S1

Neuropeptides and Neuroactive Substance in the *Bombyx mori* Brain: Allatotropin Gene and Localization, Neuronal Growth by BDNF, and Apoptosis by Edysone

Bong Hee Lee

School of Life Sciences & Biotechnology, Korea University, seoul 136-701, Korea

Allatotropin is a 13-residue amidated neuropeptide isolated from pharate adult heads of the tobacco hornworm, *Manduca sexta* and strongly stimulates biosynthesis of juvenile hormones in adults, but not larval, lepidopteran corpora allata. From a *Bombyx mori* midgut cDNA library, a cDNA that encodes a 130-amino-acid polypeptide containing *M. sexta* allatotropin sequence was isolated. The *B. mori* allatotropin cDNA consists of 1196 nucleotides. The encoded allatotropin peptide is identical to that isolated from *M. sexta* and that predicted from *Pseudaletia unipuncata*, with 84% and 81% identity in the amino acid sequence of the allatotropin peptide precursor, respectively. *M. sexta* allatotropin is flanked by two different endotproteolytic cleavage sites within the precursor of the *B. mori* allatotropin peptide. Evidence from northern blotting *B. mori* tissues showed that the allatotropin gene is expressed in the cells of midgut, head and integument with different transcription amount, but not in the fat body and silk gland. Midgut has also a number of allatotropin-immunoreactive cells and nerve fibers.

Brain-derived neurotrophic factor (BDNF) induced a significant neurite extension of antennal lobe neurons from *B. mori* in culture on laminin/concanavalin A-coated dishes, in comparison with smaller effect of 20-hydroxyecdysone (20-HE). But the effect for neurite extension by 5-hydroxytryptamine (5-HT) could not be found. A significant increase in number of new primary branches from the principal neurites of AL neurons was also shown in culture with BDNF and 5-HT, but not with 20-HE. The BDNF stimulated to outgrow more increased number of branches than the 5-HT. In culture of antennal lobe neurons with BDNF, 20-HE and 5-HT, they showed the highest survival rate in culture with BDNF. Results from the western blots and ELISA assay suggested that before its transportation from the specific neurosecretory cells to the corpora allata, BDNF has a molecular weight of 38 kDA and might be also secreted into hemolymph. Immunostaining of 5-stage pupal brains with anti-BDNF antibody revealed presence of four pairs of large median neurosecretory cells and six pairs of small lateral neurosecretory cells of which axons were innervated to the corpora allata.

To investigate programmed cell death (PCD) pattern of the neurons in metamorphic brains from the 1st instar larvae to late pupae immediately before the ecdysis to adult, a given pattern of PCDs of brain neurons could be found by TUNEL assay. In larval brains, abrupt increase of neuronal PCDs occurred in the 4th instar and the first, highest peak of neuronal PCDs was found in the 5th instar. In wandering stages brain neuronal cells formed the second, increased peak of PCDs. In pupal stages, earlier brains showed decreased PCDs with a gradual slope. However, late pupal brains exhibited more frequency of neuronal PCDs with a third peak. These neuronal PCDs could be demonstrated *in vivo* to be induced by experimental injection of 20-hydroxyecdysone in the silkworm brain.

Introduction

Insect brains have various types of neuropeptides in their neuronal cells, including allatotropin and BDNF. It has been shown that allatotropin is actively produced by specific neuronal cells, in particular during the early larval periods, and then transported to retrocerebral complex for controlling secretion of juvenile hormone in the corpora allata, suggesting that allatotropin is a neurohormone to be indirectly involved in the insect metamorphosis. Thus, it is important to demonstrate molecular characteristics of allatotropin for its clear functional roles in insect hormonal system.

Some of insect brain neurons must grow their developing axons and survive for their development *in vitro* and *in vivo*. It is well known that in vertebrates, neurotrophic factors, such as BDNF, are necessary to grow the neurites of developing neurons in vitro. The BDNF stimulates growth and survival of the neurite in the culture, but the effects of BDNF to grow the neuronal neurites and survive during their development are not yet confirmed from the insect brains *in vitro*, as well as *in vivo*.

Programmed cell death (PCD, or apoptosis) occurs frequently during the animal development. In various insects the adult brains are developed by a series of complex processes of the larval brains during the pupal periods, including PCD of neuronal cells. Neuronal PCDs found during the larval and pupal stages occur to eliminate unnecessary larval neurons to develop a new adult brain. However, neuronal PCDs in the brains throughout the postembryonic life had to be demonstrated in an insect until recently.

Results

1. Allatotropin gene and localization

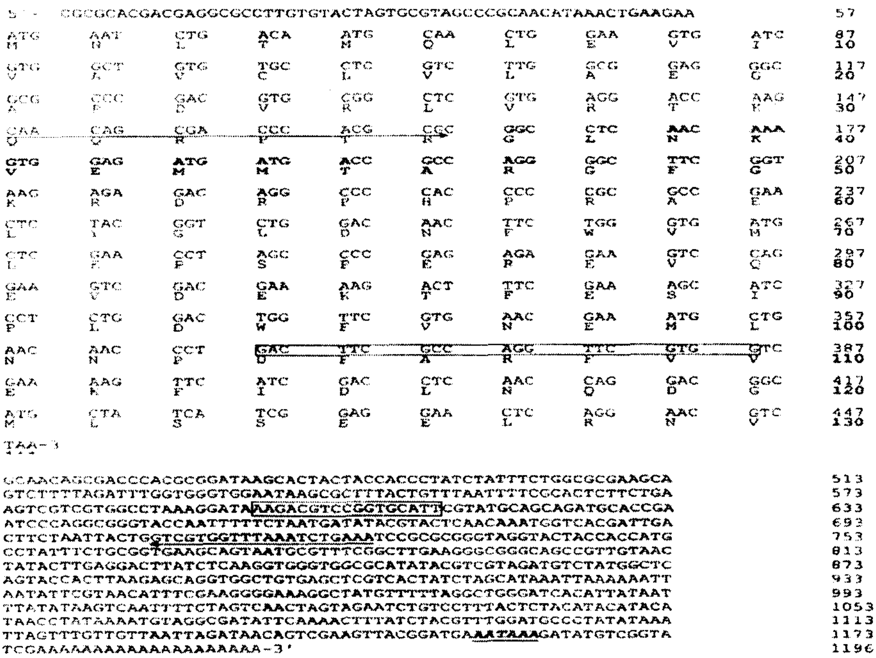

Fig. 1. Nucleotide sequence of Bombyx-AT cDNA. The translated portion of sequence is organized into codons with the deduced amino acid sequence shown just below with the capital letter. The allatotropin peptide sequence is shown in bold type. The proteolytic sites and the glycine residue required for amidation are surrounded by rectangle. The polyadenylation signal (AATAAA) is shown bold underlined.

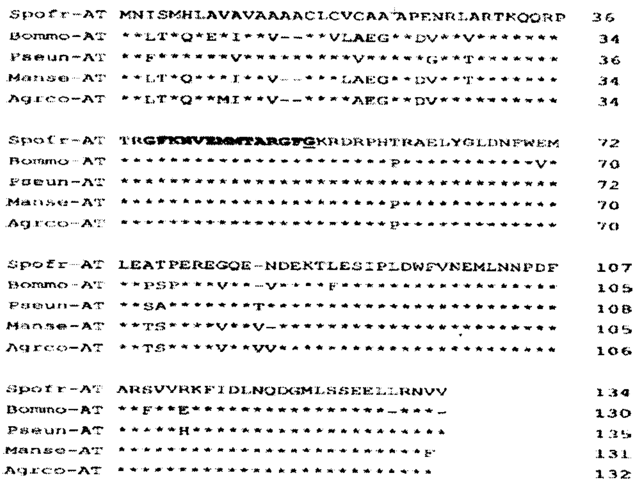

Fig. 2. Alignment of the AT precursor peptides of *S. frugiperda* (Spofr-AT), *B. mori* (Bommo-AT), *P. unipuncta* (Pseun-AT) (Truesdell et al., 2000), *M. sexta* (Manse-AT) (Taylor et al., 1996), and *A. convolvuli* (Agrco-AT). Asterisks represent amino acids identical to each of the precursors. The mature AT peptide is shown in bold type. A signal peptide cleavage site is indicated by a downward arrow.

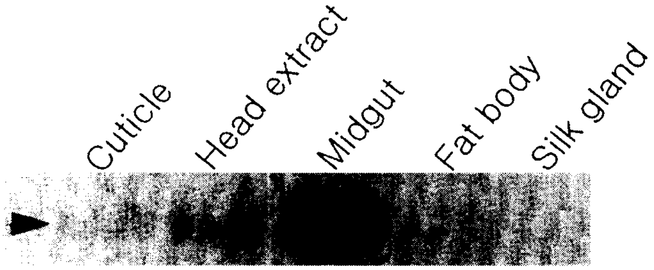

Fig. 3. Expression of Bombyx-AT gene by Northern blotting

2. Neuronal Growth by BDNF

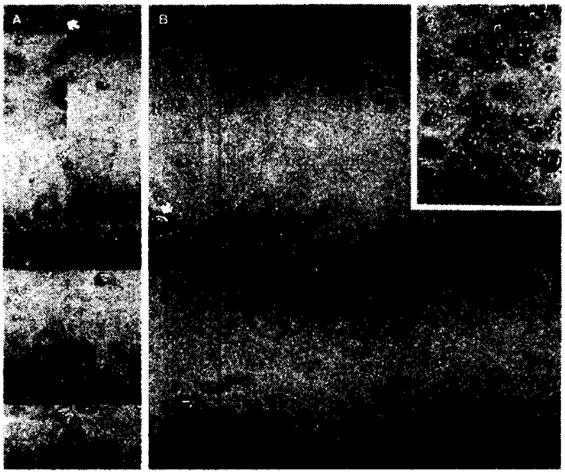

Fig. 4. The AL projection neurons in the culture with BDNF plus 20-HE or BDNF plus 5-HT combinations on laminin/ con A showing very significant extension in length or increase of primary branch from the principal neurites. The neurite length or primary branches of the two neurons are in good comparison with those in culture with BDNF alone, as shown in Fig. 3. **A**, Montage photograph of an AL projection neuron (cell body indicated by arrow) with very long principal neurite extended by culturing with a combination of 200*ng/mℓ* BDNF and 10 μg/mℓ 20-HE for 20 days. Scale bar indicates 50 μm. **B**, Montage photograph of an AL projection neuron (cell body indicated by arrow) with several long primary branches in principal neurite during culture with a combination of 200*ng/mℓ* BDNF and 50μM 5-HT for 20 days. Scale bar indicates 50 μm. **C**, Several neuroblasts with no process a few hours after the culture on laminin/con A. Scale bar indicate 40 μm.

3. **Apoptosis by Edysone**

PI FITC Merged

Fig. 5. Representative apoptosis of neuronal cell bodies in two cerebral hemispheres in 4th instar larval brain from *Bombyx mori* revealed with the TUNEL assay. In the day-6 of 4th instar larva, a large number of neurons showed apoptosis in the brain, especially in lateral protocerebrum. **A**: Apoptosis in the brain of a larva on 4 instar stage. PI (Propidium Iodine)-stained brain. **B**: FITC-labelled brain in the same stage. **C**: A and B merged. Apoptotic nuclei are located exclusively within the brain proliferation cluster. The overlap of distribution in PI stained nuclei and FITC-labelled apoptotic neurons is visible (yellow spot).

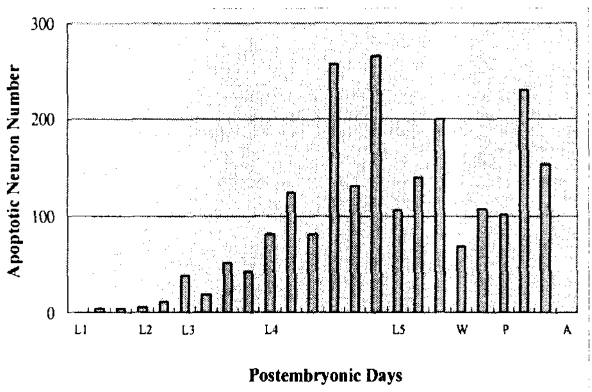

Fig. 6. Pattern of apoptotic neurons in the brain of *Bombyx mori* throughout postembryonic life. The brains in both some late larval and some late pupal stages show a number of apoptotic neurons.

References

Jones KR, Farinas I, Backus C. 1994. Targeted disruption of the BDNF gene brain and sensory neuron development but not motor neuron development. Cell 25: 76, 6: 989-99.

Kim MY, Lee BH, Kwon D, Kang H, Nässel DR. 1998. Distribution of tachykinin-related neuropeptide in the developing central nerwous system of the moth *Spodoptera litura*. Cell tissue Res 294:351-365.

Oland LA, Hayashi JH. 1993. Effects of the steroid hormone 20-hydroxyecdysone and prior sensory input on the survival and growth of moth central olfactory neurons *in vitro*. J Neurobiol 24: 1170-1186.

Park CI, Hwang JS, Kang SW, Lee BH. 2002. Molecular characterization of a cDNA from the silk moth *Bombyx mori* encoding *Maduca Sexta* allatotropin peptide. Zool Sci 19:287-292.

Park HH, Park CI, Kim KS, Kwon OS, Han SS, Hwang JS, Lee BH. 2003. Effects of 20-Hydroxyecdysone and serotonin on neurite growth and survival rate of AL neurons in pupal stage of the silk moth *B. mori in vitro*. Zool Sci 20:111-119.