Ascending pain-related pathways to the anterior cingulate cortex: retrograde transneuronal tracing with rabies virus

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Abstract

The organization of ascending pain-related projections to the anterior cingulate cortex was examined in the rat by means of retrograde transneuronal labeling with rabies virus. Three days after the virus injection into the anterior cingulate cortex, a large number of labeled neurons were observed in the thalamus (i.e., the parafascicular and centrolateral nuclei) and the limbic-related structures (i.e., the orbital cortex and the basolateral amygdaloid nucleus). The neuronal labeling in these regions became much more intense four days after the virus injection. At the four-day post injection period, the submedial nucleus of the thalamus also contained many labeled neurons. In addition, retrograde labeling simultaneously occurred in the brainstem regions, such as the parabrachial nuclei and the caudal division of the spinal trigeminal nucleus. At the same timing or a little later stage, labeled neurons were seen in the trigeminal ganglion. The present results suggest that the ascending pathways linking the trigeminal ganglion and the anterior cingulate cortex may play important roles in the processing of noxious information in association with the emotional aspect of pain.

Key words: pain, tract-tracing, retrograde transsynaptic labeling, TRG

INTRODUCTION

It has been reported that the anterior cingulate cortex (ACCx) is an important area for pain perception^{1,2,4,5,6,9,10,15,16,23,26,27,29–32}. Neurons in the ACCx respond to noxious stimuli of the cutaneous receptive field, and these nociceptive neurons receive inputs from wide areas of the body^{20–22,25,34}. Previous electrophysiological

studies^{28,34} have reported that the nociceptive neurons in the ACCx can be classified into nociception-specific neurons that respond exclusively to noxious stimuli and noxious-tap neurons that respond not only to noxious stimuli, but also to non-noxious tap stimuli of the receptive field. These two types of neurons are also distributed in the parafascicular nucleus of the medial thalamic region. Ryu and Willis⁸ have reported

that the nociceptive neurons in the parafascicular nucleus of the medial thalamic region respond to both noxious and non-noxious tap stimuli of receptive fields. These thalamic neurons have electrophysiologically been shown to send projection fibers to the ACCx²⁸⁾.

It has been considered that the ascending pain-related pathways from the peripheral ganglions are divided into medial and lateral systems. These two systems are likely to participate in different functions for pain perception. The lateral system is involved in the discrimination of pain, whereas the medial system is in the emotional and affective aspects of pain. While the primary somatosensory cortex is regarded as a component of the lateral system, the ACCx is as a component of the medial system^{11,14,19)}. Previous anatomical studies have also reported that the ACCx is strongly connected with many limbic-related regions, such as the limbic cortical areas and the amygdala^{3,25,33)}. However, the precise pattern of multi synaptic projections from the trigeminal ganglion (TRG) to the ACCx remains unclear. Using retrograde trans-neuronal labeling with rabies virus, we therefore investigated the organization of such ascending pain-related pathways in the rat.

METHODS

Animals used

Adult male SD rats were used in this study. The experimental protocol was approved by the Animal Care and Use Committee of the Tokyo Metropolitan Institute for Neuroscience, and all experiments were conducted according to the Guideline for the Care and Use of Animals (Tokyo Metropolitan Institute for Neuroscience). The animals were treated according to the guidelines of the International Association for the Study of Pain³⁵⁾.

Virus

The CVS-11 strain of rabies virus (1.4×10^8 pfu) was used in the present study. The virus

was derived from the Center of Disease Control (USA) and amplified by Dr. Satoshi Inoue (National Institute of Infectious Diseases, Japan). A virus suspension was kept in small aliquots at -80°C . Just prior to each injection experiment, each aliquot was thawed in a safety cabinet.

Virus injection

The rats were anesthetized with sodium pentobarbital (50 mg/kg, ip). The animals were fixed in a stereotaxic frame. The skull was partially removed, and the frontal lobe was exposed. A total of 1–3 μl of the virus suspension was injected into multiple portions of the ACCx (1 μl per injection) through a 10- μl Hamilton micro-syringe.

Histological procedures

After the survival periods of 3–5 days, the rats were anesthetized deeply with sodium pentobarbital (80 mg/kg, ip) and perfused trans-cardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4), followed by 8% formalin and 15% picric acid in 0.1 M phosphate buffer (PB, pH 7.4). The removed brains were post-fixed in the same fixative overnight, and then saturated with 30% sucrose in PB. The brains were cut into 50- μm -thick coronal sections on a freezing microtome. Every other section was processed for rabies virus immunohistochemistry by a standard avidin-biotin-peroxidase complex (ABC) method. The rabbit anti-rabies antibody was a generous gift from Dr. Takuya Iwasaki (National Institute of Infectious Diseases and Nagasaki University). After washes in PBS, the sections were incubated in PBS containing 1% skim milk for two hours, and then overnight in PBS containing the primary antibody (diluted at 1:10,000), 0.3% Triton X-100, and 1% normal goat serum. Subsequently, the sections were incubated with biotinylated goat anti-rabbit IgG antibody (diluted at 1:200, Vector) and processed with the ABC kit (ABC Elite, Vector). Finally, the sections were reacted with nickel-enhanced diaminobenzidine

(0.04% DAB, 0.04% nickel chloride) and 0.002% hydrogen peroxide. The sections were mounted on gelatin-coated glass slides. The adjacent series of sections were mounted on gelatin-coated glass slides and Nissl-stained with 1% Neutral red or Cresyl violet.

Safety issues

The entire experiments were carried out in a special laboratory (biosafety level 2) designated for in vivo virus experiments. Throughout the experiments, the rats were kept in individual cages which were placed in the laboratory. To avoid accidental infection with the virus, all experimenters received immunization prior to the experiments and wore protective clothes in each experimental procedure, including virus injection, feeding, and perfusion. All equipments were disinfected with 70% ethanol after each experimental session. Wastes were autoclaved prior to disposal.

RESULTS AND DISCUSSION

A total of 1–3 μ l of a rabies virus suspension was injected into multiple portions of the ACCx. A large number of labeled neurons were observed around the injection sites (**Fig. 1**). Three days after the virus injection, clusters of neurons were labeled in the thalamus, including the parafascicular and centrolateral nuclei, and in the limbic-related structures, including the orbital cortex and the basolateral amygdaloid nucleus. The number of labeled neurons in these regions was much greater four days after the injection. At the same timing, the submedial nucleus of the thalamus also contained many labeled neurons. Moreover neuronal labeling simultaneously occurred in the brainstem regions, such as the parabrachial nuclei (especially the lateral parabrachial nucleus) and the caudal division of the spinal trigeminal nucleus. In the caudal spinal trigeminal nucleus, not only large neurons in lamina I, but also small neurons with round cell bodies in laminae III–IV were labeled (**Fig. 2**).

Fig. 1 Injection site of rabies virus in the ACCx. Many neurons were labeled around the injection site.

Fig. 2 Neuronal labeling in the caudal division of the spinal trigeminal nucleus (Vc). Clusters of labeled neurons were located in both the superficial and deep layers of the Vc.

Accumulated evidence to date clearly indicates that axon terminals of the primary afferents are distributed in both lamina I and laminae III–V²⁴. This supports our findings on the arrangement of labeled neurons in the caudal spinal trigeminal nucleus.

Fig. 3 Neuronal labeling in the TRG.

Many TRG neurons were labeled in the 2nd and 3rd branches of the trigeminal nerve.

At the four-day post injection period or a little later stage, labeled neurons were seen in the TRG. The longer the post injection period was, the more TRG neurons were labeled. As shown in **Figure 3**, the vast majority of the labeled neurons were middle (A δ -range neurons) to large (A β -range neurons). Only rarely were small neurons (C-range neurons) labeled in the TRG. This suggests that TRG neurons with A δ or A β fibers might be dominantly involved in pain perception mediated by the ACCx.

The likely patterns of ascending projections from the TRG to the ACCx are summarized in **Figure 4**. Together with previous anatomical data^{17,18}, the time course of transneuronal labeling in the present study indicates that neurons in the caudal spinal trigeminal nucleus may constitute the second-order or third-order neurons, while those in the TRG constitute the third-order or fourth-order neurons. In this diagram, we have dealt primarily with the pathways consisting of the brain regions that displayed strong retrograde labeling with rabies virus injected into the ACCx. The present results suggest that the ascending pathways linking the TRG and the ACCx that are relevant to the medial system may play important roles in the processing of noxious

Fig. 4 Schematic diagram showing the patterns of ascending projections from the TRG to the ACCx.

BL: basolateral amygdaloid nucleus; OrCx: orbital cortex; Pb: parabrachial nuclei; Pf: parafascicular nucleus of the thalamus; Sm: submedial nucleus of the thalamus.

information in association with the emotional aspect of pain.

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