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Determination of the exact copy numbers of particular mRNAs in a single cell by quantitative real-time RT-PCR

Akiko Wagatsuma[1], Hisayo Sadamoto[1,2,3], Takashi Kitahashi[4], Ken Lukowiak[5], Akihisa Urano[1,4] and Etsuro Ito[1,2,*]

[1]*Division of Biological Sciences, Graduate School of Science, Hokkaido University, North 10, West 8, Kita-ku, Sapporo 060-0810, Japan,* [2]*Division of Innovative Research, Creative Research Initiative 'Sousei' (CRIS), Hokkaido University, North 21, West 10, Kita-ku, Sapporo 001-0021, Japan,* [3]*Laboratory of Functional Biology, Faculty of Pharmaceutical Sciences at Kagawa Campus, Tokushima Bunri University, 1314-1 Shido, Sanuki 769-2193, Japan,* [4]*Field Science Center for Northern Biosphere, Hokkaido University, North 9, West 8, Kita-ku, Sapporo 060-0809, Japan* and [5]*Hotchkiss Brain Institute, University of Calgary, Calgary, Alberta T2N 4N1, Canada*

*Author for correspondence (e-mail: eito@sci.hokudai.ac.jp)

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Summary

Gene expression is differently regulated in every cell even though the cells are included in the same tissue. For this reason, we need to measure the amount of mRNAs in a single cell to understand transcription mechanism better. However, there are no accurate, rapid and appropriate methods to determine the exact copy numbers of particular mRNAs in a single cell. We therefore developed a procedure for isolating a single, identifiable cell and determining the exact copy numbers of mRNAs within it. We first isolated the cerebral giant cell of the pond snail *Lymnaea stagnalis* as this neuron plays a key role in the process of memory consolidation of a learned behavior brought about by associative learning of feeding behavior. We then determined the copy numbers of mRNAs for the cyclic AMP-responsive element binding proteins (CREBs). These transcription factors play an important role in memory formation across animal species. The protocol uses two techniques in concert with each other: a technique for isolating a single neuron with newly developed micromanipulators coupled to an assay of mRNAs by quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR). The molecular assay determined the mRNA copy numbers, each of which was compared with a standard curve prepared from cDNA solutions corresponding to the serially diluted solutions of *Lymnaea* CREB mRNA. The standard curves were linear within a range of 10 to 10^5 copies, and the intra-assay variation was within 15%. Each neuron removed from the ganglia was punctured to extract the total RNA directly and was used for the assay without further purification. Using this two-step procedure, we found that the mRNA copy number of CREB repressor (CREB2) was 30–240 in a single cerebral giant cell, whereas that of CREB activator (CREB1) was below the detection limits of the assay (<25). These results suggest that the CREB cascade is regulated by an excess amount of CREB2 in the cerebral giant cells. Our procedure is the only quantitative analysis for elucidation of the dynamics of gene transcription in a single cell.

Key words: CREB, learning, *Lymnaea stagnalis*, manipulation, memory.

Introduction

Cells within the same tissue function differently (Saito and Urano, 2001; Egorov et al., 2002). The control of gene expression is, therefore, thought to be different in each cell within the same tissue (reviewed by Carey and Smale, 2000; Sonenberg et al., 2000). For this reason, to understand the control of the transcription process better it is necessary to determine the exact copy number of each mRNA in each cell.

One example where the determination of mRNA copy numbers would be advantageous is given in single, identifiable neurons that play key and/or necessary roles in transcription and translation mechanisms for new protein synthesis in memory consolidation process, particularly in simpler model central nervous systems (CNSs) of invertebrates (Kandel and Pittenger, 1999; Kandel, 2001). It therefore seems logical that we should try to determine the exact copy numbers of mRNAs of transcription factors thought to play necessary roles in the molecular cascade that leads to the formation of memory (Yin and Tully, 1996; Abel and Lattal, 2001; Carew and Sutton, 2001; Pittenger and Kandel, 2003; Sangha et al., 2003a,c).

A number of groups, including our own, have used the pond snail *Lymnaea stagnalis* to analyze the behavioral, cellular and molecular mechanisms of associative learning and subsequent memory formation (Ito et al., 1999; Benjamin et al., 2000; Lukowiak et al., 2003; Wagatsuma et al., 2004; Sakakibara et

al., 2005). The results obtained from many experiments show that two identifiable neurons play key and/or necessary roles in the memory consolidation process following different forms of associative learning: (1) the cerebral giant cell for aversive and appetitive feeding conditioning (Kojima et al., 1997, 2001; Sadamoto et al., 2000; Hatakeyama et al., 2004a; Korneev et al., 2005) and (2) the right pedal dorsal 1 cell for respiratory operant conditioning (Sangha et al., 2003b).

In the cerebral giant cell, a transcription factor, cyclic AMP (cAMP) responsive-element binding protein (CREB), was shown to be present at both the mRNA and protein levels (Ribeiro et al., 2003; Sadamoto et al., 2004a,b). CREB has been proposed to act as a molecular switch for memory consolidation (Bailey et al., 1996; Silva et al., 1998; Tully, 1998; Carew and Sutton, 2001). We hypothesized that in the paired cerebral giant cells the phosphorylation of CREB following associative training of feeding behavior initiates a cascade of altered gene activity and new protein synthesis that is necessary for memory consolidation (Nakamura et al., 1999; Ribeiro et al., 2003; Sadamoto et al., 2004a,b). However, there are no accurate, rapid and appropriate methods to determine the exact copy numbers of CREB mRNAs in a single neuron.

To develop a protocol for isolating a single cell and determining the mRNA copy numbers within it, we had to overcome two major technical difficulties. The first of these is the accurate and rapid preparation of mRNAs from a single cell, and particularly the preparation of those that turn over rapidly. The second obstacle was the determination of the exact copy number of each particular mRNA in this isolated cell.

To reliably prepare mRNAs rapidly from a single cell, there appeared to be two possibilities: (1) aspiration of the intracellular contents with a patch pipette (Monyer and Lambolez, 1995; Surmeier et al., 1996; Baro et al., 1997; Liss et al., 2001; Tsuzuki et al., 2001; Liss, 2002; Song, 2002); and (2) isolation of the cell's soma by laser microdissection (Schütze et al., 1997; Fink et al., 1998; Mawrin et al., 2003). As with any model system or experimental procedure, there are various 'pluses and minuses' that must be taken into account before proceeding. The aspiration method has some serious limitations. For example, it is not certain whether all the intracellular contents, e.g. mRNAs, can be collected completely into the patch pipette by negative pressure. On the other hand, the laser microdissection method cannot exclude the problem of contamination of other small cells beneath the target one. Furthermore, in some instances, the isolated cell required fixation, dehydration and embedding, and these steps may have disadvantageous consequences for the preparation of mRNAs.

The other obstacle was the difficulty in determining the exact mRNA copy number, which is expected to be small, in a single cell. Whereas traditional methods, such as northern blot analysis and competitive polymerase chain reaction (PCR), have yielded reproducible results (Horikoshi and Sakakibara, 2000; Silbert et al., 2003), this methodology requires a large amount of mRNA and, thus, cannot be easily used to determine the small amount of mRNA in a single cell. By contrast, the recent development of the quantitative real-time reverse transcription-PCR (qRT-PCR) method allows not only the quantification of a small amount of mRNA in cells but also the rapid analysis of multiple gene targets (Bustin, 2002; Bhandari et al., 2003; Hatakeyama et al., 2004c; Stram et al., 2004). Using qRT-PCR, research teams have begun to focus on single-cell quantitative analyses of mRNAs in identifiable cells (Spijker et al., 1999; Schacher et al., 1999; Schacher et al., 2000; Steuerwald et al., 2000; Tkatch et al., 2000; Eberwine, 2001; Becker et al., 2002; Storm et al., 2002; Parhar et al., 2003).

In the present study, we therefore used the qRT-PCR method on a single cerebral giant cell isolated with newly developed micromanipulators to determine the exact copy numbers of mRNAs of the activator and repressor types of CREB. We show here in a single cerebral giant cell isolated from a naïve snail that we are able to determine reliably the exact copy number of mRNA of the repressor type of CREB (CREB2) but not the activator one (CREB1). Because the protocol is not only relatively simple, but highly specific and reliable, we will be able in future experiments to determine if the amounts of other mRNAs change in a single cell as a result of development, immunity, learning and memory.

Materials and methods

Animals

Adult pond snails *Lymnaea stagnalis* L. (shell length, *ca.* 20 mm) from our snail raising facility (original stocks from Vrije Universiteit Amsterdam supplemented with snails from the Calgary facility that were derived from the same Amsterdam colony) were used in all experiments. Snails were fed lettuce in the laboratory and maintained on a 12 h:12 h light:dark schedule at 20°C. Before dissection, snails were anesthetized with cold *Lymnaea* saline containing (in mmol l^{-1}) 40.0 NaCl, 1.7 KCl, 4.1 $CaCl_2$, 1.5 $MgCl_2$, 0.3 D-glucose, and 10.0 Hepes (pH 8.1), as described previously (Koert et al., 2001).

Isolation of a single, identifiable neuron

The CNS was removed from the anesthetized snail, and carefully desheathed in cold *Lymnaea* saline. Then the CNS was exposed to 2 mg ml^{-1} trypsin (Sigma-Aldrich, St Louis, MO, USA) in *Lymnaea* saline at room temperature for 10 min. This CNS dissection method did not interfere with mRNA stability, as will be described later. After washing off the enzyme solution (at least three times) with cold *Lymnaea* saline, the cerebral giant cells were isolated in a cold cell culture medium [50% modified Leibovit's L-15 medium (special order; Invitrogen, Carlsbad, CA, USA) and 50% *Lymnaea* saline] containing 30 mmol l^{-1} D-glucose.

Single cerebral giant cells were bored with glass microelectrodes constructed with a tip diameter of about 10 μm that was rounded by fire-melting and capped. The glass microelectrode was advanced into the cerebral giant cell with state-of-the-art micromanipulators (Narishige Scientific

Instrument Laboratory, Tokyo, Japan) under a stereoscopic microscope, without damaging the soma because of the capped dull tip. In addition, a small length of primary neurite was also isolated with the somata (Fig. 1).

Hoechst staining of isolated neurons

The isolated neurons were fixed in 4% paraformaldehyde solution at 25°C for 10 min, and washed with phosphate-buffered solution (PBS). The nuclei were stained for 15 min with Hoechst33258 (1 μg ml^{-1} in PBS; Wako Pure Chemical Industries, Osaka, Japan). The stained neurons were observed under a fluorescence microscope (80iFU-RFL-3; Nikon, Tokyo, Japan) equipped with a CCD digital camera (Retiga Exi; Qimaging, Burnaby, BC, Canada).

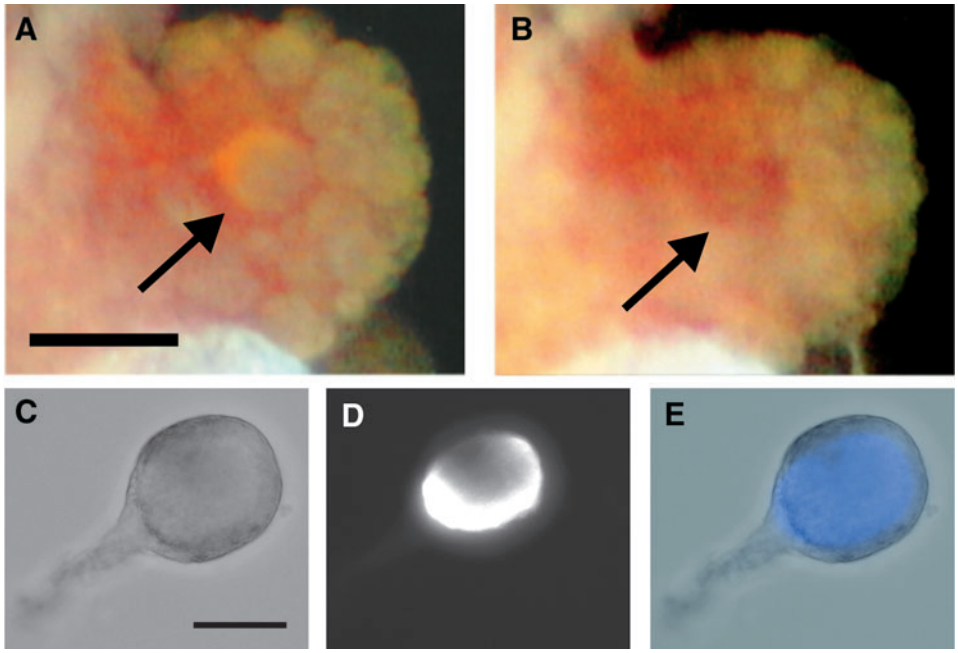

Fig. 1. Isolation of a single, identifiable neuron from the *Lymnaea* CNS. (A) Ventral view of the right cerebral ganglion in *L. stagnalis*. The arrow indicates the cerebral giant cell. Scale bar, 100 μm. (B) The same cerebral ganglion after isolation of the cerebral giant cell. (C) Light microscopic photograph of the isolated cerebral giant cell. Scale bar, 50 μm. (D) Hoechst staining of the same cell. (E) Merged image of (C) and (D). Only the nucleus of the isolated, single cerebral giant cell was observed, indicating that no other cells were removed along with the cerebral giant cell.

Lysis of isolated neurons

The soma with its primary neurite was first gently removed from the ganglion, and then it was placed into a lysis solution with an RNase inhibitor and rapidly frozen, as described below. The following procedures were performed to certify that the loss of mRNA was minimal.

The isolated cerebral giant cell in 0.5 μl of cell culture medium was transferred to a tube containing 4.5 μl of lysis solution [0.75 μl of 50 μmol l^{-1} oligo d(T)$_{16}$ primer (PE Applied Biosystems, Foster City, CA, USA), 0.2 μl of 1 mg ml^{-1} yeast tRNA (Roche, Basel, Switzerland), 0.05 μl of 30 U μl^{-1} Prime RNase inhibitor (Eppendorf, Hamburg, Germany), and 6×10^4 copies of salmon gonadotropin hormone (GTH) α2 RNA with 0.2 μl of 10% NP40 (Calbiochem, Darmstadt, Germany), all of which were stocked in DEPC water]. The salmon GTH α2 RNA was used as an internal control to check the technical variation between determinations. Although housekeeping genes, such as glyceraldehyde-3-phosphate dehydrogenase, are generally used as internal controls for qRT-PCR assays (Aerts et al., 2004), no such suitable gene has been found in *Lymnaea*. Therefore, we prepared salmon GTH α2 RNA by *in vitro* synthesis. The samples were rapidly frozen in liquid nitrogen and then stored at –80°C.

Preparation of primers, probes and standard curves for Lymnaea *CREB1 and CREB2 mRNAs and salmon GTH α2 RNA*

The nucleotide sequences of *Lymnaea* CREB1 and CREB2 mRNAs (accession numbers: AB041522 and AB083656, respectively; for details, see Sadamoto et al., 2004b) were used to design the primers and probes for qRT-PCR. We used the Primer Express software (version 1.0; PE Applied Biosystems) to design appropriate primers and fluorogenic probes for qRT-PCR assays.

For *Lymnaea* CREB1 mRNA, the primers were 5′-GTT GGT GAC GAA AAG TAC GTA ATT G-3′ and 5′-CTC ACA TGG ACC ACT GAA ATG C-3′, and the probe was 5′-FAM-TTT TCA ATG TCA GCT GTT CCA GGA CCA T-TAMRA-3′. For *Lymnaea* CREB2 mRNA, the primers were 5′-CCT AGC TAC GGC TGC TAT ATC TAC AAA-3′ and 5′-GTC AAC AAG TCC AGG TCC CAT T-3′, and the probe was 5′-FAM-CTG CCA AGC AGC AAA TCT TCG TTC CA-TAMRA-3′. The melting temperatures are described later.

For the control experiments, as mentioned above, we also determined the copy number of salmon GTH α2 RNA. The specific primers were 5′-AAT CTT CCC CAA CAT CAT ACA GTG-3′ and 5′-TCA CCG GGA AGC CAT CCT-3′, and the probe was 5′-FAM-TTG CAA CGC AGC ATG TGG CTT CAG-TAMRA-3′.

The standard RNAs were synthesized from the target cDNA templates by an *in vitro* RNA synthesis kit (MAXIscript; Ambion, Austin, TX, USA), and the copy numbers of RNA products were calculated on the basis of their absorbance values. The RNA products were serially diluted to prepare standard RNA solutions, and were subjected to reverse transcription. The volume of reaction mixture (15 μl) and the components were the same as those for test samples that consisted of the lysis and reverse

transcription solutions. Standard RNA solutions that were reverse-transcribed were referred to as standard cDNA solutions. In the qRT-PCR assay, several doses of standard cDNAs (1×10 to 1×10^5 copies for CREB1 cDNA, 1×10, 2×10, 5×10 and 1×10^2 to 1×10^5 copies for CREB2 cDNA, and 2.4×10 to 2.4×10^4 copies for GTH α2 cDNA) were applied in duplicate in each run. In cases in which there were fewer than 10 copies of standard RNA, reproducible amplification could not be shown (see Results).

Quantitative RT-PCR assay for Lymnaea *CREB1 and CREB2 mRNAs in a single neuron*

The samples that were frozen with the lysis solution and stored at –80°C were warmed to 65°C for 1 min to break the cell membrane completely, and then quenched on ice. Then 10 μl of a solution for reverse transcription was added. The reverse transcription solution contained 0.375 μl of 50 U μl^{-1} MultiScribe reverse transcriptase (PE Applied Biosystems), 1.5 μl of 10× TaqMan Buffer A (PE Applied Biosystems), 3.24 μl of 25 mmol l^{-1} $MgCl_2$ (PE Applied Biosystems), 3.0 μl of 2.5 mmol l^{-1} dNTP (Toyobo, Tokyo, Japan), and 0.1 μl of 30 U μl^{-1} Prime RNase inhibitor (Eppendorf), all of which were stored in DEPC water. The final concentrations of components in 15 μl of the reverse transcription solution were 1.25 U μl^{-1} MultiScribe reverse transcriptase, 1/10 volume of 10× TaqMan Buffer A, 5.5 mmol l^{-1} $MgCl_2$, 0.5 mmol l^{-1} dNTP, 2.5 μmol l^{-1} oligo $d(T)_{16}$ primer, 13.3 μg ml^{-1} yeast tRNA, 0.3 U μl^{-1} Prime RNase inhibitor, and 0.13% NP40 in DEPC water.

On the basis of the protocol provided by the manufacturer (PE Applied Biosystems), reverse transcription was performed at 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min. The cDNA samples (6 μl) were applied for qRT-PCR in duplicate to avoid technical variation. For real-time PCR, a PCR reaction solution (19 μl; PE Applied Biosystems) was added to the above reverse-transcribed solution (6 μl). The final components of the reaction mixture (25 μl) for real-time PCR were 0.025 U μl^{-1} AmpliTaq Gold DNA polymerase (PE Applied Biosystems), 1/10 volume of 10× TaqMan Buffer A, 3.5 mmol l^{-1} $MgCl_2$, 0.2 mmol l^{-1} dNTP, 0.1 μmol l^{-1} each of the forward and reverse primers, and 50 nmol l^{-1} fluorogenic probe (PE Applied Biosystems) in distilled deionized water. The conditions were as follows. For CREB1 mRNA, PCR consisted of 1 cycle at 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 62°C for 30 s. For CREB2 mRNA, the PCR conditions were 1 cycle at 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 58°C for 30 s.

The intensity of reporter dye fluorescence was captured at each cycle of PCR with an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems), and the amplification plots were traced. A threshold for the increase in fluorescence was arbitrarily determined in the exponential phase of the amplification plots, and a standard curve was drawn to show the starting copy number of the standard RNA *vs* the threshold cycle.

Assessment of qRT-PCR assay for Lymnaea *CREB2 mRNA in a single neuron in the reverse transcription solution with cell debris*

RT-PCR was carried out immediately after lysis of a single cerebral giant cell without centrifugation. Therefore, to examine the RT-PCR efficiency in samples containing cell debris, the efficiencies were compared between standard RNA solutions and sample mRNA solutions, which were prepared from 19 cerebral giant cells. Both standard CREB2 RNA solutions and serially diluted sample mRNA solutions were subjected to qRT-PCR in triplicate. We thus obtained curves between the starting quantity of RNA and the threshold cycle for both solutions, and checked the linear range of the curves and the parallelism between them. The amount of total mRNA in the sample was arbitrarily assigned a value of one.

In addition, we examined whether the target mRNA was degraded during the aforementioned procedures with endogenous RNase in isolated neurons, and also the effects of freeze-thaw. For this purpose, a known amount of salmon GTH α2 RNA (6×10^4 copies) was added to the lysis solution (4.5 μl) with an isolated, single cerebral giant cell, and its copy number was determined by qRT-PCR of 1 cycle at 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 58°C for 30 s.

Effects of enzyme treatment during dissection of the central nervous system on the copy numbers of mRNAs

We examined the effects of enzyme treatment for dissection of the CNS on the copy numbers of mRNAs. Total RNA was extracted from the CNS samples treated with 2 mg ml^{-1} trypsin for 10 min at room temperature or with 2 mg ml^{-1} protease type IX (Sigma) for 10 min at room temperature (Hatakeyama et al., 2004b) in *Lymnaea* saline by the acid guanidium thiocyanate-phenol-chloroform method (Chirgwin et al., 1979). The amount of total RNA extracted from a single CNS ranged from 1.4 to 3.0 μg. The copy number of CREB2 mRNA in total RNAs (120 ng each) was determined in duplicate by qRT-PCR.

Statistical analysis

The data are expressed as the means ± S.E.M. One-way analysis of variance (ANOVA) was applied to determine the statistical significance ($P<0.05$).

Results

The protocols that we developed aimed to isolate a single cerebral giant cell accurately and rapidly from the *Lymnaea* CNS and then to determine the exact copy numbers of CREB mRNAs, each of which was compared with a standard curve prepared from cDNA solutions corresponding to the serially diluted solutions of *Lymnaea* CREB mRNA. As a result, our assay system showed that the copy number of CREB2 mRNA was 30–240 in a single cerebral giant cell, whereas that of CREB1 mRNA was below the determination sensitivity.

Accurate and rapid isolation of a single, identifiable neuron

The newly developed micromanipulators with glass microelectrodes, which were fabricated with a fire-melted round and capped tip (*ca.* 10 μm diameter), were used for isolation of a single cerebral giant cell under a stereoscopic microscope. As mentioned previously, a small length of primary neurite of the cerebral giant cell was also isolated (Fig. 1). Our procedure enabled us to isolate a single cerebral giant from the CNS within 20 min. This period is much shorter than that by use of a patch pipette or laser microdissection (Monyer and Lambolez, 1995; Surmeier et al., 1996; Baro et al., 1997; Schütze et al., 1997; Fink et al., 1998; Tkatch et al., 2000; Liss et al., 2001; Tsuzuki et al., 2001; Liss, 2002; Song, 2002; Mawrin et al., 2003).

The morphology of isolated cerebral giant cells was observed under a stereoscopic microscope to confirm that the somata were intact and that no other cells were attached to the target neurons. To further confirm that we isolated only a single cerebral giant cell without any other cells, the isolated neuron was fixed and stained with Hoechst33258, followed by observation under a fluorescence microscope. Only the nucleus of the cerebral giant cell was observed, confirming that no other cells were removed with the single cerebral giant cell (Fig. 1). We therefore considered that RNAs in the somata of isolated neurons were completely maintained and that no RNAs were contaminated by other neurons or glial cells.

Validity of qRT-PCR assay for determination of the mRNA copy number in a single neuron even in the presence of cell debris

When the qRT-PCR method is used for a whole cell, sample solutions include cell debris, such as genome DNA, proteins, lipids, and so on. By comparison, standard RNA solutions are pure, as they are synthesized from the cDNA templates. Accordingly, we need to confirm the efficiency of RT-PCR for a sample mRNA solution that contains cell debris.

We first obtained the standard curve of CREB2 RNA solutions, and then obtained the curve for sample solutions of CREB2 mRNA that were prepared from 19 cerebral giant cells. The standard curve was linear within the range of 2×10 to 1×10^5 copies, and the curve of sample mRNA was parallel to the standard curve at 10 to 3×10^2 copies (Fig. 2). The slopes of the lines were –3.98 for the standard solutions, and –3.94 for the sample solutions. These results showed that RNA can be amplified with the same efficiency both in standard RNA solutions and in sample mRNA solutions by qRT-PCR.

RNA stability during the procedures

It was also important to check for any degradation of the target mRNA caused by endogenous RNase in isolated neurons or by the freeze-thaw procedure. To examine this issue, we determined the copy number of synthesized RNA of salmon GTH α2 that was added to the lysis solution with an isolated neuron (Fig. 3). The copy number of salmon GTH α2 RNA in 1 μl of a sample solution (1/15 volume of reverse-transcribed products) was 4143±71 (*N*=20 from duplicates of 10 samples,

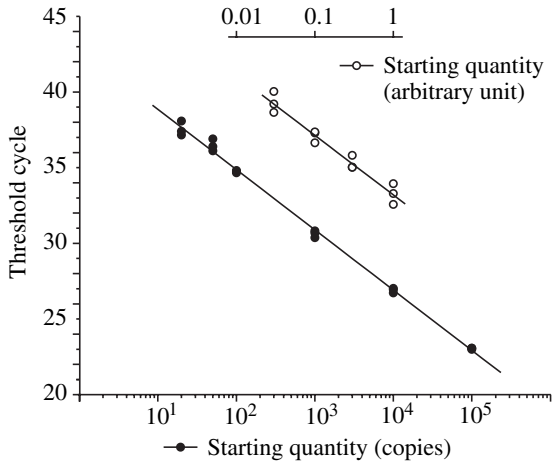

Fig. 2. Assessment of qRT-PCR assay by a comparison of RT-PCR efficiencies between standard RNA solutions and sample mRNA solutions that contain cell debris. The solid circles show the relation between the starting quantity of RNA and the threshold cycles for standard *Lymnaea* CREB2 RNA solutions. The slope of the regression line is –3.98. The open circles show the relation between the starting quantity of mRNA and the threshold cycles for the *Lymnaea* CREB2 mRNA samples obtained from the cerebral giant cells. The amount of total mRNA in the sample, when 19 cells in a tube were used, was arbitrarily assigned a value of one. The slope of the regression line is –3.94. The two parallel slopes confirmed that RNAs of standard RNA solutions and samples could be amplified with the same RT-PCR efficiency in qRT-PCR.

Fig. 3C). Therefore, the intra-assay variation of our qRT-PCR assay system is within 15%, when the copy number ranges from tens to thousands.

Furthermore, we diluted the salmon GTH α2 RNA from 100,000 copies (1, 1/10, 1/100, 1/1000, 1/10000, 1/100000), and theoretically obtained 1 copy (Fig. 3D). The error at 10 copies, that is 1/10000, shows that the S.E.M. is low enough (2.93, *N*=6 samples). These results indicated that we can disregard degradation of target mRNA by either endogenous RNase in the isolated cerebral giant cells or by the freeze-thaw procedure.

Determination of the copy number of Lymnaea *CREB2 mRNA in a single cerebral giant cell*

We determined the copy number of *Lymnaea* CREB2 mRNA in a single cerebral giant cell (Fig. 4A,B). In 6 μl of the sample solution (in duplicate from 15 μl solution for a single neuron), the copy number was determined to range from 10 to 180 by the following procedures. The threshold was set under the condition that the PCR amplifications for all the single neuron samples showed exponential patterns (Fig. 4A). The threshold cycles were then exported to the standard curve prepared with the standard RNA solutions (Fig. 4B). The copy number of 10–180 obtained from the determination of CREB2 mRNA (*N*=11 single cells) was included in the region parallel to the standard curve, as shown in Fig. 2. Thus, the copy number obtained here was considered reliable. By calculating the average value of duplicated samples for each neuron, it was

estimated that the copy number of CREB2 mRNA in a single cerebral giant cell was between 30 and 240.

Determination of the copy number of Lymnaea *CREB1 mRNA in a single cerebral giant cell*

We next determined the copy number of *Lymnaea* CREB1 mRNA in a single cerebral giant cell (Fig. 4C,D). In 6 µl of the sample solution (in duplicate from 15 µl solution for a single neuron), the copy number was less than 10 (N=31 single cells, Fig. 4D). Such a small copy number was below the detection range of our assay system, because less than 10 copies of standard RNA is too small a number to show reproducible amplification. The S.E.M. calculated from three experiments to obtain the standard curve was 0.47 for 10 copies, 0.98 for five copies and 3.77 for one copy. Therefore, while we cannot verify the exact copy number of *Lymnaea* CREB1 mRNA in a single cerebral giant cell, we can confirm that the number is small.

Effects of enzyme treatment on the copy number of Lymnaea *CREB2 mRNA*

Enzyme treatment is required for desheathing the CNS to isolate a single neuron rapidly. We therefore examined the effects of enzyme treatment on the copy number of target mRNA. Total RNAs of control CNSs and those of CNSs treated with 2 mg ml^{-1} trypsin for 10 min or 2 mg ml^{-1} protease type IX for 10 min in the *Lymnaea* saline were purified, and 120 ng of total RNA of each group was reverse-transcribed. Then, the copy number of *Lymnaea* CREB2 cDNA was determined by qRT-PCR. No significant differences were observed between the control and the sample treated with trypsin or between the control and the sample with protease (control, N=8, 102498±7065; trypsin, N=5, 110202±9312; protease type IX, N=10, 123005±10296). These results indicate that the use of enzymes is feasible for the dissection of CNS samples.

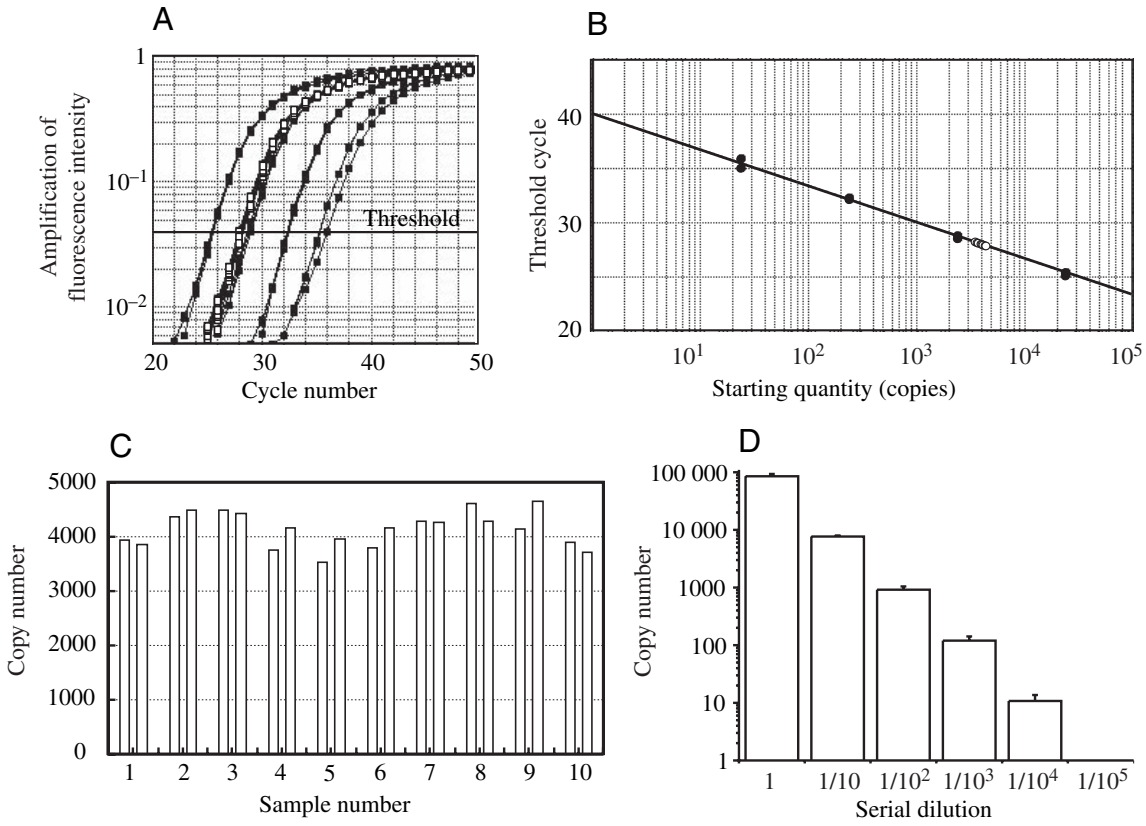

Fig. 3. Assessment of qRT-PCR assay by determination of the copy number of exogenous RNA at a known concentration in reaction solutions. (A) Amplification plots generated by the increase of reporter dye fluorescence with each cycle of PCR of salmon GTH α2 cDNA. The solid boxes show standard solutions, and the open boxes show the test samples. All reactions were performed in duplicate (1 µl each, which was 1/15 of the total volume of the reverse-transcribed products). An arbitrary threshold cycle was obtained in the exponential phase of PCR. (B) Standard curve for the starting quantity of salmon GTH α2 RNA. The solid circles show standard solutions, and the open circles the test samples. (C) Copy numbers of salmon GTH α2 RNA in 1 µl samples (1/15 of the total volume of the reverse-transcribed product). The total volume for reverse transcription was 15 µl with 6×10^4 RNA copies, and thus the detected value of 4143±71 copies (N=20 from the duplicates of 10 samples) in 1 µl of solution (i.e. 1/15 of the volume of the reverse-transcribed products) was reasonable. (D) Serial dilution of salmon GTH α2 RNA. The error at 10 copies shows that the S.E.M. is low enough (2.93, N=6 samples).

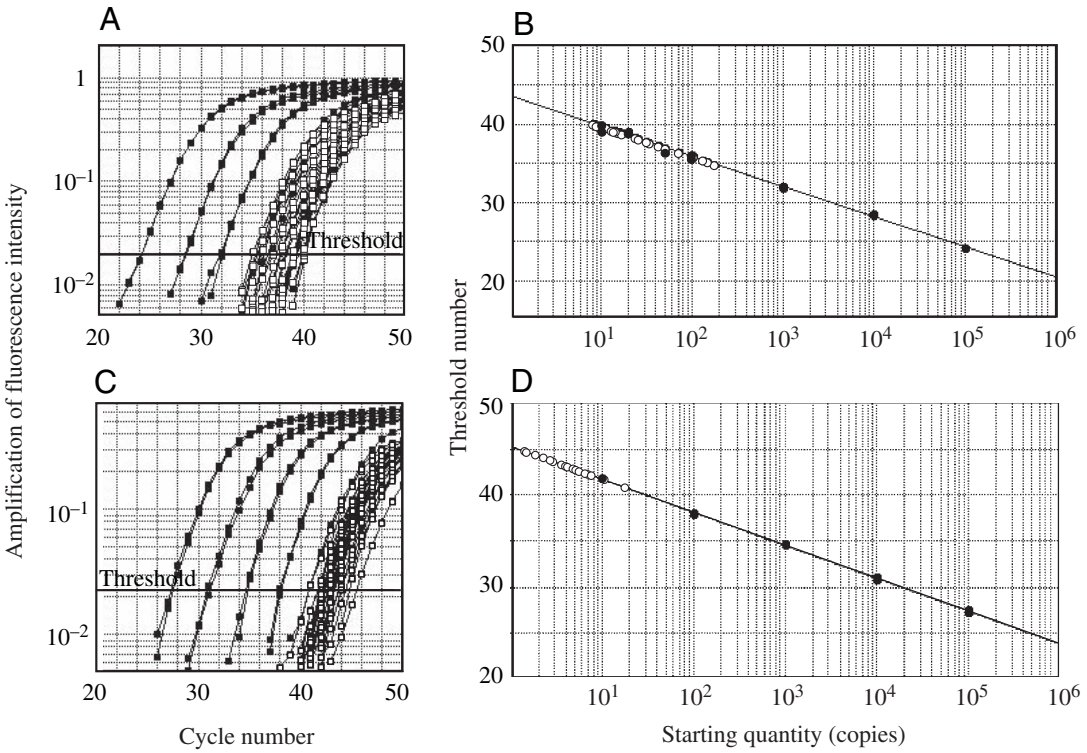

Fig. 4. The copy numbers of *Lymnaea* CREB2 and CREB1 mRNAs in single cerebral giant cells. (A) Amplification plots generated by an increase of reporter dye fluorescence with each cycle of PCR of *Lymnaea* CREB2 cDNA. The filled squares show standard solutions, and the open squares show the test samples. All reactions were performed in duplicate (6 μl each, which was 6/15 of the total volume of the reverse-transcribed products). An arbitrary threshold cycle was obtained in the exponential phase of PCR. (B) Standard curve for the starting quantity of *Lymnaea* CREB2 RNA. The filled circles show standard solutions, and the open circles the test samples. The estimated copy number of *Lymnaea* CREB2 mRNA in a single cerebral giant cell was between 30 and 240. (C) Amplification plots generated by an increase of reporter dye fluorescence with each cycle of PCR of *Lymnaea* CREB1 cDNA. The notation is the same as (A). (D) Standard curve for the starting quantity of *Lymnaea* CREB1 RNA. The notation is the same as (B). The estimated copy number of *Lymnaea* CREB1 mRNA in a single cerebral giant cell was below the determination sensitivity.

Discussion

In the present study, we used newly developed micromanipulators to perform the single-cell isolation of the cerebral giant cells followed by qRT-PCR to determine the copy numbers of mRNAs for the two CREB types. Whereas we could reliably determine the copy number of CREB2 mRNA at 30–240 copies in a single cerebral giant cell, we could not determine that of CREB1 mRNA, because it was below the sensitivity of our assay.

Reliability of our assay system

One of the first questions that must be raised concerns the reliability of our assay. On the basis of the data presented here, we concluded that our assay is reliable. We based this conclusion on the following points: (1) the standard curves were linear within the range of 10–10^5 copies; (2) the curve obtained from the serially diluted sample mRNA solutions was parallel to the standard curve; and (3) the intra-assay variation was within 15% in our assay system. We were also confident in regard to our limit of detection, which is 25 mRNA copies in a single cell. Our assay system can, therefore, reliably determine the numbers of mRNAs in a single cell as long as there are at least 25 copies of the target mRNA being assayed.

Advantages of our assay system compared with the others

As described in the Introduction section, some studies at single-cell level have been previously reported (Baro et al., 1997; Tkatch et al., 2000; Liss et al., 2001; Tsuzuki et al., 2001). First, the studies by Baro et al. (1997) and Tsuzuki et al. (2001) measured the density of PCR bands using conventional methods. Thus, the quantification was less sensitive than reported by us here. Second, Tkatch et al. (2000) and Liss et al. (2001) used qRT-PCR. In the Tkatch et al. (2000) study, brain slices from mice were made after decapitation and then incubated with artificial saline. The slices were then digested by protease and dissociated mechanically; and only then were single neurons picked up for analysis. However, these neurons were not uniquely identified. Furthermore, because the experiment took a relatively long time, it could not be used to measure mRNAs that turned over rapidly. Liss et al. (2001) used a patch pipette for aspiration of

intracellular contents. However, it is not certain whether all the contents of the cell could be collected into the patch pipette.

Recently, a few other new methods have been proposed to accomplish the single-cell task (e.g. Kamme et al., 2003; Ginsberg and Che, 2004; Korneev et al., 2005). However, our assay surpasses all of them from the viewpoints of accuracy, rapidity and relevancy. In particular, we should add some comments on the method by Korneev et al. (2005). They measured the amounts of nitric oxide synthase (NOS) and other mRNAs along with the isolated total RNA from single cerebral giant cells with an RNA purification kit. However, and this is the important difference between our work and theirs, they did not determine the exact copy numbers of target mRNAs for NOS or any other proteins in a single cerebral giant cell, whereas we have. They have only reported the relative levels of target mRNAs.

The methodology in the Korneev et al. (2005) paper is different in a number of important ways than ours. (1) To obtain the relative levels of target mRNAs, they used mRNA for β-tubulin as a reference point. However, it must be remembered that the shape of neurons changes (e.g. the dendritic tree) even with learning and memory (in fact morphological change at either the pre- or post-synaptic specialization is probably one of the most important physical manifestations of memory formation; e.g. Alkon et al. (1990) and Bailey and Kandel (1993). Because such changes involve alterations in β-tubulin activity, it is probably not the most accurate reference point to use as its mRNA levels are most likely changing. (2) There may also be differences in PCR efficiency. These differences may arise from a number of factors the most relevant being: (a) differences in the efficiency of how primers anneal to templates; (b) differences in the efficiency of polymerization of PCR products; and (c) a different affinity of SYBR Green to PCR products. (3) A loss of mRNA in their isolation procedure cannot be ruled out. Taken together, the data presented in the Korneev et al. (2005) paper are weaker than the data presented in our present paper. Because only relative amounts of mRNAs were measured in the Korneev et al. (2005) paper against a probably changing reference point, the conclusions that could and should be drawn are relatively weak regarding their biological meaning.

By contrast, the protocol developed by us and presented in the present paper has perfectly settled all these problems. (1) Our present protocol clearly gives the exact copy numbers of target mRNAs in a single cell, and this is important to elucidate the biological meaning of transcription. (2) The copy numbers of target mRNAs are determined by comparison with the standard curves prepared from the cDNA solutions corresponding to the serially diluted solutions of the target mRNAs themselves in our present protocol. This indicates that the PCR efficiency is not questionable. (3) The RNA loss is highly improbable in our RNA isolation protocol. We, therefore, claim that our present protocol completely surpasses all other published methods including that described by Korneev et al. (2005).

Why is the copy number of CREB2 mRNA variable between single neurons?

Whereas our intra-assay variation was on the order of 15% (Fig. 3), we found a much larger variation in the copy number of CREB2 mRNA between single cerebral giant cells (Fig. 4B). In addition, the estimated copy number of CREB1 mRNA in single cerebral giant cells also showed a slight variance (Fig. 4D). For CREB2, the range was from 30 to 240 mRNA copies obtained from single cerebral giant cells from different snails. The difference in the mRNA copy numbers should be regarded as meaningful and important. There are a number of possible reasons for this range in the mRNA copy numbers, particularly as regards CREB2. First, preliminary experiments showed that various environmental and experimenter-applied stimuli are able to up-regulate the expression of the CREB2 gene in the cerebral giant cells (H. Sadamoto and E. Ito, unpublished data). Second, the mammalian homolog of *Lymnaea* CREB2, ATF4, is also upregulated by many different extracellular signals (Hai and Hartman, 2001). In addition, several intracellular stress pathways are known to activate ATF4 (Rutkowski and Kaufman, 2003). Third, ATF proteins are also involved in such important homeostatic functions as glucose metabolism and trophic factor-dependent survival. Thus, various physiological and environmental stimuli can easily alter the expression of CREB2 in single cerebral giant cells from different snails, even when the snails are maintained under similar conditions.

In conclusion, the results from our study showed that it is possible to isolate a single, identifiable cell accurately and rapidly, and to determine the exact copy numbers of particular mRNAs within it. We chose to examine the CREB transcripts and found that in the cerebral giant cells there were more mRNA copies of the repressor type, CREB2, than there were mRNA copies of the activator type, CREB1.

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