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Immunohistochemical Study on the Distribution of Canonical Transient Receptor Potential Channels in Rat Cerebellum

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Abstract : Channels formed by the transient receptor potential (TRP) family of proteins have a variety of physiological functions. In the present study, we examined the localization of canonical transient receptor potential channels (TRPCs) in rat cerebellum.

Twelve adult (4 ~ 6 month old) Sprague-Dawley rats were examined in this study. We performed immunohistochemistry using specific antibodies against TRPCs to investigate the detailed and comparative distribution of six TRPCs in rat cerebellum.

There was a high density of TRPC1, TRPC3, TRPC4, TRPC5 and TRPC7, with a much lower density of TRPC6 in the rat cerebellar cortex. The somatodendritic Purkinje cell areas were intensely stained with antiTRPC3, TRPC4, TRPC5 or TRPC7 antibodies, whereas the staining intensity of TRPC6 was relatively low in the Purkinje cell bodies. In the cerebellar nuclei, the cell bodies of cerebellar output neurons showed moderate TRPC1, TRPC3, TRPC5 and TRPC7 immunoreactivities, while TRPC6 immunoreactivity was observed in the surrounding neuropil.

This study showing the differential localizations of TRPC channels in the cerebellum may provide useful data for the future investigations on the structural and functional properties of TRPCs.

Key words : Transient receptor potential (TRP) channels, TRPC, Rat, Cerebellum, Immunohistochemistry

Introduction

The transient receptor potential (TRP) superfamily of ion channels is widely expressed in eukaryotes. Of the six mammalian TRP subfamilies (TRPA, TRPC, TRPM, TRPML, TRPP and TRPV), canonical TRP (TRPC) family comprise seven different channels (TRPC1-7), which are Ca^{2+} influx channels and contribute importantly to certain Ca^{2+} signaling processes (Pedersen et al. 2005). On the basis of sequence homology and functional similarities, members of the mammalian TRPC family can be divided into four subfamilies: TRPC1, TRPC2, TRPC3/6/7 and TRPC4/5. With a few exceptions, the TRPC channels are broadly expressed, and a given cell type generally contains multiple TRPCs (Montell et al. 2002). TRPC channels are thought to be tetrameric and increasing evidence suggests heteromultimeric channel assembly. Thus, TRPC1 can form heteromers with TRPC4 and 5, and the TRPC subfamilies TRPC4/5, and TRPC3/6/7 can form heteromers among themselves, the current properties of which may be significantly different

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from those of the homotetramers (Strubing et al. 2001, Goel et al. 2002, Hofmann et al. 2002, Strubing et al. 2003, Shilling and Goel 2004). However, the channel subunit composition in vivo and the mechanisms underlying subunit assembly are still largely unknown.

Most TRPC subunits are prominently expressed in the CNS. TRPC5 has been implicated in regulating neurite outgrowth of rat hippocampal neurons (Greka et al. 2003, Bezzerides et al. 2004), whereas TRPC3 and TRPC6 seem to play a role in brain-derived neurotrophic factor (BDNF)-mediated protection in cerebellar granule cell cultures (Jia et al. 2007). The presence of TRPC1, 3, 6, and 7 in rat cerebellum had previously been reported (Mizuno et al. 1999, Goel et al. 2002, Kim et al. 2003, Li et al. 2005). Two previous studies did not find any TRPC4 or TRPC5 expression in rat cerebellum (Mizuno et al. 1999, Li et al. 2005). An essential role for both TRPC3 and TRPC6 was reported in the guidance of nerve growth cones of cultured cerebellar granule cells by BDNF but any substantial TRPC4 expression was not found in either cultured rat cerebellar granule cells or in whole rat cerebellum at the mRNA level (Li et al. 2005). However, little is known about the detailed distribution of TRPCs in rat cerebellum. Therefore, we performed immunohistochemistry using specific antibodies against TRPCs to investigate the detailed and comparative distribution of six TRPCs in rat cerebellum.

Materials and Methods

Twelve adult (4～6 month old) Sprague-Dawley rats were examined in this study. These animals were treated in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals' (NIH publication No. 80-23) revised in 1996. After the animals were anesthetized with pentobarbital (100 mg/kg, i.p.), they were perfused transcardially with cold phosphate buffered saline (PBS, 0.02 M, pH 7.4), and then with ice-cold 4% paraformaldehyde. Brains were cryoprotected in a series of cold sucrose solutions, and were cut at 40 μm in the coronal plane. Immunohistochemistry was performed in accordance with the free-floating method described earlier (Chung et al. 2006). Rabbit anti-TRPC1, TRPC3, TRPC4, TRPC5, TRPC6 and TRPC7 (AB-5446, AB5576, AB5812, AB5489, AB5574 and AB9326, Chemicon International, Inc., Temecula, CA) were used as primary antibodies with a dilution of 1 : 200, 1 : 400, 1 : 200, 1 : 200, 1 : 200 and 1 : 200, respectively. The sections were incubated using the free-floating method for 48～72 hours at 4°C in primary antiserum. After incubation, the sections were visualized according to the avidin-biotin complex (ABC) method, using an ABC kit (Vectastain™), then developed for peroxidase reactivity with 3,3′-diaminobenzidine (DAB). To

Table 1. The intensity of immunoreactivities for TRPC1, TRPC3, TRPC4, TRPC5, TRPC6 and TRPC7 in each region of rat cerebellum

Area	TRPC1	TRPC3	TRPC4	TRPC5	TRPC6	TRPC7
Cerebellar cortex						
Molecular layer	++	++	++	++	+	++
Purkinje cell layer	++	+++	+++	+++	+	+++
Granular layer	++	+++	++	+++	+	++
Deep cerebellar nuclei						
Nucleus medialis	++	++	++	++	+	++
Nucleus interpositus	++	++	++	++	+	++
Nucleus lateralis	++	++	++	++	+	++

Mean density is the sum of the gray values of all the pixels in the selection that was divided by the number of pixels within the selection. The intensity of immunoreactivities was classified into three categories according to the % of maximal mean density level (+, < 30% of maximal level; ++, < 50% of maximal level; +++, < 80% of maximal level).

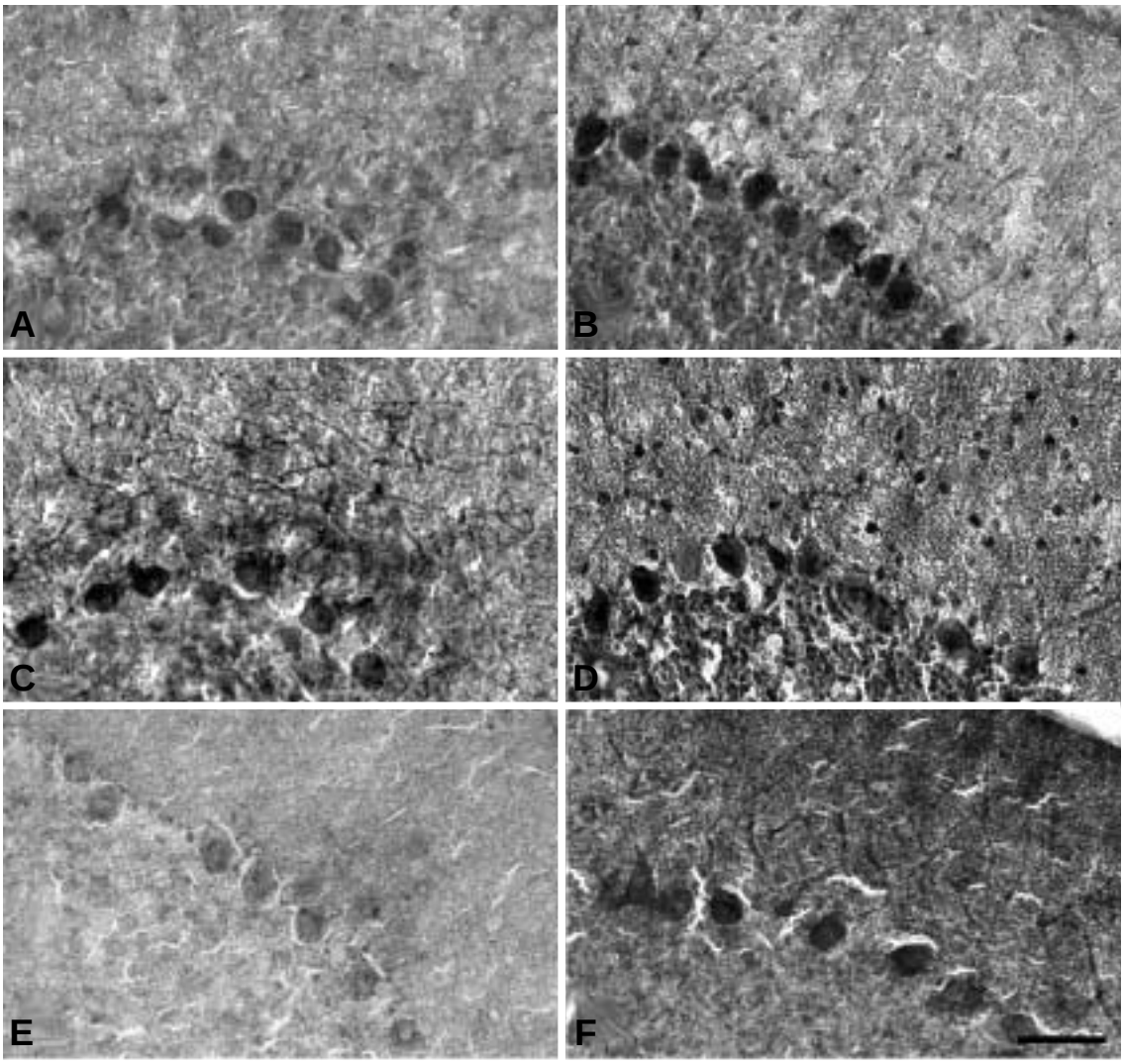

Fig. 1. Differential localization of TRPC1 (A), TRPC3 (B), TRPC4 (C), TRPC5 (D), TRPC6 (E) and TRPC7 (F) in the cerebellar cortex. Strong immunoreactivities for TRPC3, TRPC4, TRPC5 and TRPC7 were observed in the somatodendritic Purkinje cell areas, whereas TRPC1 immunoreactivity was detected in the Purkinje cell bodies, not in the dendrites (A-D, F). On the contrary, the staining intensity of TRPC6 was relatively low in the Purkinje cell bodies (E). Interestingly, TRPC5-immunoreactive nuclei were prominent in the molecular layer (D). M: molecular layer, P: Purkinje cell layer, G: granular layer. Scale bar=50 μm (A-F)

observe the stained cells, a microscope (Leica DM-4500B; Leica Microsystems, Germany) with a computer-driven digital camera (DFC320; Leica Microsystems, Germany) was used.

To show the specificity of the antibodies used in this study, a sample of sections was reacted without primary antiserum, and a different sample was exposed to a primary antiserum that had been preincubated with control antigen that was included free of charge with each antibody. The stock solution of the antigen

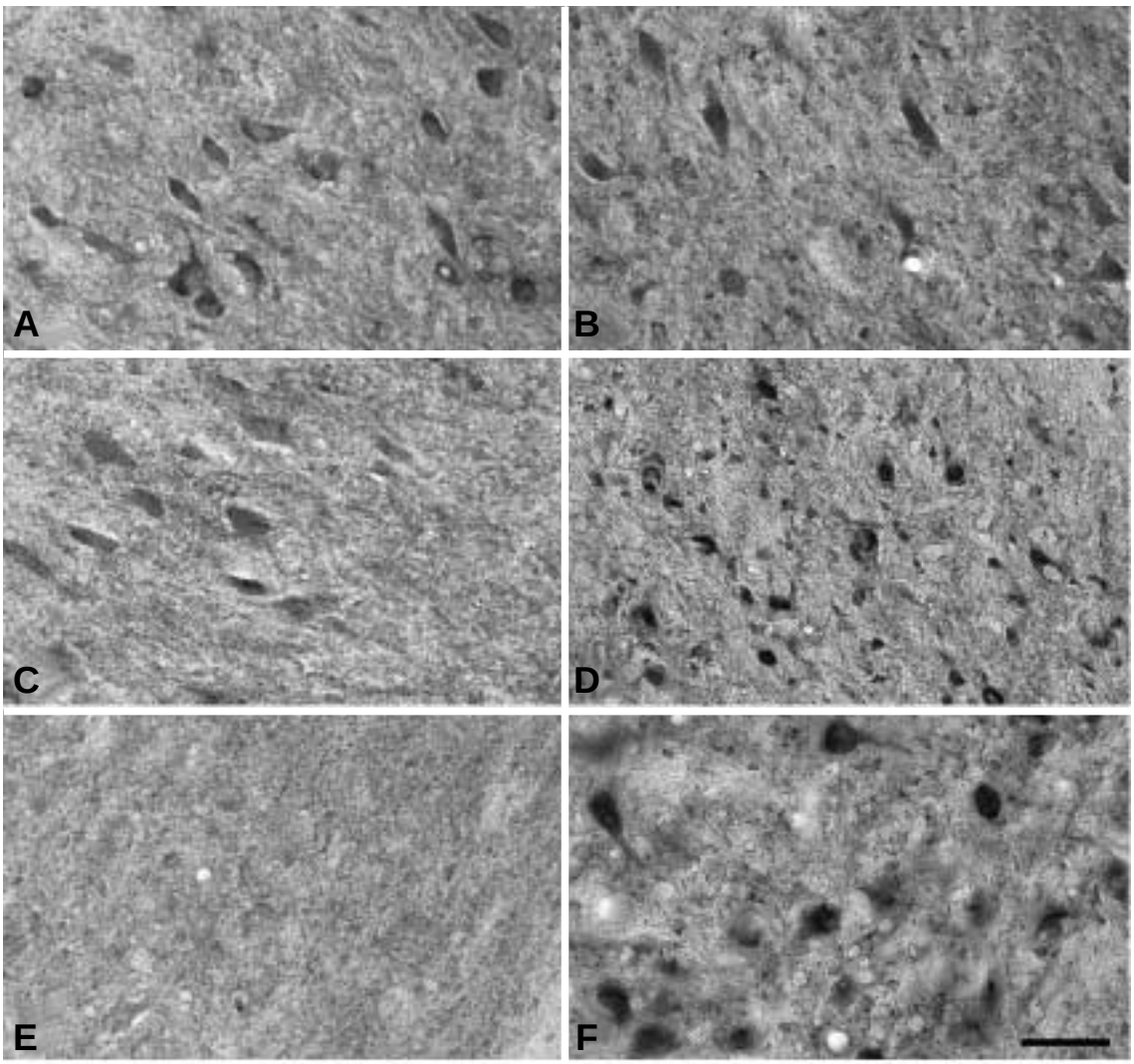

Fig. 2. Cellular localization of TRPC1 (A), TRPC3 (B), TRPC4 (C), TRPC5 (D), TRPC6 (E) and TRPC7 (F) in the cerebellar nuclei. The cell bodies of cerebellar output neurons showed moderate immunoreactivities for TRPC1, TRPC3, TRPC5 and TRPC7 in the nucleus medialis, with moderate to low staining intensity of TRPC4 in the cell bodies. On the contrary, weak immunoreactivity for TRPC6 was observed in the surrounding neuropil, not in the cell bodies. Scale bar=50 μm (A-F)

was made up using 100 μL of sterile deionized water. We preincubated 1 μg of peptide with 1 μg of antibody for one hour at room temperature. No sections from these samples exhibited any of the immunoreactivity described in this report. Visual assessment and densitometric measurement using Leica QWin Plus (Leica Microsystems Imaging Solutions, United Kingdom) determined the staining intensities (Table 1).

Results

There was a high density of TRPC1, TRPC3, TRPC4, TRPC5 and TRPC7, with a much lower density of TRPC6 in rat cerebellar cortex (Fig. 1, Table 1). The somatodendritic Purkinje cell areas were intensely stained with anti-TRPC3 (Fig. 1B), TRPC4 (Fig. 1C), TRPC5 (Fig. 1D) or TRPC7 antibodies (Fig. 1F), whereas TRPC1 immunoreactivity was detected in the Purkinje cell bodies, not in the dendrites (Fig. 1F). On the contrary, the staining intensity of TRPC6 was relatively low in the Purkinje cell bodies (Fig. 1E). Cerebellar granule cells were densely packed in the granular layer. These small cells are in fact the numerous cells in the brain. The cerebellar granule cells send ascending fibers into the cerebellar molecular layer, making *en passant* synapses with Purkinje cell dendrites. The strong immunoreactivities for TRPC3 and TRPC5 were observed in the granular layer (Fig. 1B, D), whereas TRPC6 immunoreactivity was low in the granule cells (Fig. 1E). Interestingly, TRPC5-immunoreactive nuclei were prominent in the molecular layer (Fig. 1D)

In the cerebellar nuclei, the cell bodies of cerebellar output neurons showed moderate TRPC1, TRPC3, TRPC5 and TRPC7 immunoreactivities in the nucleus medialis, interpositus and lateralis (Fig. 2A, B, D, F, Table 1). Moderate to low staining intensity of TRPC4 was found in the cell bodies, while weak immunoreactivity for TRPC6 was observed in the surrounding neuropil (Fig. 2C, E). In addition, immunoreactivities for six TRPC channels were also observed in the surrounding neuropil.

Discussion

Studies on regional localization patterns of TRPCs should provide helpful guidelines for correlating current types with particular channels. In the present study, we described the regional localization of six members of TRPC channels in rat cerebellum, for the first time. In several ways, our results were not consistent with the previous studies on the presence of TRPC mRNAs and proteins in the cerebellum (Mizuno et al. 1999, Goel et al. 2002, Kim et al. 2003, Li et al. 2005), although they were partly in accordance. We found that the somatodendritic Purkinje cell areas were intensely stained with anti-TRPC3, TRPC4, TRPC5 or TRPC7 antibodies (Fig. 1, Table 1), whereas Li et al. (2005) did not find TRPC4 expression in whole rat cerebellum. Li et al. (2005) also did not find appreciable TRPC5 or TRPC7 mRNA expression in either cerebellar granule cell cultures or whole cerebellum. TRPC7 protein expression had previously been shown to be present in rat cerebellum by Goel et al. (2002), which was confirmed in the present study. Mizuno et al. (1999) did also not find any TRPC4 or TRPC5 expression using cerebellar RNA isolated from rats at P21. The reason for these discrepancies is unclear but may result from the age of the rats they used.

TRPC channels form non-selective cation channels that can have a high permeability to Ca^{2+} (Montell et al. 2002), suggesting that Ca^{2+} permeability through TRPC channels is likely to increase cytosolic Ca^{2+} levels significantly. Recently, it was reported that activation of group I mGluRs gave rise to increases in intracellular Ca^{2+} concentration through TRPC channels in Purkinje cell soma and dendrites under conditions when intracellular Ca^{2+} stores were depleted and voltage-gated channels could not activate (Huang et al. 2007). Changes in intracellular Ca^{2+} concentration are known to be involved in such key processes as neuronal cell proliferation, differentiation, migration and cell death and hence it is tempting to speculate that the expression of the different TRPC subunits is required for proper cellular development. In the case of TRPC4 and TRPC6, their down-regulation may be required for initiation of differentiation and/or migration of

granule cells (TRPC4) or interneurons (TRPC6) whereas an increase in TRPC3 expression may be required for dendritic tree development in Purkinje cells (Huang et al. 2007). The fact that TRPC proteins undergo marked changes in expression after birth, and in a neuron-specific manner (Huang et al. 2007), suggests that this novel family of ion channels may be intimately involved in cerebellar development .

The present study showed the differential localization of TRPC channels in the cerebellum, for the first time. Jia et al. (2007) reported that TRPC3 and TRPC6 protected cerebellar granule neurons (CGNs) against serum deprivation-induced cell death in cultures and promoted CGN survival in rat brain (Jia et al. 2007). Another study showed that TRPC channels were essential for BDNF-mediated growth cone turning in CGNs (Li et al. 2005). Furthermore, BDNF triggers intracellular $[Ca^{2+}]_i$ elevation in the CGN growth cone through TRPC3 and 6 (Li et al. 2005). Because Ca^{2+} is known to mediate cell growth and survival, it is possible that Ca^{2+} influx through TRPC channels is required for the neuronal protection effect of neurotrophic factors, such as BDNF. Therefore, our study may be useful in the future investigations on the structural and functional properties of TRPCs in the cerebellum.

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흰쥐 소뇌에서 canonical transient receptor potential channel들의 분포에 관한 면역조직화학적 연구

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간추림 : Transient receptor potential (TRP) family에 속하는 채널들은 다양한 생리학적인 기능을 하는 것으로 알려져 있다. 본 연구에서는 흰쥐 소뇌에서 canonical TRP channels (TRPCs)의 분포에 관하여 조사하였다.

여섯 종류의 TRPC의 분포를 관찰하기 위해서 성숙(4 ~ 6개월령) Sprague-Dawley 흰쥐의 소뇌를 사용하였고, TRPC 각 타입에 따른 항체를 이용하여 면역조직화학을 시행하였다.

연구결과, 소뇌겉질에서는 TRPC1, TRPC3, TRPC4, TRPC5 및 TRPC7에 대한 염색 강도가 높은 반면, TRPC6에 대한 염색성은 상대적으로 낮은 경향을 나타냈다. 조롱박세포의 세포체에서 TRPC3, TRPC4, TRPC5 및 TRPC7에 대한 염색성이 높게 나타났고, TRPC6의 경우에는 낮은 면역염색성을 보였다. 소뇌핵에서는 TRPC1, TRPC3, TRPC5 및 TRPC7에 대한 중간 정도의 염색성이 소뇌핵 신경세포의 세포체에서 관찰된 반면, TRPC6에 대한 약한 염색성은 세포체에서는 발견되지 않았고 주위의 세포망에서만 관찰되었다.

소뇌에서 여섯 종류의 TRPC들의 서로 다른 분포들을 보여 준 본 연구결과는 앞으로 TRPC의 구조적, 기능적인 연구를 위한 유용한 자료로 제공될 수 있을 것이다.

찾아보기 낱말 : Transient receptor potential (TRP) channels, TRPC, 흰쥐, 소뇌, 면역조직화학

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