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Detection of gene expression in single neurons by patch-clamp and single-cell reverse transcriptase polymerase chain reaction

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Abstract

Detection and quantitation of gene expression in single cells is especially important in the central nervous system where, at the cellular level, the synapse can be considered the single functional unit. For example, the consolidation of long-term memories may be mediated by persistent changes in the strength of synaptic transmission at individual synapses. In order to investigate the requirement for de novo RNA synthesis during long-term potentiation in individual neurons, we have combined single-cell electrophysiology with single-cell gene-expression methodology. Described are methods combining whole-cell patch-clamp and single-cell RT-PCR for the detection of a single mRNA species for nitric oxide synthase, or, through a multiplex strategy, for the simultaneous detection of several mRNAs including heme oxygenase 2, protein phosphatase inhibitor 1 protein, and several isoforms of the calcium/calmodulin dependent protein kinase II. © 1998 Pubished by Elsevier Science B.V.

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1. Introduction

The ability to detect and quantitate gene expression in individual cells is becoming increasingly important. In order to completely understand human physiology at the cellular level, we need to segregate the functional heterogeneity between cells within whole tissues and organs and begin to determine the functional contribution of an individual cell in the context of its complex in vivo environment. The challenge is to be able to predict the stimulus-response of an individual cell in situ. The advantage of physiological studies at the single-cell level is the elimination of classic problematic interpretation of population effects, i.e., whether a measure of population phenomena reflects most of the cells in the population or only a few.

Such issues are especially relevant in the central nervous system. Complex behaviors such as learning require multiple levels of organization from systems and pathways, to local circuits and synapses, to membranes, molecules and ions. Memories are thought to be stored in the temporal and spatial patterns of connections maintained within networks of neurons. Neuronal connectivity is expressed as the strength of synaptic transmission at individual synapses.

Certain synapses have been shown to be plastic, that is, the strength of synaptic transmission can be altered depending upon prior activity. For example, one form of synaptic plasticity, called long-term potentiation (LTP), requires ‘‘training’’ (experimentally applied as high frequency stimulation of the presynaptic terminal), and results in ‘‘learning’’

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(experimentally measured as increases in the postsynaptic response). Many of the molecular mechanisms comprising induction of LTP have been elucidated (Fig. 1, see for review Refs. [1–3]). These include binding of the excitatory neurotransmitter glutamate to specific *N*-methyl-D-aspartate or NMDA-receptor subtypes resulting in calcium influx and signaling in the postsynaptic terminal, and postulated retrograde signaling to the presynaptic terminal. As a result of protein modification, perhaps through phosphorylation, subsequent stimulation produces augmented synaptic transmission due to increased presynaptic neurotransmitter release and/or postsynaptic responsiveness.

Persistent changes in synaptic strength, lasting days and longer, have been shown to require new protein synthesis and RNA transcription (see for review Refs. [4,5]). This longer lasting form of LTP has been correlated with the consolidation of long-term memory as opposed to short-term [6–8]. Protein synthesis may be required for more permanent structural and functional changes at the potentiated synapse and also for the formation of additional synaptic connections (see for review Ref. [9]). Since the synapse can be considered a single functional unit and LTP-induced increases in synaptic transmission are specific to the trained synapse, the necessary changes in gene expression should be specific to the presynaptic and/or postsynaptic neuron.

In order to segregate LTP-relevant events from the functional heterogeneity of the entire brain, we have combined single-cell electrophysiology with single-cell gene-expression methodology. We used the whole-cell patch-clamp technique to characterize electrophysiologically single neurons and to gain access to their cytoplasm. The cellular content was aspirated and used as template for reverse transcriptase (RT) allowing for the detection of specific mRNAs by subsequent polymerase chain reaction (PCR). Compared with immunocytochemistry or in situ hybridization, our method more easily couples function with gene expression at the single-cell level. The cell-type specificity and tremendous detection sensitivity gained by removing the background of unstimulated cells is demonstrated in experiments, described below (and in more detail in Ref. [10]), to detect nitric oxide synthase in rat hippocampus. Also described is the multiplex PCR strategy that was

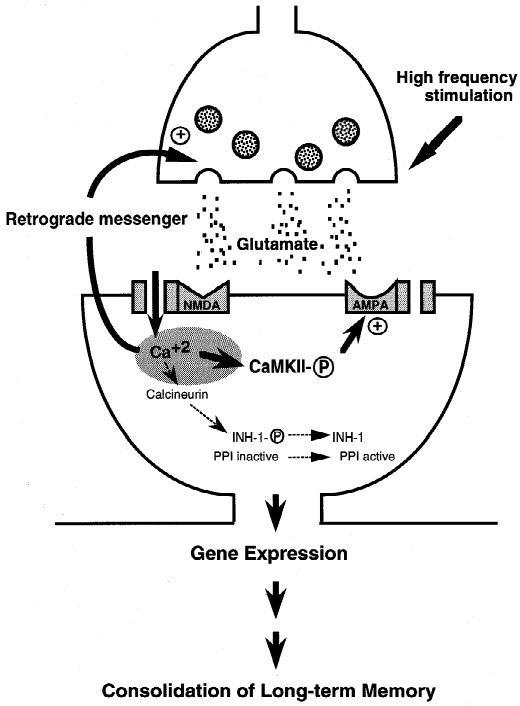

Fig. 1. Model molecular mechanisms mediating long-term potentiation. During high frequency stimulation, LTP is induced when concomitant binding of the presynaptically-released neurotransmitter, glutamate, to NMDA receptors, and depolarization of the postsynaptic terminal through non-NMDA glutamate-channels, including the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxalone propionic acid) receptor, results in high Ca^{2+} influx into the postsynaptic terminal. Calcium effects a variety of processes including activation of Ca^{2+}/calmodulin-dependent kinases, such as CaMKII, and production of retrograde messengers. These kinases are presumed to phosphorylate a plethora of substrates, including the AMPA-receptors, to effect persistent changes that enhance synaptic transmission. Nitric oxide synthase and heme-oxygenase 2 release retrograde messengers nitric oxide and carbon monoxide, respectively, which can permeate membranes and affect targets in the presynaptic terminal that increase vesicle fusion and neurotransmitter release. The consolidation of long-term memory has been equated with a longer lasting form of LTP which requires new RNA transcription and protein synthesis presumably for the formation of new synaptic connections. An opposing phenomena called long-term depression (LTD) involves low frequency stimulation (not shown), which produces a small increase in Ca^{2+} that selectively activates a phosphatase cascade including protein phosphatase 1 (PP1) and its regulator inhibitor 1 (INH-1), leading to overall dephosphorylation in the postsynatic terminal and depression of synaptic transmission.

implemented to compare simultaneously the expression of various signal transduction molecules implicated in LTP expression.

2. Nitric oxide synthase expression in single hippocampal neurons

In the rat hippocampus, at the synapse between CA3 and CA1 neurons, experiments with nitric oxide synthase (NOS) inhibitors had supported a role for nitric oxide (NO) in retrograde communication during LTP [11–14] (Fig. 1). LTP is induced in postsynaptic CA1 neurons, but at least part of the expression seems to be mediated through increased neurotransmitter release from presynaptic CA3. A requirement for postsynaptic to presynaptic (retrograde) communication could be satisfied by postsynaptically produced messenger molecules, such as NO, acting on the presynaptic terminal. However, the apparent absence of NOS detection in postsynaptic CA1 pyramidal cells by histochemical, immunocytochemical and in situ hybridization methodology had argued against NO as a retrograde messenger

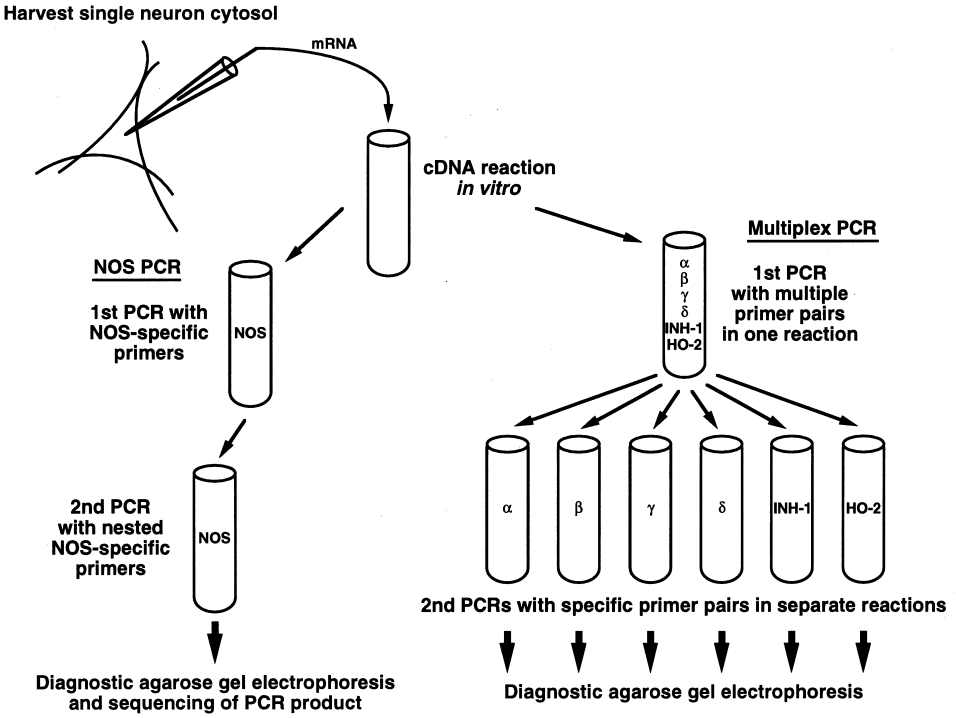

Fig. 2. Single-cell RT-PCR. Whole-cell patch-clamp provides access to the cytoplasm of individual hippocampal neurons. The usual patch-pipette buffer (with an addition of yeast tRNA at 20 μg/ml) and bath solutions are prepared RNase-free. After recording for 5 to 60 min, the cellular content was aspirated into the silanized patch-pipette and then expelled into a silanized RNase-free microcetrifuge tube containing 10 μl reverse transcription reagents [50 m*M* Tris·HCl (pH 8.3), 10 m*M* dithiothreitol, 3 m*M* $MgCl_2$, 75 m*M* KCl, 5 μ*M* random hexamer primer (New England Biolabs), 0.5 m*M* of each deoxynucleotide (Boehringer Mannheim), 2 U/μl RNase inhibitor (rRNasin, Promega) and 10 U/μl reverse transcriptase (Superscript II, Gibco BRL)]. Incubation was 15 min at room temperature followed by 1 h at 37°C. The reaction is stopped (with the addition of 250 μl ethanol, 100 μl 0.3 *M* sodium acetate and 5 μg yeast tRNA) and may be stored at −20°C for several months. Before PCR, the entire single-cell cDNA template is recovered by centrifugation, resuspended in 5 μl water after a 70% aqueous ethanol wash, and used for the first PCR. In the first reaction, the outer pair of primers for NOS (see Fig. 3) or for six genes, α, β, γ and δ CaMKII, INH-1 and HO-2 simultaneously (see Fig. 6) are used for NOS PCR and Multiplex PCR, respectively. Then 1/10th or 1/100th of the resulting NOS or multiplex amplification product, respectively, is diluted into a second PCR containing one pair of gene-specific nested primers. Gene expression is detected by agarose gel electrophoresis and ethidium bromide staining of the entire product from the second PCR.

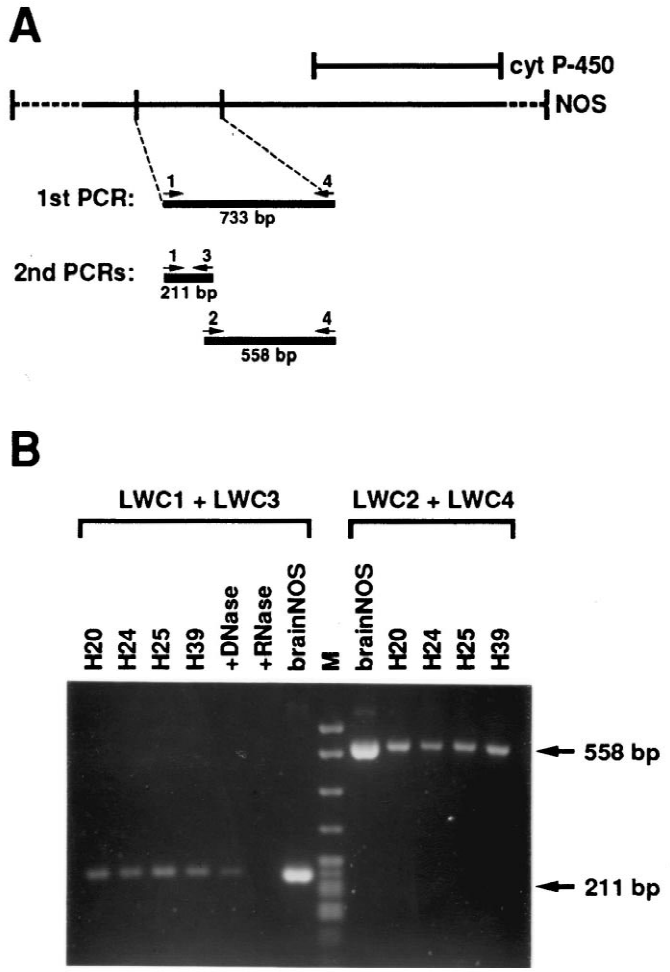

Fig. 3. NOS PCR strategy and analysis. (A) Approximate positions of nested PCR primers LWC1, LWC2, LWC3 and LWC4 are indicated by arrows (for coordinates see Ref. [10]). Dashed lines at the end of NOS represent the variable lengths of different isoforms. The predicted sizes for PCR products are indicated (for detailed amplification conditions see Ref. [10]). (B). Single-cell PCR products from hippocampal neurons H20, H24, H25 and H39 were run on an ethidium bromide-stained agarose gel. All samples were first amplified with LWC1 and LWC4. In the second PCR, the samples were amplified with LWC1+LWC3 or with LWC2+LWC4 as indicated. Control single-neurons were treated with DNase or RNase prior to first-strand cNA synthesis. Included for reference are the amplification products of the full-length cDNA clone of the cerebellar-isoform of NOS (brain NOS) and pBR322 DNA restricted with HpaII (M). bp=base pairs. From Ref. [10], with permission.

[15–19]. Two possible explanations were that NOS is present in low abundance or that it encodes for a different isoform not detected by the probes used.

A single-cell RT-PCR strategy was developed to detect NOS for two reasons. First, the isolation of RNA from single cells reduces the complexity of the message pool, thereby facilitating the detection of relevant mRNA molecules by RT-PCR. Second, single-cell RT-PCR would establish the presence of a certain mRNA in a given cell-type. Since interneurons and glia in the rat hippocampus were known to contain NOS, detection of NOS in whole hip-

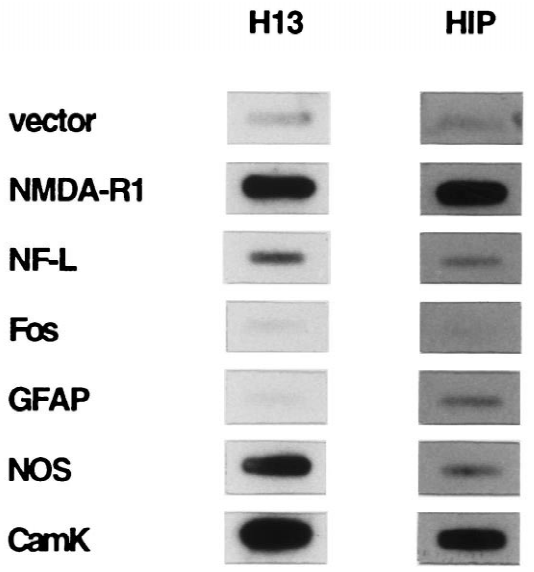

Fig. 4. Sensitivity of gene expression detection in single hippocampal neuron H13 in comparison to whole hippocampus. RNA isolated from H13 or the whole hippocampus (HIP) was labeled in vitro (see Fig. 5) and hybridized to slot blots loaded with Bluescript vector (Stratagene), various rat cDNA clones: NMDA receptor RI (NMDA-RI; pN60 [41]), neurofilament protein (NF-L; p567c [42]), immediate early gene *c-fos* (Fos; pc-*fos*(rat)-1 [43]), cerebellar isoform of NOS (full-length cDNA in *Eco*RI site of Bluescript) and a CaMKII (CaMK; full-length cDNA in *Eco*RI sith of Bluescript) and mouse glial fibrillary acidic protein (GFAP; GI [44]). Each column represents a single hybridization experiment and exposure. Relative to the other genes, expression of NOS is clearly enhanced in single neuron H13 compared to whole hippocampus. From Ref. [10], with permission.

pocampal extracts would not have been conclusive with regard to its presence and role in postsynaptic CA1 pyramidal neurons.

Whole-cell patch-clamp, a method for recording electrical activity in single neurons via a patch-pipette electrode [20–22], was used to isolate and identify CA1 pyramidal neurons by their electrophysiological signature [10] (Fig. 2). RNase-free recording conditions (RNase-free solutions, equipment and gloves) [23], silanized patch-pipettes and inclusion of yeast tRNA in the electrode buffer were used to optimize the recovery of nucleic acid. Care was taken to apply positive pressure on the patch-pipette as it was lowered into the preparation until a gigaohm seal was achieved to prevent contamination of the samples. After recording, the cell cytosol was aspirated into the patch-pipette and subsequently expelled into a silanized, RNase-free microcentrifuge tube. RT was added in vitro to copy the single-cell RNA into first strand cDNA.

Detection of NOS mRNA from single neurons required a nested PCR strategy (Fig. 3A) and optimized PCR conditions [24,25]. NOS-specific product of the expected size, visualized by agarose gel electrophoresis and ethidium bromide staining (Fig. 3B), was detected in 34 out of 45 hippocampal neurons. Control cytosols included those treated with DNase I and RNase A prior to cDNA synthesis to demonstrate that the PCR product was RNA-dependent and to exclude the possibility that the signal originated from chromosomal DNA (Fig. 3B). To monitor PCR contamination [26,27], additional controls included reactions performed in the absence of added template and mock patch-pipettes inserted into the preparation without aspiration of a cytosol (not shown). Cycle-sequencing (AmpliTaq Cycle Sequencing Kit, Perkin-Elmer Cetus, Foster City, CA, USA) of nine gel-purified single-cell PCR products indicated that the brain-isoform, previously cloned from cerebellum [28], was also expressed in hippocampal CA1 pyramidal neurons.

Since the NOS detected was identical to one of the previously cloned isoforms, NOS single-cell PCR was more sensitive than NOS-specific histochemistry, immunocytochemistry or in situ hybridization. Fig. 4 illustrates the enhancement of the NOS signal over background when RNA from a single neuron expressing a specific message is compared to RNA from whole tissue, in which the NOS signal has been diluted by RNA from other cells. In this experiment, all of the mRNAs in a single hippocampal neuron (H13) were simultaneously amplified by attaching sequences for the T7 RNA polymerase promoter to each cDNA during reverse transcription (Fig. 5); linear amplification and radiolabeling of the single-cell mRNA population was achieved by synthesizing in vitro transcripts from the T7 promoter present on single-cell cDNAs [29]. In a reverse Northern analysis, the NOS-specific hybridization signal from single-neuron H13 mRNA is clearly enhanced compared to whole hippocampus mRNA (Fig. 4).

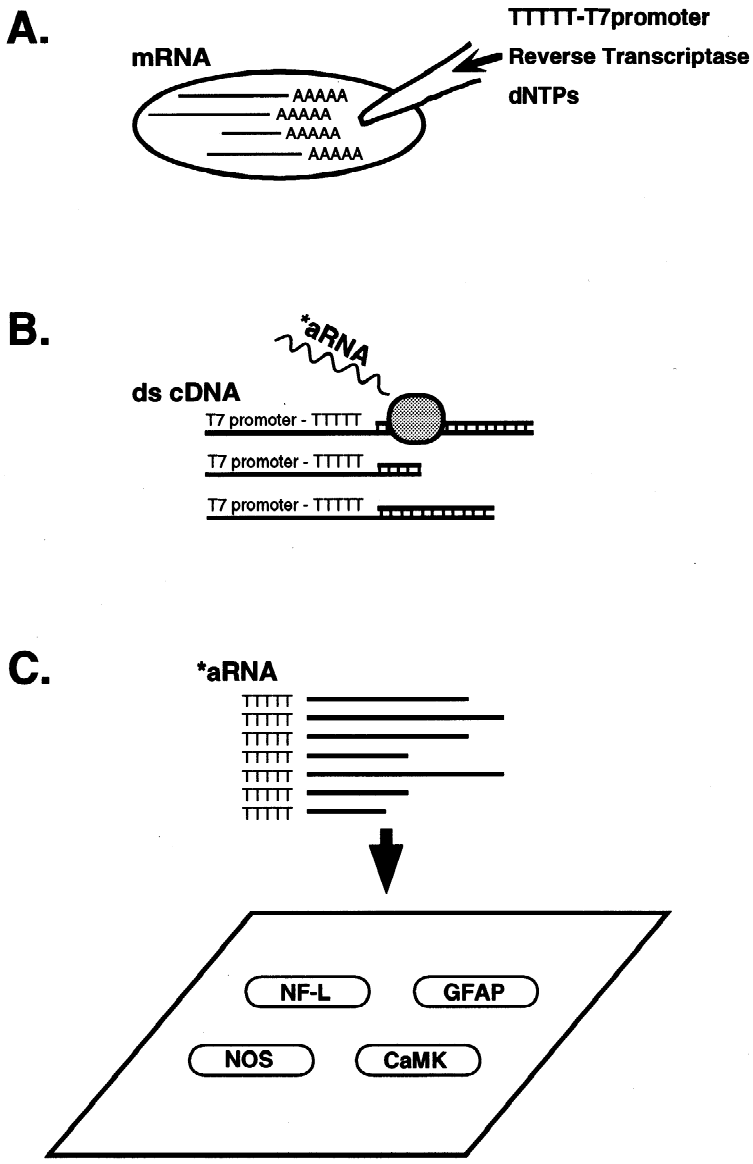

Fig. 5. Single-cell expression profiling. (A) cDNA synthesis is initiated in situ during whole-cell patch-clamp by including in the electrode buffer a mRNA-specific poly(dT) primer containing the T7 RNA polymerase promoter sequences on the 5′-end, reverse transcriptase and deoxynucleotides A, G, C, and T [29]. The cDNA reaction is completed in vitro, by aspirating the cytosol, adding it to reverse transcription reagents in an Eppendorf tube, and incubating at 37°C. (B) A series of in vitro steps generates a double-stranded cDNA copy of the mRNAs in the single cytosol. Addition of radioactive ribonucleotides and T7 RNA polymerase initiates in vitro synthesis and labeling of antisense RNAs (aRNA). The resulting pool of aRNAs is linearly amplified proportionally with respect to the original population of mRNAs in the single cell. (C) The radiolabeled aRNA probe can be applied to a slot blot containing various cloned genes of interest, in a reverse Northern analysis. The hybridization signal to each gene on the slot blot partially reflects the relative abundance of specific mRNAs.

By using single-cell PCR, we increased the sensitivity for detection of NOS mRNA and demonstrated that NOS is expressed specifically in postsynaptic CA1 pyramidal neurons. Therefore, NO could function as a retrograde messenger during LTP.

3. Co-expression of signal transduction molecules in single hippocampal neurons

Pharmacological experiments have implicated roles for specific signal transduction molecules in the induction of LTP (Fig. 1, see for review Refs. [1–3]). To reiterate, glutamate release during high-frequency stimulation depolarizes postsynaptic cells via AMPA (α-amino-3-hydroxy-5-methyl-4-isoxalone propionic acid)/kainate receptors, which enables entry of Ca^{2+} through NMDA receptors. Calcium in the postsynaptic terminal is essential for the induction of LTP; it activates Ca^{2+}-dependent kinases which can enhance AMPA currents by phosphorylating the receptors; Ca^{2+} also stimulates generation of retrograde messengers that might increase presynaptic release.

During long-term depression (LTD), low-frequency stimulation produces a small increase in Ca^{2+} that might selectively activate calcineurin, a phosphatase with high affinity for Ca^{2+}/calmodulin (Fig. 1, see for review [30–32]). Calcineurin activity predominates over Ca^{2+}-dependent kinases such as CaMKII, and then de-inhibits a broad specificity phosphatase (PP1) by dephosphorylating the PP1 regulator, inhibitor 1 (INH-1). PP1 would then antagonize activation of CaMKII and its phosphorylation of AMPA receptors leading to a sustained decrease (as opposed to increase during LTP) in synaptic transmission.

The involvement of signal transduction molecules in modulation of synaptic strength suggests that their own expression could be targets of transcriptional activation cascades required for maintenance of LTD/LTP. Single-cell RT-PCR with a multiplex strategy was developed to study the relative expression of three signaling molecules. Heme oxygenase (HO-2) produces another putative retrograde messenger [33,34]. In the postsynaptic terminal, CaMKII and INH-1 participate in Ca^{2+}-dependent pathways leading to LTP and LTD induction, respectively [35–37].

Briefly, visualized slice preparations [38] of the rat hippocampus combined with the whole-cell patch-clamp technique were used to characterize the morphology and electrophysiology of individual CA1 pyramidal neurons. After recording, the cellular content was aspirated and served as template for multiplex RT-PCR utilizing nested primer sets for HO-2, INH-1 and all four isoforms of CaMKII (Fig. 2). Multiplexing in the first PCR of the entire single-cytosol cDNA required empirical selection of primer sequences to obtain a pair of primers for all six genes that amplified optimally under the same PCR conditions [39] (Fig. 6). Subsequently, the product from the first PCR was aliquoted to six different tubes for a second round of PCR; each tube contained one nested pair of primers specific for one of the six genes. Fig. 7 illustrates the specificity obtained from single hippocampal neuron JK3 expressing INH-1, one spliceform of α and β CaMKII, and no detectable γ or δ or HO-2. Multiple RT-PCR bands observed in whole rat brain for α, β, γ and δ CaMKII reflect the expression of multiple known spliceforms in whole tissue. Interestingly, neurons of the same apparent cell-type (CA1 pyramidal neurons) demonstrated marked heterogeneity in gene expression. Most expressed mRNA for α CaMKII as detected by single-cell RT-PCR, some expressed INH-1, and/or β CaMKII, while only one expressed HO-2. None out of 27 examined expressed the non-neuronal γ or δ isoforms of CaMKII.

This apparent heterogeneity, in the absence of in vitro stimulation, is consistent with prior synaptic transmission regulating expression of these signaling molecules at the single-cell level. Since individual neurons form connections in distinct circuits, in vivo activity at individual synapses should be different prior to in vitro slice preparation. Therefore, the use-dependent threshold for transmission would be set differently at individual synapses, and individual neurons would express different levels of the critical molecules. Alternatively, the apparent heterogeneity could have arisen due to the limit of detection (LOD) of the assay. To determine the LOD, we have generated in vitro transcripts and found that 10 to 100 molecules of RNA could be detected when

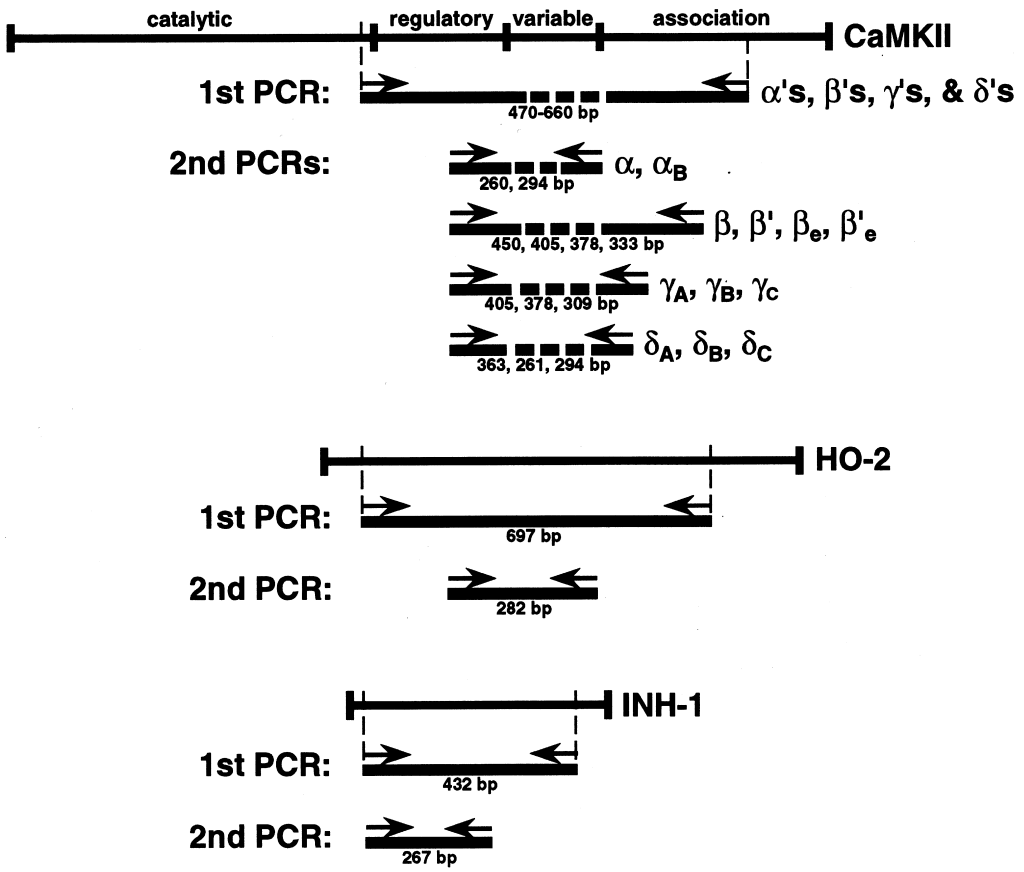

Fig. 6. Multiplex PCR. Approximate positions of nested PCR primers for HO-2, INH-1 and all known isoforms of CaMKII are indicated by arrows (coordinates will be provided upon request). Dashed lines within the CaMKII PCR products represent the variable lengths of different spliceforms as indicated by the predicted sizes. PCR conditions were individually optimized for all of the primer pairs separately. The reaction conditions for the multiplex mix were as follows: 30 cycles capillary PCR (<1 s at 94°C, <1 s at 60°C, 15 s at 72°C) in 10-μl reaction volumes containing each outer primer for α, β, γ and δ CaMKII, HO-2 and INH-1 at 1.5 μ*M*, 50 m*M* Tris·HCl (pH 8.3), 0.5 mg/ml bovine serum albumin, 0.2 m*M* each deoxynucleotide A, G, C and T, 2 m*M* $MgCl_2$ and 2 U AmpliTaq (Perkin-Elmer Cetus). The secondary PCRs were similar except that 35 cycles were performed and the gene-specific nested primer pair was present at 5 μ*M* each.

spiked into concentrations of yeast tRNA similar to the multiplex RT-PCR assay conditions (not shown). Despite the level of sensitivity, this control does not account for unequal loss of mRNA during harvest of the single cytosols. Indeed, if a single cytosol is split into two prior to RT-PCR, the expression pattern observed for the two half-cytosols does not always correlate (not shown). Inconsistencies observed include detection of a less abundant message, such as INH-1 or HO-2, in one half-cytosol and absence of detection in the other (not shown). Under the conditions of the assay (below saturation for reagent utilization), a tally of the number of single cells expressing each gene is partially related to the message level for that gene. For the abundant messages (more than 10-100 mRNAs per cell), cell-to-cell differences in detection probably do reflect cell-to-cell heterogeneity in levels of expression, especially when less abundant messages are detected simultaneously in the same cells.

The observed heterogeneity posed a problem for studying postulated activity-dependent regulation of these signaling molecules at the single-cell level. Unfortunately, the method does not permit the assay of gene expression in the same neuron before and after a particular stimulation protocol; since the

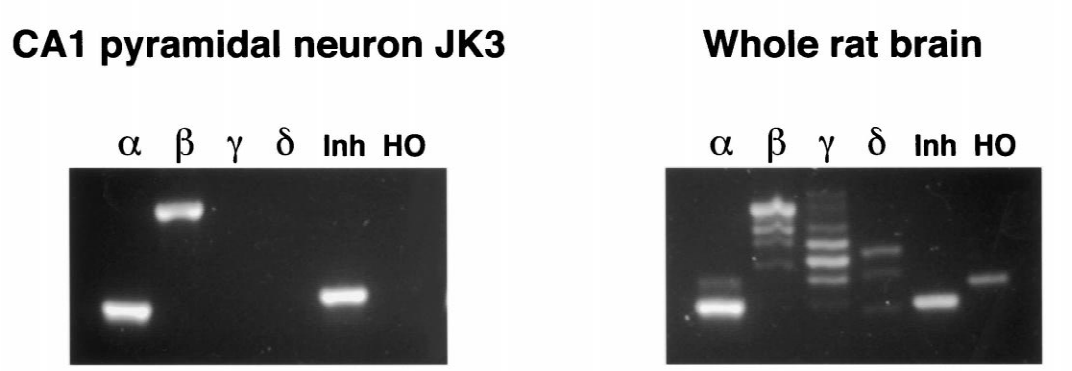

Fig. 7. Specificity of gene expression detection in single hippocampal neuron JK3 in comparison to whole rat brain. After single-cell multiplex RT-PCR, the entire product from each gene-specific (as labeled) secondary PCR was run on a 2% agarose gel in the presence of ethidium bromide. For comparison, whole rat brain RNA was analyzed by RT-PCR using each inner nested pair of primers directly on rat brain cDNA (without the first multiplex reaction). Multiple bands of the expected size for each CaMKII spliceform are observed for whole rat brain. By single-cell multiplex RT-PCR, transcripts were detected in CA1 pyramidal neuron JK3 for α CaMKII, β CaMKII and INH-1 (Inh), but not for γ or δ CaMKII or HO-2 (HO).

baseline appeared to be quite heterogeneous, many single cells would have to be tested to generate statistically significant data. In addition, because a nested strategy is required to obtain analytical amounts of material, single-cell RT-PCR is not quantitative. Therefore, we have since studied regulation using cultured hippocampal neurons. Activity-dependent regulation of α and β CaMKII was observed when stimulation was applied to the whole dish and resulting gene expression was quantified as average changes in the population [40].

4. Significance

Our experiments demonstrate the increased sensitivity and specificity of single-cell RT-PCR over other methods such as immunocytochemistry and in situ hybridization for detecting mRNA at the single-cell level. The experiments also suggest that cell-to-cell differences do exist in gene expression between individual CA1 pyramidal neurons in the central nervous system. Future directions for single-cell gene expression technology include new methods for improved quantification. Quantitative analysis will allow us to meet the challenge in neurobiology to integrate differences in gene expression in single neurons with physiologically relevant function in the context of complex neural circuits.

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