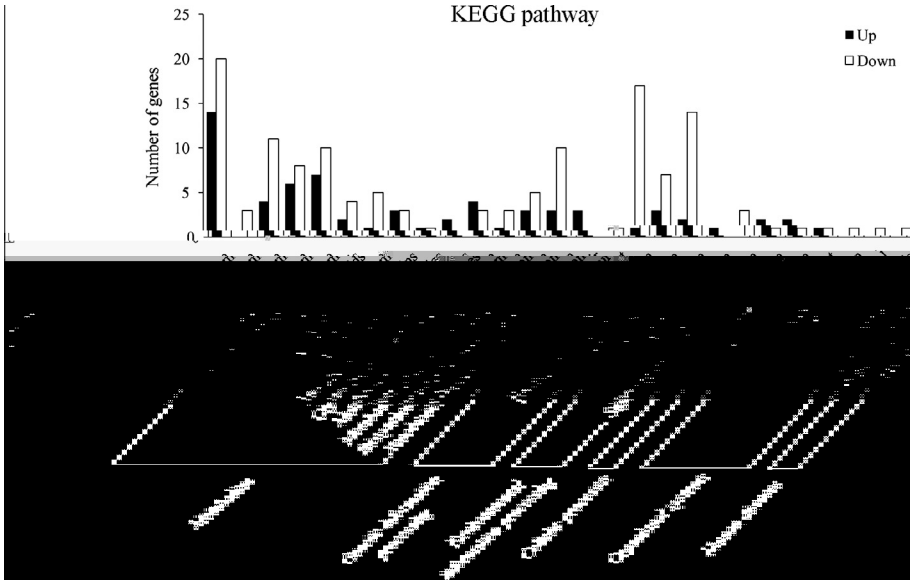
COG is a database in which orthologous gene products are classified. COG analysis of the DEGs provided informations about their possible functions (Table S5 and Fig. 4). A total of 144 DEGs with 19 functional definitions were obtained. Down-regulated genes accounted for a large proportion of this set, and observations that amino acid transport and metabolism, carbohydrate transport and metabolism and signal transduction mechanisms were prevalent in the down-regulated genes corresponded well with the results

Statistical analyses of the transcriptome sequence data.

| | | | |
|---|---|---|---|
| Clean reads | Total reads | 40,765,270 | 42,239,674 |
| | Total bases | 4,076,243,285 | 4,223,650,782 |
| | GC% | 42.79 | 44.31 |
| | Q30% | 87.50 | 91.17 |
| Mapping to genome | Mapped reads | 26,110,238 (64.05%) | 37,106,733 (87.85%) |
| | Unique mapped reads | 25,305,377 (96.92%) | 36,106,158 (97.30%) |
| | Multiple mapped reads | 804,861 (3.08%) | 1,000,575 (2.70%) |
| | Pair mapped reads | 23,239,822 (89.01%) | 34,079,941 (91.84%) |
| | Single mapped reads | 2,192,092 (8.40%) | 2,215,837 (5.97%) |




. . . GO categories of the differentially expressed genes (DEGs). The annotated DEGs were classified into the cellular component, molecular function and biological process categories by WEGO according to the GO terms. The right panel shows the gene numbers mapped to the GO terms. The left panel shows the proportions of up-regulated and down-regulated genes according to the GO terms. The right panel shows the numbers of up- and down-regulated genes.




. . . Pathway categories of the 117 DEGs according to the KEGG pathway taxonomy. The pathways were clustered into metabolism, genetic information processing, environmental information processing, cellular processes, organismal systems and human diseases. The left panel shows the numbers of genes that mapped to each of the pathways.

of the KEGG analysis. Among the up-regulated genes, nucleotide transport and metabolism, translation, ribosomal structure and biogenesis were highly represented, suggesting abnormal metabolism in the brains of the *B. mori* that were infected with BmNPV at the transcriptional and protein levels. Our COG analysis results were consistent with the results of our GO and pathway analyses.

### 3.5. Validation of the data reliability by qRT-PCR

To verify the RNA-seq results, we selected 10 DEGs by designing specific primers for quantitative RT-PCR. Our results indicated that the changes observed in the expression levels of these genes (Fig. 5) mimicked the changes measured with RNA-seq. Hence, the qPCR results confirmed the reliability of our RNA-seq data.

## 4. Discussion

*B. mori* nucleopolyhedrovirus is an exclusive pathogen of the silkworm that frequently causes serious damage to sericulture production. The BmNPV-induced ELA of *B. mori* often occurs during the late stage of infection and is characterized by irregular movements of the infected larvae. It is believed that the virus induces this behavior so that after death, the decaying cadaver will release virions and contaminate a larger surrounding area, resulting in

As the main component of the central nervous system, the brain plays essential roles in receiving and transmitting external signals and coordinating different types of behavior (Straussa and Heisenberg, 1993). Intrigued by the relationship between infection and brain activity and hence hyperactive locomotion, we used RNA-Seq to investigate the changes in the BmNPV-infected brains of *B. mori* on the transcriptional level. A high dose ($2 \times 10^4$ polyhedra per larva) was used to infect the larvae and ensure that they acquired the NPV disease during the fifth instar. The time point at which the silkworms displayed enhanced locomotor activity (96 h post-inoculation) was selected to compare the genes in the BmNPV-infected brains that were differentially expressed relative to the controls. At 96 h post-inoculation, a total of 742 DEGs were identified. These DEGs during infection provide potential insights into the complex regulatory phenomena of the response to viral infection at the transcriptional level. In this study, we focused on the DEGs that were related to the locomotion of *B. mori*, and we classified them into three categories that included circadian rhythms, synaptic transmission and the serotonin receptor signaling pathway (Table 2).

### *4.1. Differentially expressed genes related to circadian rhythms*

Disturb6.3(rel7.9702323.2062272(a1.3088R21c4fhms)]TJETE]TJ2<</MCI

sed dispersal and transmission of the virus (Hoover et al.,

that are involved in the circadian rhythms of insects, i.e. *BGIBMGA000031* and *BGIBMGA007140* (which encode ebony protein and cryptochrome-1, respectively) (Table 2). The ebony protein can be detected in the hypodermis, visual system and other brain regions (Richardt et al., 2002) and plays essential roles in cuticle tanning and sclerotization and nervous system functions such as vision and behavior (Richardt et al., 2003). Previous biochemical investigations have led to the conclusion that ebony has a β-alanyl-dopamine synthetase function and that mutations in *ebony*, among other effects, selectively perturb phototaxis and locomotor activity rhythm and cause arrhythmicity in *Drosophila melanogaster* (Newby and Jackson, 1991; Suh and Jackson, 2007). In insects, cryptochromes act as components of the circadian clock that control daily physiological and behavioral rhythms and as photoreceptors that mediate the entrainment of the circadian clock to light (Cashmore, 2003). In our study, it is possible that the down-regulations of *ebony* and *cryptochrome-1* caused by infection with BmNPV resulted in the disturbance of the locomotor activity rhythm and gave rise to the abnormal behavior of *B. mori*.

### 4.2. Differentially expressed genes in synaptic transmission

The nervous system exerts its functions through synaptic transmission. Neurons receive information from sensory organs, send information to motor organs and share information with other neurons. Neurotransmitters contained in synaptic vesicles are transmitted to the various parts of the organism via synaptic transmission and regulate the different types of behavior exhibited by an organism (Calabresi et al., 2000). In our study, nine DEGs involved in synaptic transmission were down-regulated in the BmNPV-infected brains (Table 2).

Vesicle-associated membrane protein (also known as synaptobrevin) is a type II membrane protein of small synaptic vesicles and is essential for neuroexocytosis because its proteolysis by tetanus and botulinum neurotoxins types B, D, F and G blocks neurotransmitter release (Washbourne et al., 1995). An important step in cholinergic transmission involves the vesicular storage of acetylcholine, and this process is mediated by the vesicular acetylcholine transporter (VAChT). Prado et al. (2006) found that VAChT deficiency in mice alters synaptic vesicle filling and affects Ach release and that VAChT-knockdown severely impairs object and social recognition behavior in mice. Acetylcholinesterase (AChE) is known to play important roles in the maintenance of the normal transmission of neural impulses in synaptic clefts by catalyzing the hydrolysis of the neurotransmitter acetylcholine (Lang et al., 2010). Jensen et al. (1997) reported on the relationship between AChE inhibition and locomotor behavior in the carabid beetle *Pterostichus cupreus* and noted that AChE activity affects the behavioral sensitivity of *P. cupreus*. Additionally, Gupta and Sundararaman (1991) observed a relationship between AChE activity and burrowing capability in earthworms that had been exposed to a carbamate insecticide. These results suggest critical roles of VAChT and AChE in the regulation of a variety of behaviors in animals. Syntaxin-lA is a synaptic membrane protein that is localized to axons and synapses in the nervous system. The complete absence of *syntaxin-1A* causes subtle morphological defects in the peripheral and central nervous systems that affect nonneural secretory events and entirely abolishes neurotransmitter release in *D. melanogaster* (Schulze et al., 1995). $Ca^{2+}$/calmodulin-dependent protein kinase II (CaMKII) is one of the most abundant kinases in the brain and is also a major calcium-regulated signal transducer that regulates many neuronal systems, such as the synthesis and release of neurotransmitters (Schulman, 1993). Kadivar et al. (2014) found that CaMKII activity is involved in chronic morphine-induced behavioral plasticity in the rat. Thus, we conclude that the down-regulations of the DEGs (i.e. *BGIBMGA000943*, *BGIBMGA013466*, *BGIBMGA010205*, *BGIBMGA006590*, *BGIBMGA003321*, *BGIBMGA010643*, *BGIBMGA005724*, *BGIBMGA000408* and *BGIBMGA006298*) might have affected synaptic transmission in the BmNPV-induced brains and resulted in abnormal signal transduction and hence abnormal behavior.

### 4.3. Differentially expressed genes in the serotonin receptor signaling pathway

In insects, the signaling of the biogenic amine serotonin (5-hydroxytryptamine, 5-HT) controls a variety of behaviors including those related to circadian rhythms, aggression, behavioral gregarization and phototaxis in locusts, honeybees and fruit flies (Dierick and Greenspan, 2007; Anstey et al., 2009; Thamm et al., 2010; Ott et al., 2012). The specific functions of serotonin are mediated by its binding to and subsequent activation of membrane receptors. Yuan et al. (2005) showed that serotonin acting via the *D. melanogaster* 5-$HT_{1B}$ receptor affects circadian rhythms. The activation of the 5-$HT_2$ receptor with DOI has been shown to decrease overall aggression in *D. melanogaster* (Johnson et al., 2009). Silva et al. (2014) employed the Gal4-UAS technique to pan-neuronally express RNAis directed to the transcripts of each of the cloned 5-HT receptors in *Drosophila* larvae via the use of the elav-Gal4 driver and found that the animals that expressed RNAis for 5-$HT_{1B}$, 5-$HT_2$ and 5-$HT_7$ receptors in the mushroom bodies exhibited increased motor behavior. Here, three related genes, *BGIBMGA011067*, *BGIBMGA006929* and *BGIBMGA002316* (annotated as the tyramine receptor, serotonin receptor 4 and octopamine receptor, respectively) were down-regulated in the BmNPV-infected brains. Previous studies have reported that tyramine and octopamine modulate the locomotion of *D. melanogaster* larvae (Saraswati et al., 2004) and behavioral responses to addictive drugs, such as ethanol (Scholz, 2005). We speculate that the down-regulation of these DEGs might have altered the levels of biogenic amines (BA) in the brain. The increased locomotion observed in the BmNPV-infected silkworm might be involved in the modification of BA levels.

Additionally, a rather large number of DEGs were involved in some other biological processes and functional categories (Table S3), which indicates a series of major physiological and pathological changes in the silkworm brain following infection with BmNPV. In conclusion, our results provide new avenues to explore the molecular mechanisms underlying the ELA phenomenon caused by BmNPV in *B. mori*. Furthermore, we obtained genes with unknown functions, which should not be neglected because they might make important contributions to this pathogen-host interaction.

## A

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## A A.

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