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Abstract

Sexually dimorphic neural circuits are essential for reproductive behavior. The molecular basis of sexual dimorphism in the silkworm moth brain, however, is unclear. We conducted cDNA subtraction screening and identified *nervous wreck* (*Bmnwk*), a synaptic growth regulatory gene, whose expression is higher in the male brain than in the female brain of the silkworm. *Bmnwk* was preferentially expressed in the brain at the late pupae and adult stages. *In situ* hybridization revealed that *Bmnwk* is highly expressed in the optic lobe of the male moth brain. These findings suggest that *Bmnwk* has a role in the development and/or maintenance of the optic lobe in the male silkworm brain.

Introduction

Many sexually reproductive species show sexual differences in morphology and behavior. The sexual differences in behavior, especially in mating behavior, play important roles in successful reproduction. Sexual differences in behavior are thought to depend on the activation of sexually dimorphic neural circuits (Demir & Dickson 2005; Jazin & Cahill 2010; Kimchi *et al.* 2007; Kimura *et al.* 2008; Wu *et al.* 2009; Yamamoto 2007).

In insects, sex is determined by genetic mechanisms that produce distinct males and females. Sex hormones, which are the major sex determinants in vertebrates, have not been identified in insects (Fujii & Shimada 2007; Jazin & Cahill 2010). Recent studies have elucidated diverse mechanisms of sex determination in insects, such as the numeric ratio of sex chromosomes to autosomes in the fruit fly, the epistatic W chromosome in the silkworm, and the haplodiploidity in the honeybee (Beye *et al.* 2003; Cline 1993; Fujii & Shimada 2007; Gempe *et al.* 2009). In the brain of the fruit fly, *Drosophila melanogaster*, sex-specific splicing of *fruitless* produces sex-specific neural circuits (Demir & Dickson 2005; Kimura *et al.* 2008; Yamamoto 2007). Due to the diverse mechanisms of sex determination in insects, however, the molecular basis of the sexually dimorphic neural circuits remains unclear in the brains of other insects.

Male moths of the silkworm *Bombyx mori* show stereotypic sexual behavior in response to sex pheromones released from female moths (Obara 1979). Although the neural circuitry that transmits the pheromone information and triggers the stereotypic behavior in the male moth have been extensively identified (Kanzaki & Shibuya 1986; Kanzaki & Shibuya 1992; Kanzaki *et al.* 2003; Seki *et al.* 2005; Wada & Kanzaki 2005), the molecular mechanisms that underlie sexually dimorphic neural circuits are unknown. To elucidate the molecular basis of sexual dimorphism in the moth brain, we aimed to identify genes that are expressed in a sex-dependent manner. In the present study, we used a cDNA subtraction strategy to identify the silkworm homologue of

nervous wreck, termed *Bmnwk*, as the gene whose expression is increased in the male moth brain compared to the female moth brain. *Bmnwk* was preferentially expressed in the male brain at the late pupae and adult stages. *In situ* hybridization analysis revealed that *Bmnwk* is also highly expressed in the optic lobe.

Results

Screening and identification of genes that are expressed in a sex–differential manner in the silkworm moth brain.

cDNA subtraction analysis was used to search for genes whose expression in the brain differs between male and female moths. After suppression subtractive hybridization of the male and female brain cDNA, we conducted primary and secondary polymerase chain reactions (PCR), subcloned the PCR products into vectors, and transformed them into *Escherichia coli*. We randomly selected and sequenced 64 clones from the numerous transformed clones. After excluding redundant clones, we designed primers for each clone. Using semi-quantitative reverse-transcription (RT)-PCR, we identified three candidate clones, termed *Clone36, 44*, and *49*, which were preferentially expressed in the male brain (Fig. 1A). We conducted the same screening in the opposite direction (screening for female-biased genes) and identified two candidate clones, termed *Clone54* and *56* (Fig. 1A). To accurately measure the gene expression level, we then conducted a real-time RT-PCR analysis (Fig. 1B). Because only *Clone49* differed significantly between the male and female brains, we focused our analysis on *Clone49*. An open-reading frame was not found in the *Clone49* sequence and there were no predicted genes within 5 kb upstream or downstream of *Clone49* on the silkworm genome, therefore, we performed 5'- and 3'- rapid amplification of cDNA ends (RACE) and RT-PCR utilizing the KAIKOBLAST database. With the combination of RACE and RT-PCR, we identified approximately 7 kb of cDNA sequences and found that *Clone49* resides in the 3'-untranslated region (UTR) of the cDNA. The cDNA sequence spanned 125 kb on chromosome 1 (Z chromosome) of the silkworm genome and encoded a protein with high similarity to the nervous wreck protein (Fig. 2A and B). A database search revealed that two genes, *BGIBMGA001996* and *BGIBMGA001997*, are predicted within the cDNA sequence, suggesting that the prediction

by the database was incomplete. In the course of sequence analysis of the clones isolated by RT-PCR, we identified three splicing variants encoding different proteins. We termed these clones *Bmnwk L*, *M*, and *S*, after the name of the silkworm (*Bombyx mori*) and the length of protein encoded by each cDNA sequence. The sequence of *Clone49* corresponded to the +5945/ +6421 region of *Bmnwk L* sequences. The Nwk protein functions as a negative regulator of synaptic growth at *Drosophila* neuromuscular junctions and has distinctive domains, the F-BAR domain and SH3 domains (Coyle *et al.* 2004; O'Connor-Giles *et al.* 2008; Rodal *et al.* 2008). The *Bm*nwk proteins also had an F-BAR domain and two SH3 domains, with high similarities to those of *Drosophila* Nwk (Fig. 2B), indicating that *Bmnwk* is the silkworm *nwk* homologue. The *Bmnwk L, M,* and *S* sequences were deposited into the DNA Data Bank of Japan with the accession numbers AB600238, AB600239, and AB600240, respectively.

Quantification of Bmnwk *expression in various tissues and in the brains at various developmental stages.*

To confirm the differential expression of *Bmnwk* in the brains of male and female moths, we performed real-time RT-PCR analysis using specific primers designed to amplify the region corresponding to *BGIBMGA001997*, the common region among the three variants. The *Bmnwk* expression level was approximately 2.5-fold higher in male brains than in female brains (Fig. 2C). We next investigated whether *Bmnwk* is expressed in tissues in the moth other than the brain (head [without brain], thorax, and abdomen) and in the V5 (fifth-instar day-5) larvae (fat body, midgut, silk gland, and testis; Fig. 3A). In addition to the brain, *Bmnwk* expression was detected in the adult head, thorax, and larval testis. The expression levels in these tissues, however, were quite low compared with that in the brain, suggesting that *Bmnwk* is expressed in an adult brain-preferential manner. This tendency was observed in both males (upper panel in

Fig. 3A) and females (lower panel in Fig. 3A). Combined with the finding that *Bmnwk* expression is higher in the male brain than in the female brain (Fig. 2C and Fig. 1B), these findings suggest that *Bmnwk* expression is preferential to the male moth brain. Because *Bmnwk* expression was significantly higher in adult brains than in larval brains, we next investigated the developmental changes in *Bmnwk* expression in male and female brains at V5, G0 (gut purge day-0), P0 (pupae day-0), P4, and P8 stages (Fig. 3B). *Bmnwk* expression levels gradually increased with age and peaked at P8 in both male and female brains. The preferential male brain expression became detectable at P8. These findings indicate that *Bmnwk* expression is developmentally regulated to peak at the late pupae and adult stages.

Localization of Bmnwk *transcripts in moth brains.*

Next, to localize *Bmnwk* expression, we conducted *in situ* hybridization studies using the brain sections of male moths. A strong signal was detected in the optic lobe (OL) cells (Fig. 4A and B: surrounded by white lines). In particular, the ventral OL cells had strong signals. In addition, the signal was detected in cells at the edge of the neuropil (arrowheads). We also investigated *Bmnwk* expression in female moth brains (Fig. 4C). Although the signal intensity was weaker in the female brain than in the male brain, the staining pattern was similar to that in the male moth brain. These findings were obtained with riboprobes prepared from *BGIBMGA001997* sequences, and we detected a staining pattern similar to that using riboprobes prepared from *Clone49* sequences (data not shown). These findings implythat the difference in the *Bmnwk* expression levels, not the spatial regulation, between sexes is important for OL neural circuit development and/or maintenance.

Discussion

In the present study, we identified *Bmnwk* as a gene whose expression is higher in the male silkworm moth brain than in the female silkworm moth brain. *Bmnwk* expression was significantly higher in the brain than in other tissues, and was developmentally regulated, peaking at the late pupae and adult stages. *In situ* hybridization revealed that *Bmnwk* is expressed preferentially in the OL cells. These findings suggest that *Bmnwk* has roles in OL development and/or OL function in the adult.

nwk was originally identified by screening temperature-sensitive paralysis mutants in *Drosophila* and was revealed to function as a negative regulator of synaptic growth at the neuromuscular junction (Coyle *et al.* 2004). The nwk protein interacts with Wiscott-Aldrich Syndrome Protein (Wasp), thick veins (the bone morphogenic protein [BMP] receptor), and endocytic machinery proteins, and functions as an adaptor that transmits muscle-derived growth signals to the endocytic machineries and actin cytoskeletons (Coyle *et al.* 2004; O'Connor-Giles *et al.* 2008; Rodal *et al.* 2008). Despite extensive studies of nwk function in *Drosophila* neuromuscular junctions, the functional importance of *nwk* in the brain was not known and the only published expression analysis was in the *Drosophila* embryo (Coyle *et al.* 2004). The present study showed, for the first time, that *Bmnwk* expression is increased in the male moth brain and is relatively restricted to the OL cells, providing insight into *nwk* function in the brain. Because the OL structure is complicated and the projection of the OL neurons is precise (Otsuna & Ito 2006), we speculate that *Bmnwk* has a role in the fine-tuning and/or maintenance of synaptic growth through cytoskeletons in the developing OLs. In the hover fly, the dorsal lobula-plate neuron is sexually dimorphic, and the visual receptive field is smaller in the male than in the female (Nordstrom *et al.* 2008). In addition, the male small white butterfly has a male-specific fluorescing pigment in the retina that acts as a selective visual filter, enabling

efficient discrimination of females (Arikawa *et al.* 2005). These findings indicate that sexual dimorphism exists in the visual neural circuits of insect brains and some visual information is processed in a sex-specific manner. These lines of studies led us to speculate that the sexually dimorphic gene expression in the OLs is the essential molecular basis for sexually dimorphic visual recognition in insects.

In *Bombyx mori*, dosage compensation, which is a mechanism to equalize the gene expression levels from the Z chromosome, does not seem to occur (Fujii & Shimada 2007). Thus, the level of Z-linked gene expression tends to be higher in males than in females (Koike *et al.* 2003). A recent study using microarray data also showed that the expression of Z-linked genes is significantly higher in males than in females in various tissues, strongly suggesting the lack of a global dosage compensation mechanism (Zha *et al.* 2009). Because *Bmnwk* is located on the Z chromosome, it is reasonable that there is higher expression in males than in females. On the Z chromosome, there are many muscle-related genes, which are important for flight muscle development. In fact, heterozygous partial deletion of the Z chromosome causes haploinsufficient phenotypes in the male moths, such as the muscular dystrophy and flapless phenotypes (Fujii *et al.* 2007; Koike *et al.* 2003). In addition, Gene Ontology (GO) analysis of Z-linked genes using microarray data showed that the genes related to motor function is expressed in a male-biased manner, as well as the genes classified as an enzyme regulator and biological regulation (Zha *et al.* 2009). Thus, we speculate that the Z chromosome has evolved to carry important genes for the male-preferential trait. From this viewpoint, we speculate that the higher expression of *Bmnwk* in the male brain has some relevance to male-preferential brain function. By *in situ* hybridization, we detected increased *Bmnwk* expression levels in the male brain, but no difference in the spatial distribution pattern between sexes. These findings also

support our notion that the increased *Bmnwk* expression levels in male brains have a role in male-specific brain function.

To elucidate the functional importance of *Bmnwk* in the male brain, we tried to knockdown *Bmnwk* expression by injecting double-stranded RNA (dsRNA) into the pupal head. We observed no change in phenotype, however, probably due to the low efficiency of the dsRNA delivery to the brain by the extensive trachea surrounding the brain. We expect future studies using transgenic silkworms or viral vectors will reveal the functional importance of *Bmnwk* in the brain.

In the present study, we identified *Bmnwk* as a gene whose expression is male-biased. The sex differential gene expression in the brain is an essential basis of the sexually dimorphic behavioral traits and a huge number of genes are considered to be involved in sex-specific brain functions (Jazin & Cahill 2010). Our subtractive screening method was technically limited and did not provide comprehensive screening of sex-differential genes, but future studies using microarray or mRNA-sequencing technology will provide further insight into the neural basis of sexually dimorphic brain function. Future studies to elucidate the involvement of *Bmnwk* in male-brain function are necessary.

Experimental Procedures

Insect

Eggs of a racial hybrid of *Bombyx mori*, Kinshu × Showa, were purchased from a local dealer (Ueda Sanshu, Nagano, Japan). Larvae were reared on an artificial diet (Silkmate II, Nihon Nosan Kogyo, Yokohama, Japan) at 25°C under a 12-h light/12-h dark photoperiod cycle. Adult moths were used within 0 to 4 days after eclosion.

cDNA subtraction

Whole brains of male and female silkworm moths (100 brains each; wet weight = ca. 250 mg) were surgically collected. Poly A^+ RNA was purified using Micro-FastTrack 2.0 mRNA Isolation Kit (Invitrogen, Carlsbad, CA) and subjected to cDNA subtraction using the PCR-select cDNA subtraction kit (Clontech, Mountain View, CA) according to the manufacturer's instructions. The subtracted cDNA fragments were subcloned into pGEM-T Easy Vectors (Promega, Madison, WI), and 64 clones were randomly selected and sequenced. The primers were designed based on each clone sequence, and the initial screening was conducted using semi-quantitative RT-PCR.

Real-time RT-PCR

Total RNA of tissues was extracted using TRIzol Reagent (Invitrogen). For each sample, three to five moths or larvae were used. RNA was treated by DNase I (Invitrogen) and reverse-transcribed using PrimeScript RT Master mix (Takara, Kyoto, Japan). Real-time RT-PCR was performed with SYBR Premix Ex Taq™ II (Takara) according to the manufacturer's protocol, using gene-specific primers [*ribosomal protein L3* (*rpl3*); 5'-AGCACCCCGTCATGGGTCTA-3' and 5'-CAGTTTTTGTAGAAGCGACG-3', *Bmnwk*;

5’-AGTTGAGCTGCCAAGTGCTG-3’ and 5’-AACGGTAGTTCCGGCTCCTC-3’]. *Bmnwk* expression levels were normalized with that of *rpl3*. To compare the expression levels among tissues or stages, normalized values were again divided by the mean value of that in the adult brains. As a negative control, real-time PCR without reverse-transcription was performed and confirmed that there was no genomic contamination in all experiments. All data are presented as the mean ± SEM. The primers used for the screening was listed as Primer list in Supporting Information.

cDNA cloning

To identify the full-length cDNA sequence of *Bmnwk*, 5’- and 3’-RACE were performed repeatedly using the SMART RACE cDNA Amplification Kit (Clontech). RNA samples were prepared from the male moth brains and RACE was conducted according to the manufacturer’s protocol.

In situ hybridization

In situ hybridization was conducted as described previously (Kiya *et al.* 2008; Kiya & Kubo 2010). Briefly, frozen coronal brain sections (10-μm thick) were fixed, treated with proteinase K (10 μg/ml), acetylated, and hybridized with digoxigenin (DIG)-labeled riboprobes overnight at 42°C. After hybridization, slides were washed in 50% formamide and 2× saline sodium citrate (SSC) for 30 min at 42°C, treated with 10 μg/ml RNase A (Sigma-Aldrich, St. Louis, MO) for 30 min at 37°C, and washed twice at 42°C in 2×SSC for 20 min and 0.2×SSC for 20 min. DIG-labeled riboprobes were detected immunocytochemically with alkaline phosphatase-conjugated anti-DIG antibody (1:2000, Roche) according to the manufacturer’s protocol. Sense probes were used as negative controls and the signals were confirmed to be

antisense probe-specific in every experiment. Intensity and brightness of the micrographs were processed with Photoshop software (Adobe, San Jose, CA).

Synthesis and injection of dsRNA

The DNA templates for dsRNA of *Bmnwk* and *EmGFP* were prepared by using gene-specific primers congaing T7 polymerase site at the 5' regions (*Bmnwk*: 5'-TAATACGACTCACTATAGGGTACTGGAGACACGTCTACTGTATAAGGGACC-3' and 5'-TAATACGACTCACTATAGGGTACTGAGCTGCCAAGTGCTGGCGA-3', *EmGFP*: 5'-TAATACGACTCACTATAGGGTACTGACGGCGACGTAAACGGCCA-3' and 5'-TAATACGACTCACTATAGGGTACTCGTCGCCGATGGGGGTGTTC-3') and plasmids containing *Bmnwk* cDNA and pREST EmGPF (Invitrogen). PCR was conducted with KOD-Plus2- (Toyobo, Osaka), the resulting products were purified, and dsRNA was synthesized using MAGAscript RNAi kit (Ambion, Austin, TX) according to the manufacturer's protocol. The *Bmnwk* dsRNA (10 μg: final volume was 5-10 μl) was injected at the head of 1-day-old or 2-day-old pupae (n = 6). As negative controls, injection of *EmGFP* dsRNA or H_2O was also conducted (n = 6 each). The injected pupae were kept under the normal condition.

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Figure legends

Fig. 1 Screening of genes whose expression is sex differential. (A) A representative picture of primary screening. Semi-quantitative RT-PCR was conducted and the resulting PCR products were electrophoresed in agarose gels. Clones whose band intensities differed between males and female are highlighted by the squares. (B) The expression levels of candidate genes were quantified by real-time RT-PCR. n = 4, each. A significant difference was observed only in *Clone49* (Student's *t*-test).

Fig. 2 Gene structure of *Bmnwk* and quantification of *Bmnwk* transcripts in the silkworm moth brain. (A) Genomic organization of *Bmnwk L*, *M*, and *S*. Exon (closed boxes) and intron (lines) structures are shown. The open-reading frame regions are shown in black and the 3'-UTR in white. The 5'-UTR (112 bp in length) is not shown due to its short length. The structure and position of the predicted genes and *Clone49* are shown in gray boxes. Approximate genome length is shown at the bottom. (B) Comparison of the domain structures of *Bm*nwk L, M, S, and *Dm*Nwk. Homology of F-BAR and SH3 domains to *Dm*Nwk are shown. (C) The *Bmnwk* expression level in the brains of male and female moths. Amount relative to that in the female is shown. Expression was significantly higher in the male brain than in the female brain, based on Student's *t*-test. n = 4, each.

Fig. 3 Analysis of *Bmnwk* expression in various tissues (A) and at various developmental stages (B). (A) The *Bmnwk* expression level (*Bmnwk* / *rpl3*) in various tissues was quantified using moths and V5 (fifth-instar day-5) larvae. n = 3, each. To compare the *Bmnwk* expression levels among tissues, the value relative to that in adult brain was calculated for each tissue (values relative to that of the adult moth brain are shown). Experiments were conducted using tissues

from males (upper panel) and females (lower panel). Significantly higher expression was observed in only the adult brain [*, $P < 0.0001$; Tukey-Kramer's multiple comparison after one-way analysis of variance]. (B) The *Bmnwk* expression level in the male and female brain was quantified at various developmental stages (V5, fifth-instar day-5 larvae; G0, gut purge day-0; P0, pupae day-0, P4, and P8). n = 3, each stage. Amount relative to that in the adult male moth brain is shown. The expression at P8 was significantly higher than that at the other stages in both sexes [**, $P < 0.001$; Tukey-Kramer's multiple comparison after two-way analysis of variance (F1: developmental stages, F2: sex)].

Fig. 4 *In situ* hybridization of *Bmnwk*. (A and B) Expression pattern of *Bmnwk* in the male moth brain. Coronal brain sections of the caudal (A) and rostral (B) brain regions are shown. A strong signal was detected in the optic lobes (surrounded by white dotted lines). Some of the cells that reside at the edge of neuropil were also stained (white arrowheads). Consecutive sections were differentially hybridized to antisense (shown in upper panels in A and B) and sense probes (shown in lower panels in A and B). Signal was obtained only in the antisense probes. (C) Expression pattern of *Bmnwk* in the female brain. Coronal brain sections of the caudal (upper panel) and rostral (lower panel) brain regions are shown. Signals were weakly detected in the female brain, but the pattern was similar to that in the male brains. Asterisks indicate non-specific staining. OL: optic lobe, Oe: oesophagus, SOG: sub oesophagus ganglion.

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