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Differential Localization of mRNAs for Mammalian *trp*s, Presumptive Capacitative Calcium Entry Channels, in the Adult Mouse Brain

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Otsuka, Y., Sakagami, H., Owada, Y. and Kondo, H. *Differential Localization of mRNAs for Mammalian trps, Presumptive Capacitative Calcium Entry Channels, in the Adult Mouse Brain.* Tohoku J. Exp. Med., 1998, **185** (2), 139-146 —— Mammalian homologues for *Drosophila trp* are likely to be candidates for capacitative calcium entry channels. By *in situ* hybridization histochemistry, we have demonstrated that the mRNAs for four species of the mouse homologues (M*trp*s-1, -3, -4, -6) were differentially expressed in the adult mouse brain. M*trp*-1 mRNA was expressed widely throughout the gray matters, while the expression for M*trp*-3 was dominant in the cerebellar Purkinje cells, the olfactory mitral cells and the striatal large-sized intrinsic neurons. M*trp*-4 mRNA was evident in the olfactory bulb, the septum, the hippocampal neuronal layers, and the cerebellar granule cell layer, while the expression for M*trp*-6 was rather confined to the dentate granule cell layer. Their differential localization suggests that the individual homologues exert their functions in region-specific and neuron-specific manners in the calcium signaling. ———— capacitative calcium entry channel; in situ hybridization histochemistry; mouse; Purkinje cell

Binding of external agents with G-protein- or tyrosine kinase-linked receptors initiates a cascade that generates inositol 1, 4, 5-trisphosphate (IP_3), a diffusible intracellular second messenger, to release calcium ions from the endoplasmic reticulum through IP_3 receptors. This transient increase in the intracellular free calcium concentration is followed by a more sustained response which is attributed to the influx of calcium ions from the extracellular space through a set of channels in the plasma membrane. This latter calcium entry is termed

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capacitative calcium entry (CCE). The channels involved in CCE have been also referred to as store-operated calcium channels (SOCs), or calcium release-activated calcium channels (CRACs).

There have been suggestions that one of the most likely candidates for CCE channels is a mammalian homologue for the transient receptor potential (*trp*) gene product in *Drosophila* photoreceptors (Montell and Rubin 1989). On the assumption that there is a significant homology in amino acid sequences between the *trp* gene product and mammalian counterparts, recent gene cloning analyses have uncovered the existence of at least six non-allelic *trp* genes (*trp*-1 through -6) in the mouse genome (Zhu et al. 1996). Full-length cDNAs for mammalian homologues have been identified from human, mouse, rat, and bovine sourses (Petersen et al. 1995; Wes et al. 1995; Zhu et al. 1995, 1996; Birnbaumer et al. 1996; Funayama et al. 1996; Philipp et al. 1996; Zitt et al. 1996). Among them, the functional expression analysis has so far been performed for human *trp*-1 and -3, bovine *trp*-4 and mouse *trp*-6, indicating that calcium entry activity is increased in transfected cells with either of the four cDNAs (Birnbaumer et al. 1996; Philipp et al. 1996; Zhu et al. 1996; Zitt et al. 1996, 1997), while the detailed information on the mRNA localization in the brain is available only for rat *trp*-4 (Funayama et al. 1996). The present study was attempted to clarify by in situ hybridization histochemistry the spatial localization of mRNAs for four species of mouse *trp*s (M*trp*s) in the adult brain, based on the general idea that the localization represents the first important information for understanding the functional roles of given molecules.

Materials and Methods

As in situ hybridization probes, 45-mer antisense oligonucleotides were synthesized on a DNA synthesizer, which were complementary to parts of coding sequences of individual mouse *trp* homologues partially identified by Zhu et al. (1996). The nucleotide sequences of the probes were as follows: M*trp*-1, 5′ GCTGTTCGCAGAAGATGCCCACACAGTCCTTCTGCTCTTTGGAAG3′; M*trp*-3, 5′TTTGTGATCATATTTGAGGACAACAGAAGTCACTTCAGACAGTCC3′; M*trp*-4, 5′GCTAGCAGCACGAGGCAGTAGATGAACAAGAACTTCAGGATGTCC3′; M*trp*-6, 5′CTTTCCTCAACTGTTGTGAATGCTTCATTCTGTTTTGCGCCAATG3′. For control templates, sense oligonucleotides were also synthesized.

Fresh frozen brains from male Balb/c mice at the postnatal 7th week were sectioned at 30 μm thickness on a cryostat and mounted on silane-coated glass slides. The sections were immersed in 4% paraformaldehyde/0.1 M sodium phosphate buffer (PB, pH 7.2) for 10 minutes, followed by acetylation with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 7.0) for 10 minutes at room temperature.

After dehydration, the sections were incubated at room temperature for 1

hour in the prehybridization solution containing 50% deionized formamide, 4x SSC (1xSSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.4), 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 0.02% Ficoll, 1% sodium N-lauroyl sarcosinate (Sarkosil, Wako Pure Chemical, Osaka), 0.1 M PB, and 250 μg/ml heat-denatured salmon sperm DNA.

After prehybridization, the sections were hybridized overnight at 42°C with

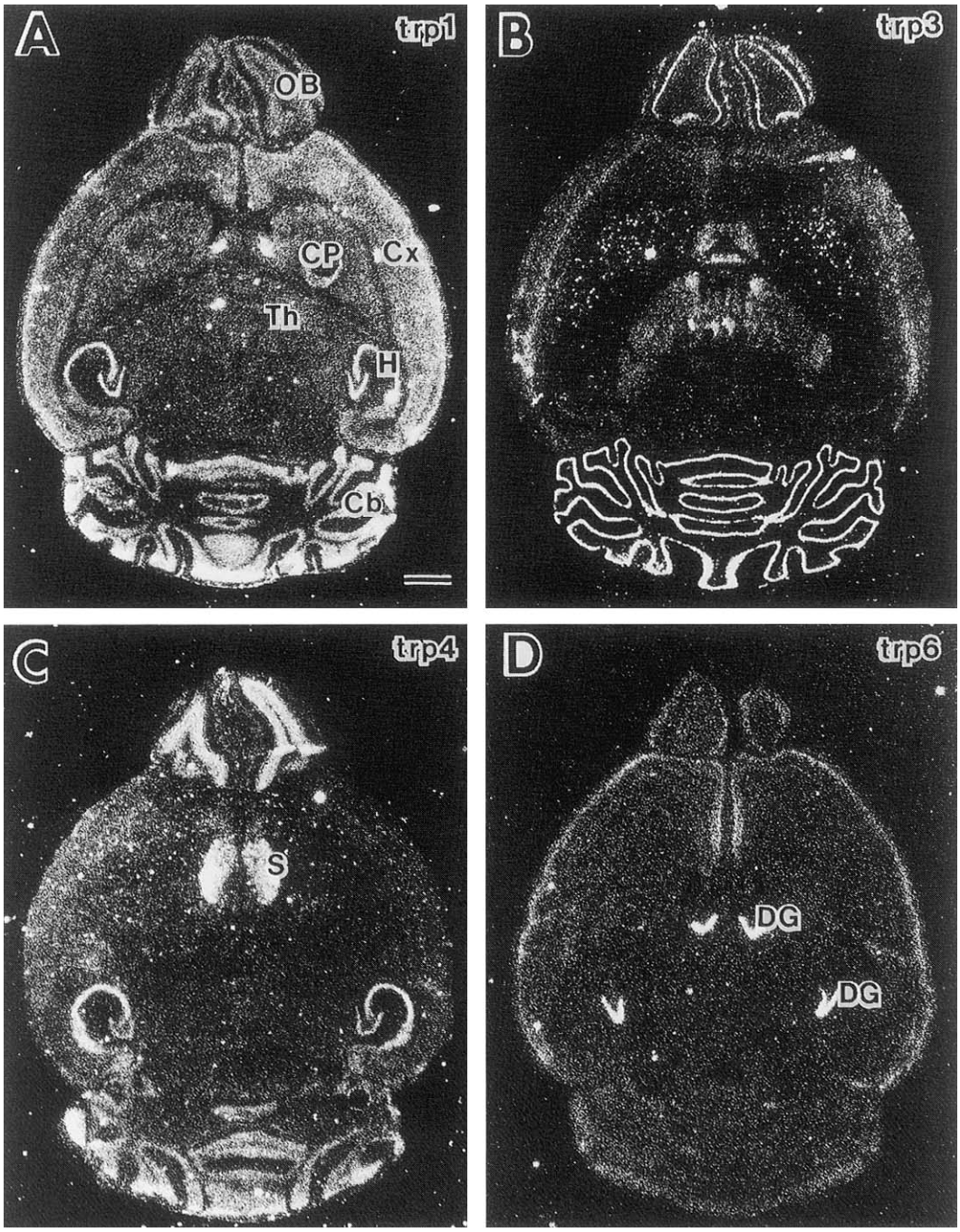

Fig. 1. General expression patterns of M*trp*-1 (A), -3 (B), -4 (C), and -6 (D) mRNAs in the adult mouse brain, showing differential expression of individual M*trp* mRNAs. Cb, cerebellum; CP, caudate putamen; Cx, cerebral cortex; DG, dentate granule cell layer; H, hippocampal formation; OB, olfactory bulb; S, septum; Th, thalamus. Bar = 1 mm.

^{35}S-labeled oligonucleotides diluted in the solution, which consisted of the pre-hybridization solution with the addition of 10% dextran sulfate and 100 mM dithiothreitol. The sections were then washed with 0.1x SSC/0.1% Sarkosil at 50°C four times for 30 minutes.

They were exposed to Hyperfilm β-max (Amersham, Arlington Heights, IL, USA) for 2 weeks at room temperature. They were subsequently autoradiographed using NTB2 nuclear track emulsion (Kodak, Rochester, NY, USA) for 3 weeks at 4°C. No significant expression signals were detected above the background levels in the control experiment in which the hybridization was performed with ^{35}S-labeled sense oligonucleotide probes.

Results

The expression signals for M*trp*-1 were detected more or less throughout the gray matters, while those in the white matters such as the corpus callosum were

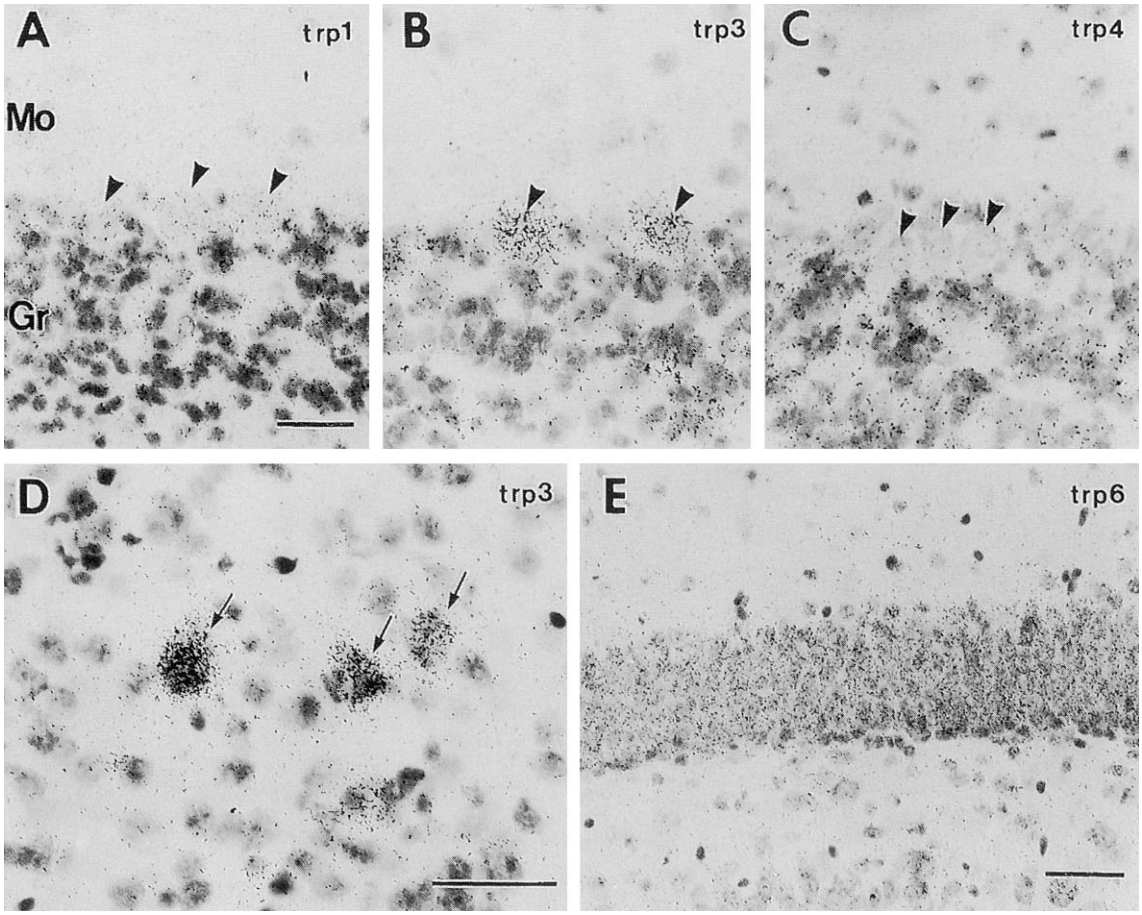

Fig. 2. Bright-field photographs showing that the gene expression of M*trp*-1 (A), M*trp*-3 (B), and M*trp*-4 (C) in the cerebellum, that of M*trp*-3 (D) in the caudate putamen, and that of M*trp*-6 (E) in the dentate granule cells. Note that Purkinje cells (arrowheads) express M*trp*-1 weakly and M*trp*-3 intensely, but not M*trp*-4, while the granule cells (Gr) express M*trp*-1 and -4 weakly, but not M*trp*-3. Arrows show intense gene expression for M*trp*-3 in the striatal large-sized intrinsic neurons. Mo, molecular layer. Bars = 50 μm.

negligible (Fig. 1A). The expression was also negligible in the cerebral layer I, the cerebellar molecular layer and the external plexiform layer of the olfactory bulb. The weak expression was seen rather evenly through the layer II-VI in the cerebral cortex, the caudate putamen, the diencephalon and the brain stem without any distinct signals concentrated on specific cells. The expression was slightly higher in the hippocampal pyramidal and dentate granule cell layers than that in other gray matters, but no regional differences in the intensity were discerned in this region. In the cerebellum, the weak expression was seen in the Purkinje cell and granule cell layers (Figs. 1A and 2A).

The expression pattern for M*trp*-3 in the brain was most evident among the four homologous molecules (Fig. 1B). The expression was most intense in the cerebellar Purkinje cells, while no significant expression was discerned in the granule cell and molecular layers of the cerebellum (Fig. 2B). Intense expression was also seen in the olfactory mitral cells and less intense expression was seen in the periglomerular cells, while no significant expression was discerned in the internal granular layer or the external plexiform layer. Distinct expression was detected discretely in large-sized intrinsic neurons scattered rather evenly throughout the caudate putamen, while no significant expression was seen in numerous medium-sized neurons in this brain region (Fig. 2D). Positive, though weak, expression was seen in the cerebral cortex with the layer V expressing it at a slightly higher level. The weak expression was also discerned in the septal nuclei and thalamic nuclei. No significant expression was detected in the hippocampal pyramidal and dentate granule cells. Moderate to weak expression signals were found in several brain stem nuclei, such as the dorsal cochlear nuclei, the inferior olive and the locus ceruleus.

The expression for M*trp*-4 was evident in the olfactory mitral and granule cells, the septal nuclei, the subiculum, and the hippocampal pyramidal and dentate granule cell layers (Fig. 1C). No significant expression was detected in other gray matters or white matters of the cerebrum and brain stem. In the cerebellum, weak expression was seen in the granule cell layer without distinct signals in the Purkinje cell layer (Fig. 2C).

The expression for M*trp*-6 was detected at moderate to intense levels in the dentate granule cell layer (Figs. 1D and 2E), while no significant expression was seen in the hippocampal pyramidal cell layer. The expression was weak in the layers II-III of the cerebral cortex. However, no significant expression was discerned in other gray matters or white matters of the brain.

Discussion

In general, an oligonucleotide sequence selected from the non-coding region of cDNA for a given molecule is regarded as a specific probe for in situ hybridization histochemistry. When only portions of the coding sequences of cDNAs for molecules forming a family are available, it is difficult to obtain specific probes,

because of higher risks of cross-hybridization to each other among the cDNAs. Admittedly, such a risk cannot be ruled out completely in case of the present probes, because of the same reason as above. However, consequent hybridization patterns for the individual M*trp*s were quite discrete. This strongly suggests that all four oligonucleotide probes are specific to the individual M*trp*s. In addition, no significant hybridization signals after incubation of brain sections with the sense control oligonucleotide probes also support the specificity of each probe itself. Further cDNA cloning of their full-length sequences and in situ hybridization histochemistry with several probes from different regions of their cDNAs will confirm our present findings retrospectively.

As stated in the introduction, there have been data strongly indicating that CCE channels are composed of subunits encoded in mammalian *trp*-homologous genes (Zhu et al. 1996). Sequence similarities between some of the hydrophobic regions of individual *trp*-homologous gene products and transmembrane segments of voltage-gated Ca^{2+} and Na^{+} channels suggest that a CCE channel may be a tetramer (Birnbaumer et al. 1996; Zhu et al. 1996). In the present study, highly discrete expression patterns of the four *trp*-homologous genes are disclosed in the brain of adult mice: The expression for M*trp*-1 is ubiquitous in the gray matter, while that for M*trp*-3, 4, and 6 is more or less confined to certain neuronal populations with marked differences in the intensity in given neuronal populations among these multiple genes. Assuming that the expression intensity at transcriptional levels represents that at translational levels, the present finding on discrete expression of M*trp*s suggests the possibility of both hetero- and homotetrameric compositions of the channels. For example, the cerebellar Purkinje cells express M*trp*-3 intensely and M*trp*-1 weakly, but not the other genes, which suggests that a homotetrameric M*trp*-3 is the dominant channel with a lesser possibility of heteromers composed of the two genes. On the other hand, the cerebellar granule cells express both M*trp*-1 and -4 weakly, which suggests a possibility of heteromers composed of both two gene products in addition to the individual homomers.

Transfection studies in cultured cells of the individual *trp*-homologous genes have shown diverse phenotypes in terms of Ca^{2+} selectivity and sensitivity to store depletion (Birnbaumer et al. 1996; Philipp et al. 1996; Zhu et al. 1996; Zitt et al. 1996, 1997). In this regard, it may be instructive to compare the present expression patterns for individual M*trp*s in the brain with those for molecules involved in the cascade of G-protein-, or tyrosine kinase-coupled phospholipase C/IP_3 receptor. A family of IP_3 receptor mRNA has now been identified and generated both by differential splicing and from different genes, and the type I IP_3 receptor is a major form in the brain (Danoff et al. 1991; Nakagawa et al. 1991; Ross et al. 1992). Although the IP_3 receptor mRNA is widely distributed throughout the gray matters in the adult brain, it is highly enriched in the cerebellar Purkinje cells without significant expression in the cerebellar granule

cells (Mailleux et al. 1992). This expression well corresponds to the dominant expression for M*trp*-3 in the cerebellum, which suggests that M*trp*-3 and IP_3 receptor may function in a concerted manner with each other, resulting in some specific Ca^{2+} signaling in the Purkinje cells.

The intense expression for M*trp*-3 in the striatal large-sized intrinsic neurons is also noticeable, because such a selective expression in the striatum has been found for phospholipase C β-4 (Tanaka and Kondo 1994). According to their report, the expression for phospholipase C β-4 is also selectively evident in the Purkinje cells, which is in accord with the expression pattern for M*trp*-3 in the present study. This concerted expression between the two molecules suggests their intimate functional linkage in the receptor-Ca^{2+} channel cascade, resulting in some specific Ca^{2+} signaling in these cells. In addition, selective expression for M*trp*-6 in the dentate granule cells and relatively dominant expression for M*trp*-4 in the septal nuclei may indicate the presence of some specific molecules involved in the cascade which play in a paired fashion with the two M*trp*s in these discrete loci.

Further localization of M*trp*s at protein levels by immunohistochemistry remains to be elucidated for more clear understanding of the functional significance of the molecular heterogeneity of *trp* homologues in the Ca^{2+}-signaling in the central nervous system.

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