Acta Biologica Hungarica 55 (1–4), pp. 163–166 (2004)

THE EXPRESSION PATTERN OF CREB GENES IN THE CENTRAL NERVOUS SYSTEM OF THE POND SNAIL *LYMNAEA STAGNALIS**

HISAYO SADAMOTO,[1,2]** SACHIYO AZAMI[2] and E. ITO[1,2]

[1] Division of Innovative Research, Creative Research Initiative "Sousei" (CRIS), Hokkaido University, Sapporo 060-0810, Japan
[2] Division of Biological Sciences, Graduate School of Science, Hokkaido University, Sapporo 060-0810, Japan

(Received: August 31, 2003; accepted: December 1, 2003)

To analyze the expression pattern of genes of cAMP responsive element binding protein (CREB), we performed *in situ* hybridization for the whole central nervous system (CNS) of the pond snail *Lymnaea stagnalis.* The CREB1 (activator) and CREB2 (repressor) homologues have already been cloned in *L. stagnalis,* and they are referred to as LymCREB1 and LymCREB2. Using the frozen sections and the whole mount preparations of the CNS, we mapped the distribution of LymCREB1 and LymCREB2 mRNA containing neurons. The present findings showed that the LymCREB1 mRNA containing neurons are a relatively few, whereas LymCREB2 mRNA is contained ubiquitously in the whole CNS of *L. stagnalis.*

Keywords: CREB – *in situ* hybridization – CNS – *Lymnaea*

INTRODUCTION

Previous studies indicated that the memory formation needs new gene expression and protein synthesis, and in which transcription factors play pivotal roles. Among them, cAMP responsive element binding proteins (CREBs) are considered to play a critical role in the consolidation of long-term memory (LTM). The transcription activator, CREB1, is necessary for the initiation step of gene expression for LTM, whereas CREB2 acts as the repressor for CREB1 [3]. Since then, the ratio of activator/repressor CREBs has been proposed to act as a 'molecular switch' in determining whether LTM is formed. As the first step to investigate the gene regulation mechanism by CREBs, such occurred in LTM formation, we performed *in situ* hybridization with frozen sections and whole mount preparations, and analyzed the expression pattern of CREB genes in the central nervous system (CNS) of the pond snail *Lymnaea stagnalis*, which is widely used for elucidation of basic mechanisms underlying learning and memory [4, 6]. The CREB1 and CREB2 homologues have been recently cloned in *L. stagnalis* [9].

* Presented at the 10th ISIN Symposium on Invertebrate Neurobiology, July 1–5, 2003, Tihany, Hungary.
** Corresponding author; e-mail: sada@sci.hokudai.ac.jp

0236-5383/$ 20.00 © 2004 Akadémiai Kiadó, Budapest

MATERIALS AND METHODS

The *in situ* hybridization study for cryostat sections was performed according to the same method of Sadamoto et al. [7, 9]. The whole mount *in situ* hybridization procedure was a modification of previously described protocols [1]. Briefly, after anesthetizing the snails on ice, the CNS was isolated and set in a small dish filled with *Lymnaea* ringer [24 mM NaCl, 2 mM KCl, 2 mM $MgCl_2$, 4 mM $CaCl_2$, 0.3 mM D-glucose, 0.1 mM NaH_2PO_4 and 35.4 mM HEPES-NaOH (pH 7.9)]. The hybridization procedure was essentially the same as that by Bogdanov [1], with exception in Proteinase K treatment. After fixation and sequential methanol treatment, the samples were treated with Proteinase K (10 μg/ml) in PBS containing 0.1% Tween 20 (PTW) for 60 min at 37 °C.

Digoxigenin (DIG)-labeled antisense RNA probes were synthesized with T7 RNA polymerase and DIG RNA labeling mixture (Roche, Indianapolis, IN, USA) using CREB1 (414 bp) and CREB2 (528 bp) cDNA plasmids. Denatured RNA probes were added to the hybridization buffer (the final concentration: 0.2 to 0.5 μg/ml) and the hybridization was performed at 50 °C overnight. The samples were washed in 5× SSC, 50% formamide and 1% SDS at 60 °C for 30 min, washed in 2× SSC, 50% formamide and 1% SDS at 60 °C for 30 min, and washed twice in 1× SSC in falcon tubes at 60 °C for 30 min. Hybridization signals were visualized using a nucleic acid detection kit (Roche diagnostics, Indianapolis, IN, USA) with alkaline-phosphatase conjugated antidigoxigenin antibody and nitroblue tetrazolium (NBT), according to the manufacturer's instructions. After washing in PTW, the samples were incubated sequentially in 50% ethanol for 10 min, 100% ethanol for 5 min, 50% ethanol for 5 min. After washing the samples in PTW for 5 min, the samples were observed under the microscope.

RESULTS AND DISCUSSION

The expression of LymCREB1 gene was observed in a relatively few neurons (Fig. 1), whereas LymCREB2 gene was expressed ubiquitously in the whole CNS (data not shown). In the right and left pleural ganglia, the left parietal ganglia and the buccal ganglia, the hybridization signals for LymCREB1 mRNA were too faint to be detected. In the cerebral ganglia, the cerebral giant cell (CGC) showed strong hybridization signal of LymCREB1 mRNA. The CGC is playing a role in feeding behavior, and also reported as the key neuron for conditioned taste aversion, a kind of associative learning in *L. stagnalis* [5]. In the pedal ganglia, the hybridization signal of LymCREB1 mRNA was observed in the right pedal dorsal 1 cell (RPeD1) and the left pedal dorsal 1 cell (LPeD1). The RPeD1 is an identified dopaminergic interneuron [2] and the key neuron for respiratory behavior and learning [6]. Each pedal ganglion also possessed a cluster of LymCREB1 mRNA containing cells on the medial surface close to the pedal commissure and one relatively large neuron on the anterior margin. In each of the visceral ganglion and right parietal ganglion, one clus-

ter of the cells, located on anterior surface, showed the hybridization signals for LymCREB1 mRNA. In summary, the identified LymCREB1 mRNA containing cells are almost all the interneurons, such as the CGC, the RPeD1 and LPeD1, but not the motoneurons. For example, no strong hybridization signal of LymCREB1 mRNA was observed in the buccal ganglia, which contains many motoneurons. Therefore, the ratio of CREB1 and CREB2 would decide the ability of gene expression, resulting in characterization of the function of the interneurons. The present findings will stimulate the future studies in behavior and development of *L. stagnalis,* because CREB is possibly crucial to the mechanisms controlling associative learning in *L. stagnalis* [4, 5, 8, 9].

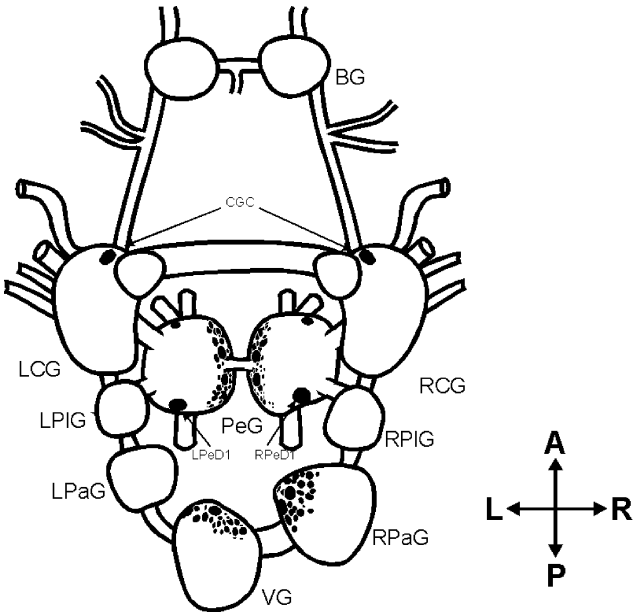

Fig. 1. Summary of LymCREB1 mRNA containing neurons in the whole CNS of *L. stagnalis.* Dorsal view of all the ganglia. BG: buccal ganglia, RCG: right cerebral ganglion, LCG: left cerebral ganglion, RPlG: right pleural ganglion, LPlG: left pleural ganglion, RPaG: right parietal ganglion, LPaG: left parietal ganglion, VG: visceral ganglion, PeG: pedal ganglia, CGC: cerebral giant cell, RPeD1: right pedal dorsal 1 cell, LPeD1: left pedal dorsal 1 cell

REFERENCES

1. Bogdanov, Y. D., Balaban, P. M., Zakharov, I. S., Poteryaev, D. A., Belyavsky, A. V. (1996) Identification of two novel genes specifically expressed in the D-group neurons of the terrestrial snail CNS. *Invertebrate Neurosci. 2,* 61–69.
2. Cotterel, G. A., Abernathy, K. B., Barrand, M. A. (1979) Large amin-containing neurons in the central nervous system in *Lymnaea stagnalis*. *Neuroscience 4,* 685–689.

3. Guan, Z., Giustetto, M., Lomvardas, S., Kim, J. H., Miniaci, M. C., Schwartz, J. H., Thanos, D., Kandel, E. R. (2002) Integration of long-term-memory-related synaptic plasticity involves bidirectional regulation of gene expression and chromatin structure. *Cell 111,* 483–493.
4. Ito, E., Kobayashi, S., Kojima, S., Sadamoto, H., Hatakeyama, D. (1999) Associative learning in the pond snail, *Lymnaea stagnalis. Zool. Sci. 16,* 711–723.
5. Kojima, S., Nakamura, H., Nagayama, S., Fujito, Y., Ito, E. (1997) Enhancement of an inhibitory input to the feeding central pattern generator in *Lymnaea stagnalis* during conditioned taste-aversion learning. *Neurosci. Lett. 230,* 179–182.
6. Lukowiak, K., Adatia, N., Krygier, D., Syed, N. (2000) Operant conditioning in *Lymnaea*: evidence for intermediate- and long-term memory. *Learn. Mem. 7,* 140–150.
7. Sadamoto, H., Hatakeyama, D., Kojima, S., Fujito, Y., Ito, E. (1998) Histochemical study on the relation between NO-generative neurons and central circuitry for feeding in the pond snail, *Lymnaea stagnalis*. *Neurosci. Res. 32,* 57–63.
8. Sadamoto, H., Yamanaka, M., Hatakeyama, D., Nakamura, H., Kojima, S., Yamashita, M., Ito, E. (2000) Developmental study of anatomical substrate for conditioned taste aversion in *Lymnaea stagnalis. Zool. Sci. 17,* 141–148.
9. Sadamoto, H., Sato, H., Kobayashi, S., Murakami, J., Aonuma, H., Ando, H., Fujito, Y., Hamano, K., Awaji, M., Lukowiak, K., Urano, A., Ito, E. (2004) CREB in the pond snail *Lymnaea stagnalis.* Cloning, gene expression and function in identifiable neurons of the central nervous system. *J. Neurobiol. 58,* 455–466.