Studies on the ecdysone-inducible genes in the brain of the silkworm, Bombyx mori : expression and functional analysis during development

氏　　　　名	MD.Monwar Hossain
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論文審査委員（主査）	岩見　雅史（自然科学研究科・教授）
論文審査委員（副査）	櫻井　勝（自然科学研究科・教授），福森　義宏（自然科学研究科・教授）， 中西　義信（医学系研究科・教授），山口　正晃（自然科学研究科・准教授）

Abstract

Ecdysone, a steroid hormone that directs insect molting and metamorphosis, is synthesized in and released from the prothoracic glands (PGs) by the stimulation of a brain neuropeptide, prothoracicotropic hormone (PTTH). To elucidate the role of ecdysone at a molecular level during post-embryonic development, I performed screening of 20-hydroxyecdysone (20E) -induced genes using a brain subtraction cDNA library of silkworm, *Bombyx mori* and identified ten such genes, which were designated *bombeil-1* to *-10*. Three of them did not show any sequence similarity with the previously identified *Bombyx* genes. Whole mount *in situ* hybridization revealed that all these *bombeil* genes were exclusively expressed in two pairs of lateral neurosecretory cells (LNCs) in the larval brain known as PTTH-producing cells (PTTH-cells). To reveal the function of these genes, RNAi gene knockdown technique was used, and the results showed that knockdown targeting *bombeil-2* gave defects in larval to pupal transformation and severe wing and leg mal-formation in adult. These results together with the cell-specific co-expression with *PTTH* suggest that *bombeil* genes could play important roles during larval-pupal-adult development.

20E transduces its signal through binding with ecdysone receptor (EcR) and Ultraspiracle (USP). Analysis of *EcR* and *usp* isoform-specific gene expression during larval-pupal development showed that these genes were up-regulated by 20E in the *Bombyx* brains. Whole-mount *in situ* hybridization and immunohistochemistry revealed that during the last larval (fifth) instar, EcR and USP isoform mRNAs and proteins were exclusively located in PTTH-cells. In the pupal brain, the expressions of these genes were detected in the tritocerebral cells (TCCs) and the optic lobe cells (OLCs) in addition to PTTH-cells. Because PTTH plays a central role in the endocrine network for insect developmental control and regulates directly ecdysone secretion by PGs, 20E-responsive PTTH-cells are the master cells of insect metamorphosis.

Although PTTH plays a crucial role in ecdysteroidogenesis by PGs, its function still remains illusive. Therefore, knockdown of *PTTH* gene is expected to reveal functions of PTTH. Using RNAi gene knockdown technique, it showed for the first time that disruption of *PTTH* gave the mal-transformation of larvae to pupae and the adult appendages deformation. The present results extend our understanding, at molecular basis, of PTTH function in the control of insect development.

Introduction and Conclusion

Insect brain is the center of endocrine network that produces many neuropeptides and plays critical roles in orchestrating developmental process. A brain neuropeptide, prothoracicotropic hormone (PTTH) stimulates the synthesis and release of ecdysone by the prothoracic glands (PGs). The released ecdysone is converted to 20-hydroxyecdysone (20E) by fat bodies, and then 20E circulates throughout the body and controls the gene expression needed for growth, molting and metamorphosis. Brain is also a target organ of 20E. 20E induces dynamic morphological changes and rearrangement of the neural network during developmental processes such as the optic lobes and mushroom bodies during metamorphosis (Champlin and Truman, 1998a; Kraft et al., 1998). Hence, the expression and functional analyses of 20E-inducible genes in the brain including PTTH, are of critical importance in understanding insect growth and development. Therefore, the present study is focused on the gene expression triggered by 20E in the brain during metamorphosis and the descriptions of the ten identified 20E-induced novel genes designated as *bombeil-1* to *-10*. All the identified genes were expressed in PTTH-cells, two pairs of lateral neurosecretory cells (LNCs) in the brain (Agui et al., 1979). This finding indicates a novel insight into ecdysone function during development. To reveal the functions of these genes, RNAi gene knockdown technique was used and showed that knockdown targeting *bombeil-2* resulted defects in larval to pupal transformation and severe wing and leg mal-formation in adult **(Fig. 1)**. This result together with the cell-specific co-expression with *PTTH* gene, suggests that *bombeil* genes could play important roles during larval-pupal-adult development. These results will give a better understanding, at molecular basis of ecdysone action in development.

The 20E signal is mediated intrinsically through binding with its heterodimeric nuclear receptor consisting of the ecdysone receptor (EcR) and Ultraspiracle (USP) (Thomas et al., 1993; Yao et al., 1993). The 20E and its receptor complex induce directly expression of several early genes, and the products of these genes activate late effector genes that control stage- and tissue-specific developmental responses (Thummel, 1995, 1996). Two *EcR* isoforms, *EcR-A* and *EcR-B1*, and two *usp* isoforms, *usp-1* and *usp-2*, have been identified in *Bombyx* (Tzertzinis et al., 1994; Swevers et al., 1995; Kamimura et al., 1996, 1997; Takaki et al., 2004) that are involved in tissue-specific responses to 20E. *EcR-A* gene expression regulates neuronal maturation, while *EcR-B1* expression controls neuronal regression both in *Drosophila melanogaster* (Brown et al., 2005; Davis et al., 2005) and *Manduca sexta* (Truman et al., 1994). Mutational analysis of EcR isoforms in *Drosophila* identified several different lethal phases, while USP mutation analysis in *Drosophila* has proven its critical roles in larval and pupal development (Perrimon et al., 1985; Oro et al., 1992; Hall and Thummel, 1998; Martin et al., 2006). Although the effects of 20E on neurons of the central nervous system has been extensively studied in *Drosophila* and *Manduca*, little information is available on the role of *EcR* and *usp* expression in the brain. I therefore tried to analyze *EcR* and *usp* expression in *Bombyx* brain and found that expressions of both isoform genes were co-localized exclusively in PTTH-cells in the fifth instar larval brain **(Fig. 2)**. Because PTTH controls ecdysone secretion by PGs, these results indicated that 20E-responsive PTTH-cells were the master cells of insect metamorphosis (Hossain et al., 2006).

PTTH is synthesized in the two pairs of LNCs of the insect brain (Kawakami et al., 1990) and acts as a primary stimulator of ecdysteroidogenesis in PGs (Gilbert et al., 2002). It has been believed since the work of Truman (1972) that PTTH release is controlled by a circadian clock in the brain (Mizoguchi et al., 2002; Helfrich-Forster, 2003). The PTTH titer in *Bombyx* larval hemolymph is, however, not exclusively controlled by photoperiod and circadian clock mechanisms (Mizoguchi et al., 2001; Mizoguchi et al., 2002), rather a signaling pathway of muscarinic acetylcholine receptor is involved in the control of PTTH release (Aizono et al., 1997). At the transcriptional level, *Bombyx PTTH* is regulated by trans-regulatory factors such as myocyte enhancer factor 2 (Shiomi et al., 2005). Besides all of these correlative data, a conclusive understanding of regulatory factors of PTTH synthesis and release remain elusive (Kim et al., 1997; Rybczynski, 2005).

PTTH plays as the primary activator of ecdysteroidogenesis in PGs (Gilbert et al., 2002) and acts via a signaling cascade that is so complexed to understand (Rybczynski, 2005). Besides its prothoracicotropic effect, PTTH may act as a growth factor as it shares a common ancestor with the vertebrate growth factor superfamily peptides (Noguti et al., 1995) showing promotion of the silk production in *Bombyx* (Trivedy et al., 2000). On the other hand, lowering of PTTH production is responsible for induction of pupal diapause in many insects (Xu and Denlinger, 2003; Wei et al., 2005). Despite these knowledge, the molecular basis of PTTH functions are still at an early level of understanding in spite of the critical importance in insect developmental control (Rybczynski, 2005). Therefore, I tried to elucidate PTTH function using RNAi gene knockdown technique and showed the important role in the transformation of larvae to pupae and differentiation of adult appendages **(Fig. 3)**.

In summery, I identified ten 20E-induced genes, proposed that PTTH-cells are the master cells of insect metamorphosis, and revealed the involvement of PTTH in the post-embryonic differentiation in *Bombyx*. The present study gives an opening of a new era for the study of ecdysone action as well as various neuropeptides with unknown functions in the control of insect development.

学位論文審査結果の要旨

提出された論文と公聴会での口頭発表及び質疑応答の結果をふまえて審査を行い，審査委員会において以下の通り決定した。

本論文は，これまで未知であった変態ホルモン（エクジソン）のカイコガ脳内での作用を明らかにするため，エクジソンにより脳内で誘導される遺伝子の解析を系統的に行ったものであり，以下の成果が示されている。

1）脳サブトラクションライブラリーから，エクジソン誘導性遺伝子を10同定した。多くは機能未知の新規な遺伝子であった。

2）同定した遺伝子は脳内で前胸腺刺激ホルモン（PTTH）産生細胞でのみ発現することを示した。

2）bombeil-2遺伝子およびPTTH遺伝子について，RNA干渉法により発現量を低下させたところ，異常な変態形質，翅や脚の形成異常が見られたるを示した。bombeil-2遺伝子は変態過程で重要な機能が、PTTH遺伝子には未同定の機能が担われていることが示された。

3）エクジソン受容体遺伝子及びultraspiracle遺伝子の発現部位もPTTH産生細胞であることを示した。これは，PTTH産生細胞が昆虫変態のマスター細胞であることを示唆することとなった。

提出論文の一部は既に論文として公表され，掲載雑誌で表紙にも成果が掲載されるなど多大なインパクトを与えた。本論文に出された多くの成果は，今後，昆虫変態を研究する上で不可欠な道しるべの役割を果たすものであり極めて高く評価される。以上の結果，本論文は博士（理学）に値すると判定した。