Nov. 2008 24(6) 575~580

神 经 解 剖 学 杂 志
(Chinese Journal of Neuroanatomy)

Co-localization of metabotropic glutamate receptor 2 with ASIC3 or TRPV1 in the dorsal root ganglion of rat①

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Abstract Metabotropic glutamate receptor (mGluR) 2/3 plays an important role on the nociceptive transmission from periphery to spinal cord. The previous studies demonstrated that mGluR2 can contribute to mechanical hypersensitivity and thermal hypersensitivity in rat. Therefore, in the present study, by using the immunofluorescence histochemical technique, we try to explore that whether mGluR2 is co-localized with acid-sensing ion channel 3 (ASIC3), a multi-modulator of mechanosensation, or transient receptor potential/vanilloid receptor subtype-1 (TRPV1), which responses for thermosensation in dorsal root ganglion (DRG). Morphological observations showed that mGluR2-immunoreactivity was mainly distributed in cellular plasma of neurons in DRG. The counting number results indicated that 35.84% of DRG neurons were mGluR2-immunoreactive (ir). On the other hand, 82.61% of mGluR2-ir cells were the small-diameter neurons (diameter: <30 μm), 5.79% of which were the medium-diameter neurons (diameter: 30 – 50 μm) and 11.59% of which was the large-diameter neurons (diameter: >50 μm). Furthermore, 42.45% and 79.78% of mGluR2-ir cells was individually co-localized with ASIC3- or TRPV1-ir in small-diameter neurons in the double-labeled immunofluorescence sections. The present results suggest that mGluR2 mainly exists in small neurons of the DRG, which are regarded as nociceptors consisting of Aδ- and C-fibers. While mGluR2 is highly co-localized with ASIC3 and TRPV1, implying their potential relationship in DRG may be involved in mechanical hypersensitivity and thermal hypersensitivity.

Key words metabotropic glutamate receptor 2, acid-sensing ion channel 3, transient receptor potential/vanilloid receptor subtype-1, dorsal root ganglion, rat

背根神经节内代谢型谷氨酸受体2与酸敏感性离子通道3和辣椒素受体1的共存研究

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摘 要 代谢型谷氨酸受体(mGluR)2/3在伤害性信息从外周向脊髓传递的过程中发挥着重要作用。以往研究证明在大鼠中mGluR2参与了机械性超敏和热超敏的形成,因此本研究拟采用免疫荧光组织化学染色技术揭示背根节(DRG)中mGluR2和酸敏感性离子通道3(ASIC3),一个多觉机械性感受器,或者和热伤害性感受器辣椒素受体(TRPV1)的共存情况。结果显示:mGluR2主要存在于DRG神经元的胞浆中。计数结果显示DRG中35.85%的神经元呈mGluR2免疫阳性。在这些阳性神经元中,82.61%为小细胞(直径小于30 μm);5.79%为中等细胞(直径为30~50 μm);11.59%为大细胞(直径大于50 μm)。进一步在免疫荧光双重标记切片上可观察到分别有42.45%和79.78%的小型mGluR2阳性神经元同时表达ASIC3或TRPV1免疫阳性。以上结果提示mGluR2主要存在于DRG中的小神经元中,这些神经元通常被认为是外周Aδ-或C-纤维传入的伤害性感觉神经元,在这类神经元中mGluR2与ASIC3或TRPV1均有大量共存,提示这些共存可能与机械性或热超敏的产生或者维持有着重要的联系。

① 国家自然科学基金(No. 30500154, 30871004)资助项目

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关键词 代谢型谷氨酸受体2 酸敏感性离子通道3 辣椒素受体1 背根节 大鼠

Sensory neurons in DRG play crucial roles in nociceptive apperceive and transmission, and DRG is one mainly location of mGluR2/3 production[1]. After synthesized in DRG, mGluR2/3 is transported to periphery and spinal cord, accordingly, peripheral inhibitory role[2-5] and spinal facilitatory role[6] of mGluR2/3 have reported by previous studies, these lines of evidence further reflect the importance of mGluR2/3 in DRG. However, whether the neurons containing mGluR2/3 also express other pain-related substances in DRG are not known.

ASIC3 is a key component of receptor complexes that detect some cutaneous touch and painful stimuli[7]. Loss of ASIC3 increases the sensitivity of mechanoreceptors detecting light touch and reduces the sensitivity of a mechanoreceptor responding to noxious pinch and decreases the response of acid- and noxious heat-sensitive nociceptors[7]. Furthermore, ASIC3 modulates the moderate- to high-intensity pain sensation[8]. It is notably that ASIC3 is especially expressed in DRG[9,10]. The multiple roles and peculiar location in DRG of ASIC3 combined the function of mGluR2/3 in mechanical hypersensitivity drive us to take both them into account.

TRPV1 is a non-selective cation channel and functions as a transducer of painful thermal stimuli *in vivo*[11]. It can be activated by vanilloid compounds, proton, or heat (> 43℃)[11,12]. Although TRPV1 knockout mice show normal responses to noxious mechanical stimuli but exhibit no vanilloid-induced pain behavior and are impaired in the detection of painful heat, and show little thermal hypersensitivity in the setting of inflammation[13]. TRPV1 is expressed in both the peptide-containing and the nonpeptide primary afferent neurons in DRG neurons[11,12]. Therefore, considering of the functions of TRPV1 on normal thermal sensation and thermal hypersensitivity combine with the role of mGluR2/3 in the thermal hypersensitivity, we are going to detect whether mGluR2 is co-localized with TRPV1 in DRG neurons.

Materials and Methods

1. Animals and tissue preparation

Adult male SD rats (body weight 200 g) from the Animal Centre of Fourth Military Medical University were employed in the present study. Four of rats were anesthetized deeply by intraperitoneal injection of sodium pentobarbital (40 mg/kg body weight). The anesthetized animals were firstly perfused transcardially with 0.01 mol/L PBS and followed with 0.1 mol/L PB (pH7.3) containing 4% paraformaldehyde and 0.2% picric acid. Afterwards, L4 - L5 DRGs were removed and cut into 10 μm thick sections longitudinally on a cryostat. The sections were consecutively collected and divided into three sets. The first and second sets of the sections were detected double-labeled results of mGluR2/ASIC3 or mGluR2/TRPV1. The third set of the sections were stained with 0.1% cresyl violet.

2. Immunofluorescence histochemical staining

For immunofluorescence histochemical staining, the sections were incubated with a mouse-anti mGluR2 antibody (1:500, GeneTex) and rabbit-anti ASIC3 (1:200, Sigma) or rabbit-anti TRPV1 (1:500, Sigma) 72 h at 4℃. Subsequently, the sections were incubated with biotinylated donkey-anti mouse IgG (1:200, Chemicon) and FITC labeled donkey anti-rabbit IgG (1:200, Chemicon) for 4 h. Lastly, the sections were incubated with Cy3 combined avidin complex (1:1000, Jacksons) for 1 h. In the control experiments, the primary antibodies against mGluR2, ASIC3 or TRPV1 were replaced with normal IgG and in these control experiments no mGluR2-ir and ASIC3-ir or TRPV1-ir were detected.

3. Quantitative analysis

The images were captured by using a confocal laser-scanning microscope (FV1000; Olympus) and an light microscope (AH-3, Olympus). Cell body diameters of DRG neurons were measured under the light microscope by using an imaging system equipped with an

eyepiece micrometer (Q500MC; Leica) and the confocal laser-scanning microscope. The average diameters of fluorescently labeled and cresyl violet-stained DRG neurons were calculated by averaging the major diameter with the minor diameter; the major and minor diameters were the longest and shortest axes through the nucleus, respectively. Eight of the biggest sections of each DRG were selected to count and the averaged count number for each section was used as the mean. Percentages of single-labeled neurons were calculated by dividing the numbers of single-labeled neurons by the total cresyl violet-stained DRG cell number × 100. Percentages of double-labeled neurons were calculated by dividing numbers of double-labeled neurons by numbers of single-labeled neurons × 100. These data are presented as the percentage of the total and the labeled population in each section.

Results

1. Single-labeled for mGluR2

Under the confocal laser-scanning microscope, mGluR2-ir was mainly observed in cellular plasma of soma in small-diameter neurons in DRG. Few large- and medium-diameter neurons were detected to be mGluR2-ir. Combined with Nissl staining, the count numbers displayed that 35.84% (138/385) of DRG cells were mGluR2-ir. Among them, 82.61% (114/138) of mGluR2-ir cells were small-diameter neurons (diameter: < 30 μm), 5.79% (8/138) of which were medium-diameter neurons (diameter: 30 - 50 μm) and 11.59% (16/138) of which were large-diameter neurons (diameter: > 50 μm) (Figs. 1A, 1D).

2. Co-localization of mGluR2 and ASIC3

Many previous studies proof that mGluR2/3 is involved in modulation or facilitation of mechanical hypersensitivity[3,4,14,15]. To explore the likely mechanism, we studied the co-localization of mGluR2 and ASIC3 in DRG. ASIC3-ir cells were mainly observed in cellular plasma in the small-diameter neurons in DRG, Also, few large-diameter neurons and medium-diameter neurons were ASIC3-ir (Figs. 2B,2E). The results of counting cell numbers displayed that 22.08% (85/385) of DRG neurons were ASIC3-ir. Among them, 75.29% (64/85) expressing ASIC3-ir cells were small-diameter neurons, however, only 9.41% (8/85) and 15.29% (13/85) expressing ASIC3-ir cells were medium- and large-diameter cells, respectively. Furthermore, 42.45% expressing mGluR2-ir in small-diameter cells were ASIC3- ir (Figs. 2C, 2F; Table 1). Although the percentages of co-localization of mGluR2 and ASIC3 were largely higher in large- and medium-diameter cells than in small-diameter cells, the total number of co-localization of mGluR2-ir and ASIC3-ir individually in large- and medium-diameter cells should be taken into account (Table 1).

Table1 Percentages of co-localization of mGluR2-ir with ASIC3-ir neurons

	Single-labeled neurons		Double-labeled neurons	Percentage of double-labeled	Percentage of double-labeled
	mGluR2	ASIC3	mGluR2/ASIC3	neurons in mGluR2 neurons (%)	neurons in ASIC3 neurons (%)
small-diameter	61.83 ± 8.98	19.67 ± 5.35	45.00 ± 3.66	42.45	70.31
medium-diameter	2.67 ± 1.51	1.33 ± 1.11	6.83 ± 1.01	71.89	83.70
large-diameter	7.5 ± 4.36	5.00 ± 1.41	8.83 ± 2.46	54.07	63.85

Table2 Percentages of co-localization of mGluR2-ir with TRPV1-ir neurons

	Single-labeled neurons		Double-labeled neurons	Percentage of double-labeled	Percentage of double-labeled
	mGluR2	TRPV1	mGluR2/TRPV1	neurons in mGluR2 neurons (%)	neurons in TRPV1 neurons (%)
small-diameter	25.00 ± 4.04	33.17 ± 7.12	97.33 ± 4.24	79.78	74.58
medium-diameter	4.17 ± 1.45	0.33 ± 0.61	3.67 ± 1.17	46.87	91.75
large-diameter	8.00 ± 1.32	0.33 ± 0.46	7.67 ± 1.78	48.95	95.87

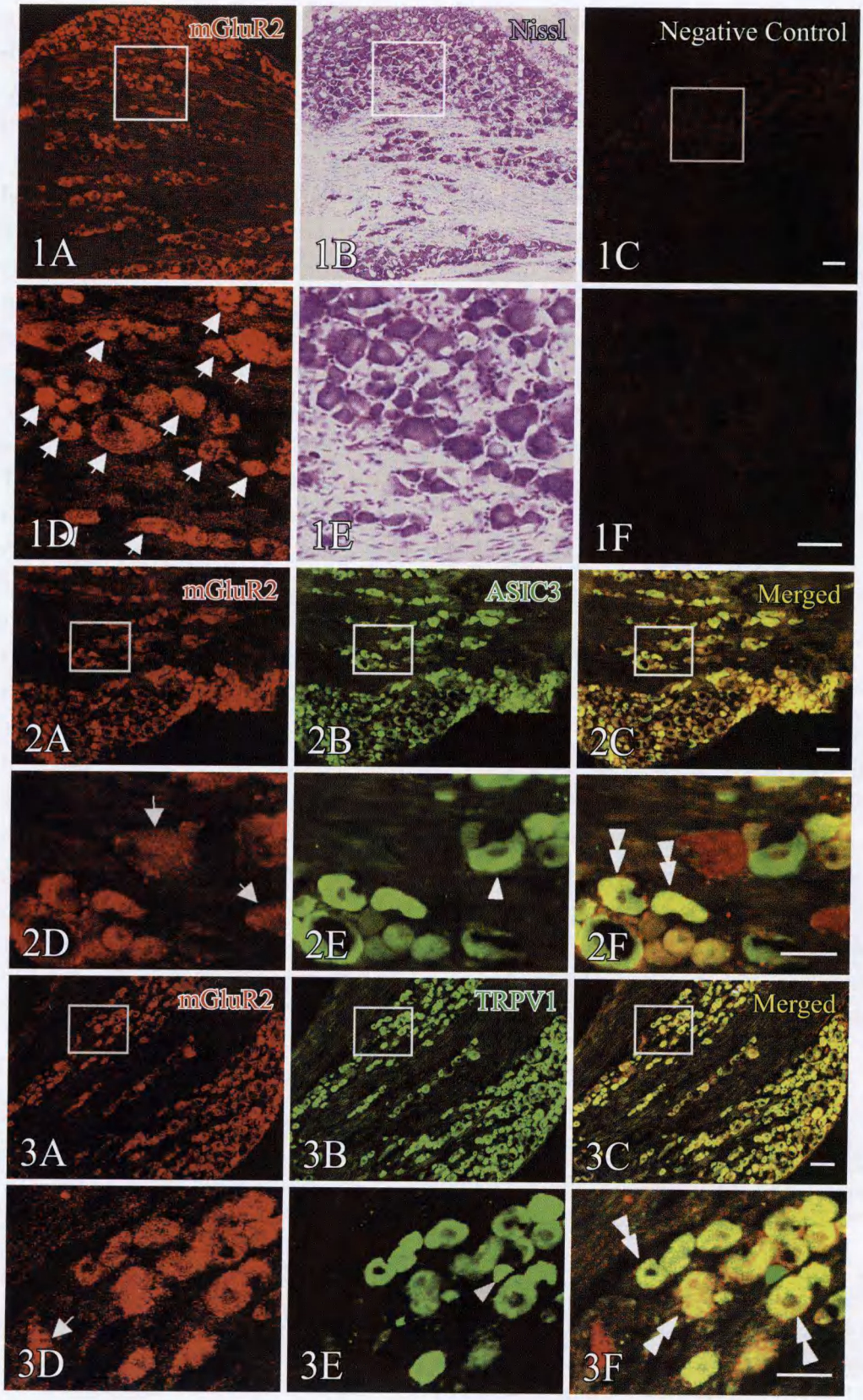

Fig. 1 Microphotographs showing immunofluoroscent histochemical staining of single-labeled mGluR2 (Figs. 1A,1C,1D,1F), double-labeled in co-localization of mGluR2 with ASIC3 (Figs. 2A – 2F) or TRPV1 (Figs. 3A – 3F) and Nissl staining (Figs. 1B,1E) in L5 DRG of rats. The framed areas in 1A – 1C, 2A – 2C, 3A – 3C are magnified in 1D – 1F, 2D – 2F, 3D – 3F, respectively. Immunoreactivities for mGluR2, ASIC3 or TRPV1 are visualized with Cy3 (red) and FITC (green), respectively. Arrows, arrowheads and double arrowheads in 2D – 2F, 3D – 3F indicate the neurons singlely labeled with mGluR2 (red), singlely labeled with ASIC3 or TRPV1 (green), and double labeled with mGluR2/ASIC3 or mGluR2/TRPV1 (yellow), respectively. Bar = 100 μm in 1A – 1C, 2A – 2C, 3A – 3C; 50 μm in 1D – 1F, 2D – 2F, 3D – 3F.

3. Co-localization of mGluR2 and TRPV1

Participation of mGluR2 in thermal nociception has been reported, especially in peripheral site[2]. Hereby, we also detected whether mGluR2 is co-localized with TRPV1. Similar to the previous study that TRPV1-ir was distributed in cellular plasma of soma in the small-diameter neurons in DRG (Figs. 3B, 3E). Very few large- or medium-diameter neurons were TRPV1-ir. The results of counting cell number showed that 36.62% (141/385) of DRG cells were expressed TRPV1-ir. Among them, 92.20% (130/141) expressing TRPV1-ir cells were small-diameter cells. It is notably that 79.78% expressing mGluR2-ir were TRPV1-ir in small-diameter neuron, which is dramatically higher percentage than that of mGluR2 co-localization with ASIC3 (Figs. 3C, 3F; Table2).

Discussion

Differently from previous reports in which mGluR2/3 positive results were shown, in the present study mGluR2 subtype was investigated to indicate the percentage of DRG neurons that was single-labeled for mGluR2. Similar to the previous research[1,16] that in the present study mGluR2 is mainly distributed in small-diameter neurons, and also few large- and medium-diameter neurons containing mGluR2. The proportion of co-localization of mGluR2 and ASIC3 is almost half of mGluR2-containing cells and 70% of ASIC3-containing cells in small-diameter neurons. The large-diameter neurons-containing both mGluR2 and ASIC3 should be notebly because ASIC3-ir small and large neurons are responsible for hyperalgesia induced by spinal nerve ligation[17]. The proportion of co-localization of mGluR2 and TRPV1 is almost 80% of mGluR2-containing cells and 75% TRPV1-containing cells in small-diameter neurons. These suggesting the potential involvements of mGluR2 in mechanical hypersensitivity and thermal hypersensitivity are mediated by ASIC3 and TRPV1 individually in DRG

1. Functional relevance of co-expressing mGluR2 and ASIC3 in small-diameter neurons

The involvement of mGluR2/3 in mechanical hypersensitivity has been well-documented. Peripheral administrations of agonists of mGluR2/3 produce a reversal of capsaicin-, PGE2-, carrageenan- and inflammatory soup-induced respectively mechanical sensitization and modulate endogenous anti-allodynia effects[3-5,14]. Although there is evidence that this inhibitory effect might be mediated via C-mechanoheat fiber[5], the other primary afferent fiber, such C-mechanonociceptors and Aδ-fiber, even Aβ-fiber are not excluded to the involvement of the mechanical hypersensitization. In periphery, ASIC3 exists in some, but not all, free nerve ending (which belongs to myelinated or unmyelinated nociceptors) running in the epidermal layer of the mouse paw pad[7]. In addition, ASIC3 mRNA is expressed in 50% of SP-positive and 43% of IB4-positive cells and complete Freund's adjuvant-induced inflammation increases ASIC3 transcript in small DRG neurons[10]. Furthermore, there is a significant increase in the response frequency for the ASIC3-/- mice 3 h after carrageenan-induced inflammation[7] and dominant-negative ASIC3 mice display lower threshold force to von-Frey filament stimuli and greater mechanical hypersensitivity after zymosan injection[18]. Therefore, ASIC3 as a proton-gated cation channel, combined the previous evidence with the present co-localization of mGluR2 and ASIC3, we postulate that the involvement of mGluR2/3 in mechanical hypersensitivity potentially mediated by activation of ASIC3 in DRG.

2. Functional relevance of co-expressing mGluR2 and TRPV1 in small-diameter neurons

The involvement of mGluR2/3 in thermal hypersensitivity has been mentioned in the previous researches. Peripheral activation of mGluR2/3 agonists blocks thermal hyperalgesia induced by inflammatory soup, carrageenan, PGE2, individually[2,5,14]. Electrophysiology evidence indicates that mGluR2/3 blocks PGE2 enhancement of capsaicin receptor function via a picrotoxin-sensitive G-protein and inhibits adenylyl cyclase[2]. TRPV1 as the capsaicin receptor, can be directly activated by >42℃ heat stimuli[11,13]. It is specifically expressed in sensory neurons which are both peptide- and nonpeptide-containing[11,12]. TRPV1-deficent mice show impaired behavioral responses to noxious thermal stimuli and reduced thermal hyperalgesia after complete Freund's adjuvant injection[13]. Therefore, combining

with the high proportion of co-localization of mGluR2 and TRPV1, it is reasonable to deduce that the involvement of mGluR2/3 in thermal hypersensitivity potentially related to activation of TRPV1 in DRG.

Taken all together, our present study supplies the first evidence about co-localizations of mGluR2 with ASIC3 or TRPV1. The high proportion of co-localization of mGluR2 with ASIC3 and TRPV1 in small-diameter neurons suggest the probably mechanism on the contributions of mGluR2/3 in mechanical and thermal hyperalgesia in primary afferent level.

(Received Mar. 29, 2008)

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