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Development of a novel photoaffinity probe for labeling nocistatin receptor

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ABSTRACT

Nocistatin (NST) is a neuropeptide produced from the same precursor protein of opioid peptide nociceptin/orphanin FQ, and it is involved in a broad range of central functions including pain transmission in the nervous system. However, the composition and structure of the receptor(s) for NST remain unclear. Here, we developed NST photoaffinity probe to identify NST receptor. The NST photoaffinity probe contains an azide moiety for the tagging of the binding protein as well as biotin for protein detection. Intrathecal administration of a NST photoaffinity probe, biotin-$(AC_5)_2$-[Y^6,azF^{14}]bNST, inhibited the nociceptin/orphanin FQ-evoked tactile pain allodynia in a manner similar to that of NST. The biotin-$(AC_5)_2$-[Y^6,azF^{14}]bNST-binding proteins were primarily localized in the gray matter of the spinal cord. After photo-crosslinking of the protein complex with biotin-$(AC_5)_2$-[Y^6,azF^{14}]bNST, two dominant binding protein bands were observed at 58 and 64 kDa. Thus, biotin-$(AC_5)_2$-[Y^6,azF^{14}]bNST has pharmacological activity and is useful for characterizing the NST receptor.

1. Introduction

Nocistatin (NST) is a neuropeptide produced from the same precursor protein of nociceptin/orphanin FQ (N/OFQ), and it is involved in a broad range of central functions including pain, anxiety, learning, memory, and feeding [1–3]. N/OFQ is an opioid-like neuropeptide, and it binds to N/OFQ receptor (NOP), a member of the G protein-coupled receptor superfamily, in a manner highly homologous with that of opioid receptors [4–7]. NST has opposite effects on various central functions evoked by N/OFQ [1–3]. We reported that intrathecal (*i.t.*) administration of N/OFQ induces touch-evoked allodynia and heat-evoked hyperalgesia [1], and that the *i.t.* administration of NST blocks the N/OFQ-induced nociceptive transmission [6]. We previously identified a 4-nitrophenylphosphtase domain and non-neuronal SNAP25-like protein homolog 1 (NIPSNAP1) as a protein that interacted with NST [8]. The inhibition of N/OFQ-evoked allodynia by NST was lacking in NIPSNAP1-deficient mice. In contrast, several biological activities of NST suggest that NST may bind other proteins in addition to NIPSNAP1 such as G protein-coupled receptor. NST induces nociception through PTX-sensitive $G_{i/o}$ [9], suppresses inhibitory neurotransmission such as glycine in the rat spinal cord [10], and inhibits 5-hydroxytryptamine release in the mouse neocortex synaptosome [11]. NST has been reported to depolarize the projection neurons of periaqueductal gray through PTX-insensitive $G_{q/11}$ [12,13]. Although the identification of a novel NST receptor will contribute to a better understanding of the biological responses evoked by NST, the composition and structure of the receptor(s) for NST remain largely unclear.

Photoaffinity labeling forms a covalent bond between a ligand and a receptor by a photochemical reaction. Photoaffinity labeling has been used in variety of ways to identify the receptors for orphan ligands and to determine the ligand-binding site on a receptor [14,15]. Therefore, we used a photoaffinity probe for NST, biotin-$(AC_5)_2$-[Y^6,azF^{14}]bNST, and performed a photolabeling experiment. This study shows the in situ labeling of the target protein for NST.

2. Materials and methods

2.1. Animals

This study was conducted with the approval of the Animal Care Committees of Osaka Institute of Technology and Osaka Medical

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College. Animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the ethical guidelines of the Ethics Committee of the International Association for the Study of Pain [16]. Five-week-old male ddY mice were purchased from the Japan SLC (Hamamatsu, Japan). Behavioral analyses and other experiments were used 5-week-old mice (330 mice) and 5-8-week-old mice (12 mice), respectively.

2.2. Peptides and photoaffinity probes

Bovine NST (bNST) and N/OFQ were provided by Peptide Institute, Inc. (Osaka, Japan). Tyrosine substitution of bovine NST ([Y]bNST), NST incorporated with 4-azido-L-phenylalanine (azF) for the photoaffinity probe, and biotinylated-NST photoaffinity probes were kindly provided by Nippon Shinyaku Co. Ltd. (Kyoto, Japan).

2.3. Allodynia analysis

The allodynia analysis was carried out as described previously [6]. Each mouse was placed in an individual $13 \times 8.5 \times 13$ cm Plexiglas enclosure with wood chips on the floor and acclimated to the testing environment for 1 h. After *i.t.* injection of peptides (5 μl) into the subarachnoid space between the L5 and L6 vertebrae, tactile allodynia was assayed by light stroking with a paintbrush. The allodynia response was ranked as follows: 0, no response; 1, mild squeaking with attempts to move away from the stroking probe; or 2, vigorous squeaking evoked by the stroking probe, biting at the probe, or strong efforts to escape. The maximum possible scores of 6 mice were $2 \times 6 = 12$. To evaluate the effects of NST-derived peptides and photoaffinity probes on allodynia, we assessed the effect at maximal score of allodynia obtained 10 min after *i.t.* injection of 50 pg N/OFQ.

2.4. Spinal cord slice preparation

Spinal cord slices were prepared as described previously [17,18]. In brief, mice were deeply anaesthetized with isoflurane and sodium pentobarbital (50–75 mg/kg, intraperitoneal). The mice were intracardially perfused with phosphate-buffered saline (PBS), and the spinal cords were removed. The spinal cords were embedded in Tissue-Tek O.C.T. Compound, immediately frozen on a mixture of ethanol and dry ice, and stored at −80 °C until sectioning. Tissues sections (20-μm thick) were prepared using a cryostat under a −20 °C setting, and transfer six sections were transferred to a glass slide. The glass slides were dried inside the cryostat for 1–2 min, and stored at −80 °C.

2.5. Fluorescent binding analysis

The spinal slices were incubated at room temperature for 30 min in the PBS containing 0.3% protease-free bovine serum albumin and 0.2% Triton X-100 (PBS-AT buffer), and then were incubated with biotinylated-NST photoaffinity probes at 4 °C for the indicated time. After the washing with ice-cold PBS-AT buffer, the slices were incubated with Alexa488-conjugated streptavidin (1:500, Thermo Fisher Scientific, MA, USA) for 2 h at room temperature. The slices were washed three times with ice-cold PBS-AT buffer and once with ice-cold PBS, and then were mounted in Vectashield mounting medium (Vector Laboratories, CA, USA). Digital images were captured with a Nikon A1 confocal laser microscope equipped with an appropriate filter (Nikon, Tokyo, Japan). Control and experimental tissues were concurrently analyzed, and the images were captured using the same conditions. The region of interest was set to the spinal dorsal horn, and image quantification was carried out using ImageJ software. The fluorescence intensity (arbitrary units) was subtracted from the background intensity of the area outside the tissue section, and the fluorescence intensity of experimental tissue was normalized by that of control tissue. The experiments were carried out three times, and similar results were obtained.

2.6. Photoaffinity labeling and SDS-PAGE

The spinal slices were incubated at room temperature for 30 min in the PBS-AT buffer, and then were incubated with the biotinylated-NST photoaffinity probe at 4 °C for 24 h. The slices were photolyzed by placing a UV lamp (254-nm wavelength) at a distance of 1-cm for 3 min at room temperature. After washing with ice-cold PBS-AT buffer, the slices were suspended with 100 μl of lysis buffer (10 mM Tris-HCl pH 7.4, 0.5% SDS) and then centrifuged at 20,000 g for 5 min. The resultant supernatant was determined by using a DC protein assay (Bio-Rad). The samples (4 μg) were heated at 95 °C for 3 min, and were loaded into the SDS-PAGE gel, electrophoresed, and transferred electrophoretically to a polyvinylidene difluoride membrane Immobilon-P (Merck Millipore, MA, USA). After having been blocked for 1 h at room temperature

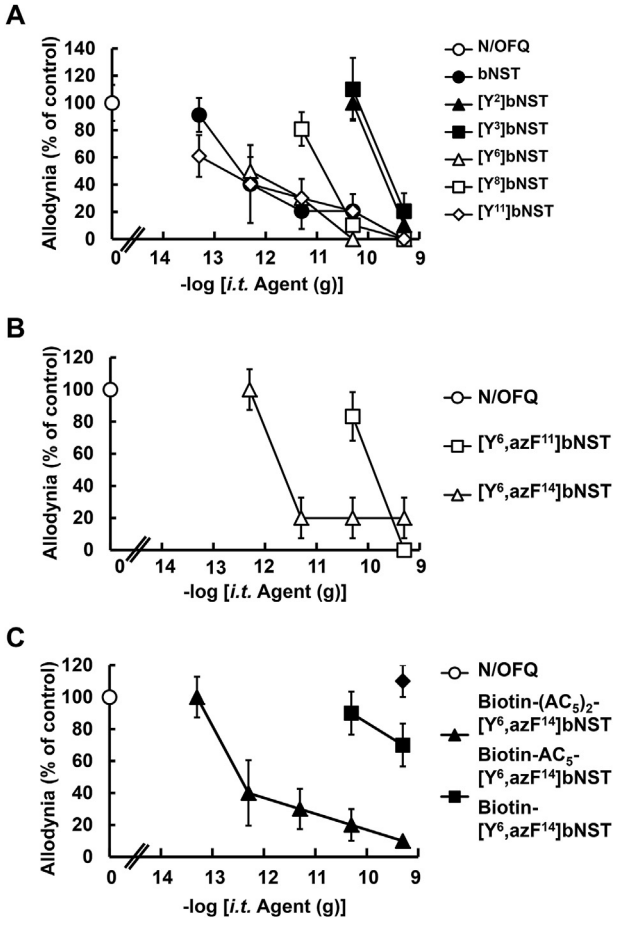

Fig. 1. Effect of tyrosine-substituted NST and NST photoaffinity probes on the N/OFQ-induced allodynia. (A) N/OFQ (50 pg) and the indicated concentration of tyrosine-substituted bNST ([Y]bNST) were simultaneously injected *i.t.* into mice. (B,C) N/OFQ (50 pg) and the indicated concentration of azF-substituted bNSTs (B) or biotin-tagged azF-substituted bNSTs (C) were simultaneously injected *i.t.* into mice. Allodynia was assessed 10 min after *i.t.* injection as described under Materials and Methods. The allodynia score at 10 min after *i.t.* injection of N/OFQ was 83.3% of the maximum possible score and was taken as 100%. The data shown are the mean ± SEM (n = 6).