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Research report

NMDA receptor activation enhances the release of a cholinergic differentiation peptide (HCNP) from hippocampal neurons in vitro

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Abstract

Hippocampal cholinergic neurostimulating peptide (HCNP) is a novel undecapeptide purified from the hippocampus of young rats. The peptide stimulates cholinergic phenotype development in the rat medial septal nucleus in vitro. Here, we have focused on the mechanism of release of the peptide from the hippocampus, by applying tissue culture techniques. Quantitation of HCNP in the culture supernatant after chemical stimulation was carried out by RIA, and by a combination of HPLC and RIA. We found that the *N*-methyl-D-aspartate (NMDA) receptor specifically mediates release of the deacetylated form of HCNP from the culture. Our results suggest that during the early development of hippocampal neurons, the peptide is released by NMDA receptor activation, and that it may be involved in mediating the effect of activity-dependent cues on developing septal cholinergic neurons. © 1998 Elsevier Science B.V.

Keywords: Hippocampal cholinergic neurostimulating peptide; HCNP; Peptide; NMDA receptor; Hippocampus; Activity-dependent cue

1. Introduction

It has been suggested that target-derived cues, via the actions of neurotrophins and other factors, play a crucial role in neuronal differentiation as well as in synaptic plasticity [19,35]. Of these factors, nerve growth factor (NGF) [4,41] and the factor(s) involved in rodent sweat glands [9,37] have been best studied. However, the molecules involved and their mechanism of action are not fully understood.

The septo-hippocampal cholinergic system, which is an important structure for memory formation and learning [13,43], has been extensively studied with respect to its anatomical connections [8,10,21], developmental neurogenesis [21,23,27], and synaptic plasticity [7,16], and for its involvement in Alzheimer-type dementia [12]. In this system, NGF produced in hippocampal neurons is released from their dendrites and play a role as a retrograde messenger in both the development and neuronal plasticity of postsynaptic cholinergic neurons [4,41].

We have previously demonstrated that a novel undecapeptide (acetyl-Ala-Ala-Asp-Ile-Ser-Gln-Trp-Ala-Gly-Pro-Leu)—known as hippocampal cholinergic neurostimulating peptide (HCNP) and derived from the hippocampus of young rats—enhances acetylcholine synthesis in explant cultures of rat septal nuclei [29,31,33]. Further, we have found that, while HCNP-mediated cholinergic stimulation is independent of the effects of nerve growth factor (NGF), both substances act cooperatively in cholinergic phenotype development in the septal nucleus [32]. Subsequent experiments have demonstrated firstly that although the expression of HCNP-related components [26] and HCNP-precursor mRNA [40] is ubiquitous among certain types of neuron, this expression is extremely high in neurons in the hippocampus, and secondly that the amount of HCNP and its precursor protein present in brain tissues is inversely related to the age of the animal, the amount being highest in the neonatal period and strikingly decreased in aged rats [30]. HCNP aligned at the N-terminal region of its 21 kDa precursor protein [42] is cleaved by a specific processing enzyme in the hippocampus, although no basic amino acid residue is situated at or near the cleaving point [34]. Moreover, we have shown that while anti-HCNP antibody recognizes all types of eosinophilic refractile rod-like inclusions (Hirano bodies; HBs)—which occur preferentially in the CA1 field of the hippocampus in some elderly individuals and patients with degenerative dementia, including Alzheimer's disease [15]—it does not recognize normal tissues or other abnormal structures in the human

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brain at autopsy [24,25]. This suggested that an accumulation of HCNP-related components is present in the inclusions, and also that unknown functional disturbance(s) involving HCNP might play a part in certain disease processes. However, the exact mechanism by which the peptide acts in the hippocampus is uncertain, and it is unclear whether HCNP is released from neurons onto the septal cholinergic neuron, because no signal sequence has been found near the N-terminal region of the HCNP precursor protein [42]. To clarify this point, we performed experiments using explant cultures of the rat hippocampus, and the radioimmunoassay (RIA) method. Here, we demonstrate that HCNP can be released from a culture of hippocampal tissue by NMDA receptor activation.

2. Materials and methods

2.1. Animals and tissue culture

Our animal experiments were approved by the Animal Care and Use Committees of the Medical School, Nagoya City University, and conformed to guidelines for the use of laboratory animals published by the Japanese government (Law No. 105, Oct. 1, 1973). All the animals were kept on a 12-h light/dark schedule (lights on 07:00–19:00) and given free access to food and water.

The methods used for tissue culture have been described previously [29,31,32]. Briefly, the hippocampus from Wistar rat embryos at 17 days of gestation was removed and dissected into approximately 0.2 mm diameter pieces. Thirty to thirty-five of these pieces were explanted onto poly-L-lysine (Sigma)-coated 35-mm Falcon plastic culture dishes, and were maintained in 2 ml of modified N2-defined medium [29] supplemented with 3% fetal calf serum (Gibco). The cultures were incubated in an atmosphere of 6% CO_2/94% air at 37°C, and the medium was changed every 3 days.

2.2. Peptide synthesis and generation of anti-free HCNP antibody

The synthesis of peptide fragments and the generation of antisera have been described previously [30,31]. Briefly, HCNP, the deacetylated form of HCNP (free HCNP), and peptide$_{63-79}$ (the amino acid sequence at positions 63–79 in the rat HCNP precursor protein) were synthesized by the solid-phase method using a Beckman 990 peptide synthesizer, and the peptides were purified by HPLC using a YMC-SH-363-5 ODS column. Free HCNP and peptide$_{63-79}$ were conjugated to keyhole limpet hemocyanin (Sigma), and the antisera were then raised in rabbits. Anti-free HCNP antibody for use in the immunohistochemical staining procedure was purified by a free HCNP-coupled affinity column [31], and γ-globulin fractions of anti-free HCNP antiserum and anti-peptide$_{63-79}$ antiserum for use in the RIA were affinity purified using an Ampure PA kit (Amersham) [30].

2.3. Peptide radioiodination and RIA

The method of iodination using Bolton and Hunter's reagent, and the procedure for RIA have been described previously [30]. Briefly, 20 pmol of *N*-succinimidyl-3-(4-hydroxy-3,5-di[^{125}I]iodophenyl)propionate (Amersham) and 2 nmol of free HCNP or peptide$_{63-79}$ in 35 μl of 50 mM borate buffer (pH 8.0) were incubated for 4 h at 4°C. The iodinated peptides were purified using a Sep-Pak C_{18} cartridge (Waters). The specific activity of the iodinated peptides was approximately 370 to 420 GBq/mmol. Mixtures of 100 μl of sample, 50 μl of radioiodinated peptide ($\approx$ 4000 cpm/assay tube), and 50 μl of the γ-globulin fraction (1:200 dilution) of anti-free HCNP antiserum or of anti-peptide$_{63-79}$ antiserum were incubated overnight at 4°C. To separate the bound from the free fraction, Amerlex-M donkey anti-rabbit IgG (Amersham) was used. Net radioactivity was calculated by subtracting the mean cpm of duplicate blank tubes containing only iodinated peptide from that of duplicate tubes with or without (total bound) sample. The net radioactivity of the sample was divided by that of the total bound fraction, and the content of HCNP analogue and HCNP precursor protein in a given sample was derived from the standard curves generated by separately assaying 100 μl of increasing concentrations of free HCNP and peptide$_{63-79}$, respectively.

2.4. Immunostaining

Culture tissues in dishes were washed with ice-cold sodium/potassium phosphate-buffered saline, fixed with Bouin's solution for 1 h, dehydrated with ethanol, cleared in xylene, and embedded in paraffin. Subsequently, 3-μm thick consecutive sections were prepared and deparaffinized. Every other serial section was incubated overnight at 4°C with affinity-purified anti-free HCNP antibody (1:500 dilution) or mouse anti-MAP2 monoclonal antibody (Chemicon International). Visualization of antibody binding was carried out as previously described [26] using the strepto-avidin-biotin peroxidase (LSAB, DAKO) method; haematoxylin was used as a counterstain.

2.5. Detection of HCNP analogue

Each dish containing a culture was washed three times with ice-cold MES–NaOH buffer solution (150 mM NaCl, 3 mM KCl, 1 mM MgCl, 12 mM glucose, 20 mM MES, pH 7.0), and explant numbers were counted. Then the cultures were incubated with 1 ml of incubation solution (3 mM CaCl containing MES–NaOH buffer solution) supplemented with or without (control) other chemicals. To estimate the effect of the hydrogen ion concentration of the buffer, the cultures were incubated with 500 μM glutamate in the incubation solution buffered with 20 mM

MES–NaOH (pH 5.5 to 7.0) or 20 mM TAPES–NaOH (pH 7.5 to 8.5). After an appropriate incubation period, the incubation solution was totally and quickly collected and lyophilized, and then the samples were re-suspended in 450 μl of RIA buffer (1 mM EDTA and 50 mM Tris–HCl, pH 7.8), duplicate samples (100 μl of each) being used for the measurement of HCNP analogue and HCNP precursor protein by RIA. The amount of HCNP analogue and HCNP precursor protein in the RIA was converted to total content in the collected solution. The levels of the released antigens were expressed as fmol per explanted tissue specimen (fmol/specimen) or fmol per protein of the cultures in each dish (fmol/μg protein). The tissue specimens in the dish were harvested with 0.6 ml MES–NaOH buffer, sonicated, and the amount of protein was then quantified by the protein-dye binding method [5] using crystalline bovine serum albumin as a standard. To identify the specific HCNP-related component, the collected supernatants were acidified with trifluoroacetic acid (TFA) to a 0.1% concentration and partially purified with a Sep-Pak C_{18} cartridge, as previously described [31]. The eluate of the Sep-Pak preparation containing HCNP analogues was lyophilized, re-suspended in 200 μl 0.1% TFA solution, and subjected to HPLC using a C4 reverse-phase column. The specific HCNP analogue in the HPLC fractions (collected every 2 min from the start to 50 min) was identified by RIA using anti-free HCNP antibody, and the amount of antigen was expressed in fmol per specimen of the culture tissues in the dishes from which the supernatant had been collected. A Student's *t*-test was used for statistical analysis where appropriate.

3. Results

Because the pyramidal cells of the rat hippocampus originate at embryonic day 14 (E14) and are in their proper location by between E17 and E19 [2], we used hippocampal tissues from E17 rats for the culture. Further, we have previously found that in RIA, antibody to free HCNP reacts with free HCNP as well as with HCNP itself, but does not react with their precursor protein, whereas antibody to $peptide_{63-79}$ does react with the precursor protein, though both antibodies recognize the precursor protein in Western blotting [30]. Consequently, using RIA we are able to measure (i) HCNP and its analogue (free HCNP) and (ii) HCNP-precursor protein independently in the supernatant after chemical stimulation of such cultures. Preliminary experiments suggested that the cultures grew well and exhibited extended processes (Fig. 1A), and that the antigen to the anti-free HCNP antibody appeared in the cytoplasm of neuronal cells by the 9th day in vitro at least (Fig. 1B).

When the cultures were incubated with buffer solution supplemented with or without glutamate, the antigen to the anti-free HCNP antibody was released from the tissues and was detectable in the supernatant of each of the incubation solutions. The antigen levels were dependent on the incubation time, linearly increasing over the period from 0 to 5 min and reaching a plateau between 5 and 10 min. The age of the culture was another important variable. A clear increase in the antigen release occurred in cultures kept for more than 6 days in vitro (Fig. 2A). Further, the release of

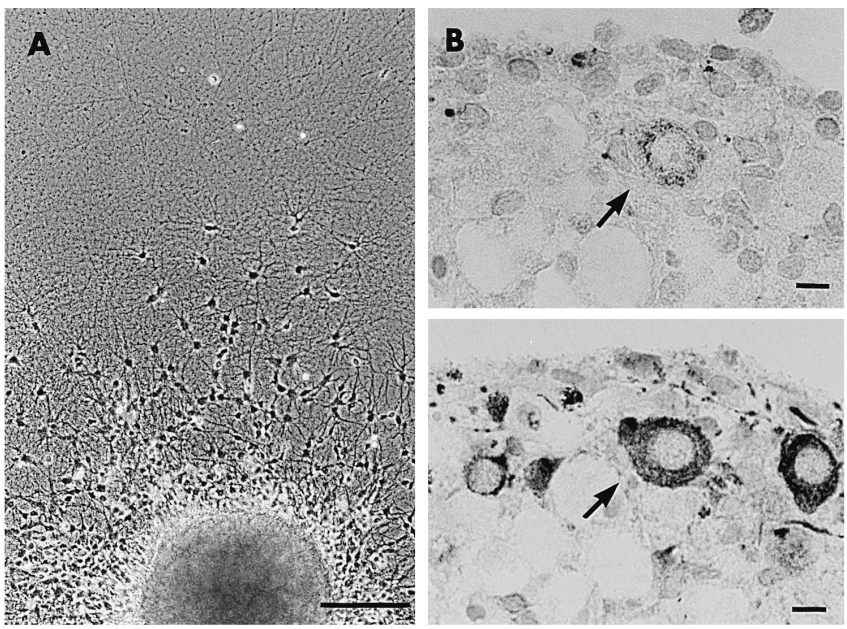

Fig. 1. Morphology of the explanted tissue culture from the hippocampus of a Wistar rat at embryonic day 17. (A) Phase-contrast photomicrograph of an explant tissue culture at 9 days in vitro. Note that growth has proceeded well in culture and that excellent processes are present. Bar, 0.2 mm. (B) Immunohistochemical staining of consecutive sections from hippocampal tissue at 9 days in vitro. A cell (indicated by arrow) showing positive cytoplasmic staining with anti-rat free HCNP antibody (upper) was also stained with neuron-specific anti-MAP2 antibody (lower). Bars, 10 μm.

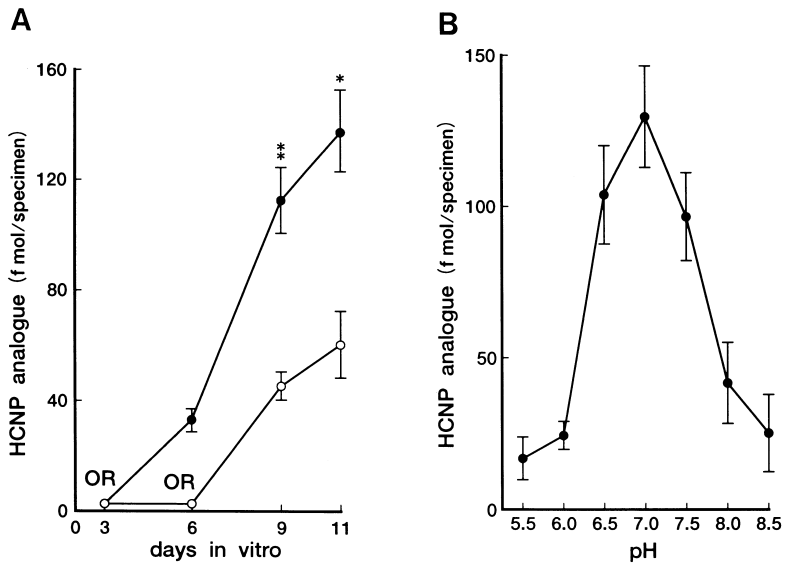

Fig. 2. Effect of experimental conditions on the release of HCNP analogue from hippocampal explant tissue culture. (A) The release of HCNP analogue is dependent on the age of the culture. Glutamate (500 μM, solid circle) induced a greater release than that seen in control (open circle) at any age of hippocampal culture examined. (B) When the culture (at 9 days in vitro) was incubated with appropriate buffer containing 500 μM glutamate, the release was dependent on the hydrogen ion concentration, being maximal at pH 7.0. Each point represents the mean of the number of fmol of HCNP analogue per culture specimen in each of three dishes. The vertical bars represent SD. $* p < 0.01$, $** p < 0.001$ vs. control. OR, out of range of the assay.

the antigen was dependent on the hydrogen ion concentration, being maximal at pH 7.0 (Fig. 2B), and on the temperature of the incubation buffer, no release being detectable at 4°C. In these preliminary experiments and the following investigations, HCNP precursor protein was not detectable in the supernatant in any trials when the RIA method was employed and a specific antibody used. Hence, in the following experiments, we used 9-day old cultures, and carried out incubations at 37°C for 10 min in pH 7.0 buffer solution. Moreover, we only show data relating to the release of the antigen to the anti-free HCNP antibody.

When the cultures were incubated with a variety of transmitters, only glutamate significantly ($p < 0.001$) increased the release of the antigen, whereas other transmitters (at both low and high concentrations) as well as a high potassium solution (55 mM) failed to affect the release

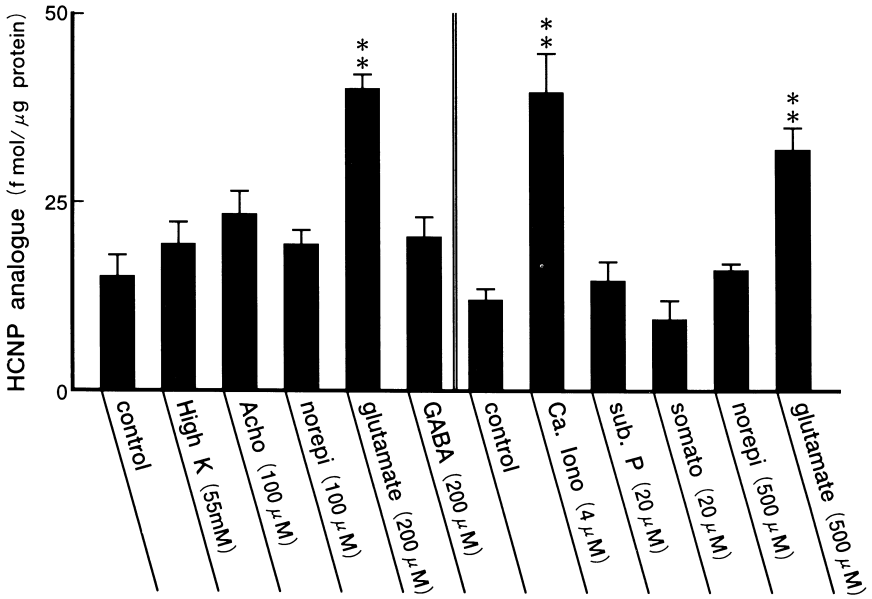

Fig. 3. Effect of various chemicals on the release of HCNP analogue from hippocampal explant cultures. The cultures were incubated for 10 min at 37°C with or without a high concentration of potassium (High K), acetylcholine (Acho), norepinephrine (norepi), glutamate, GABA, substance P (sub. P), somatostatin (somato), and calcium ionophore A23187 (Ca. Iono.). Then, the HCNP analogue in the incubation solution was measured by RIA. Note that only glutamate and calcium ionophore A23187 stimulated the release of HCNP analogue from the culture. $** p < 0.001$ vs. the corresponding control.

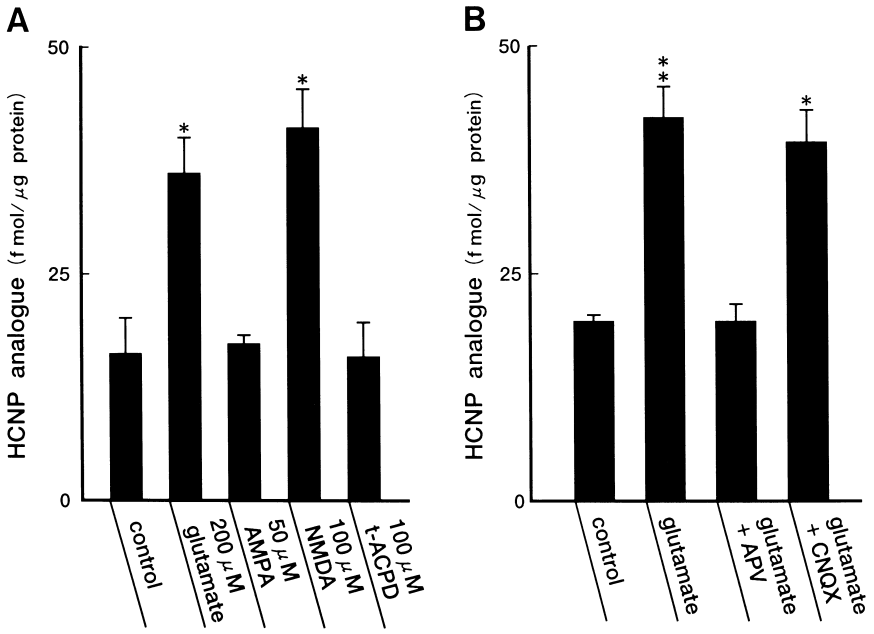

Fig. 4. Characterization of the glutamate receptor mediating release of HCNP analogue. (A) One of three agonists of the glutamate receptor, in the concentration depicted, was added to the incubation solution, and the release of HCNP analogue was estimated. Only NMDA mimicked the effect of glutamate. (B) The effect of glutamate receptor antagonists. Cultures were incubated with 200 μM APV or 4 μM CNQX in the presence of 200 μM glutamate, and the amount of HCNP analogue released was compared with that released on incubation with 200 μM glutamate alone or without any chemicals (control). Note that APV inhibited the effect of glutamate. Each column represents the mean of the number of fmol of HCNP analogue released per μg protein in each of three dishes. Vertical bars represent SD. $* p < 0.01$, $** p < 0.001$ vs. the corresponding control.

(Fig. 3). To test what type of glutamate receptor mediates the antigen release, the cultures were incubated with one of several receptor agonists or antagonists. The release of the antigen was stimulated by *N*-methyl-D-aspartate (NMDA), which is similar to glutamate, but not by either α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) or the metabotropic glutamate-receptor agonist, *trans*-(±)-1-amino-1,3-cyclopentane-dicarboxylic acid (*t*-ACPD) (Fig. 4A). The glutamate-mediated antigen release was completely eliminated by the NMDA-receptor antagonist, D-2-amino-phosphonovaleric acid (APV), but not by the non-NMDA-receptor antagonist, 6-cyano-7-nitroquinoxaline-

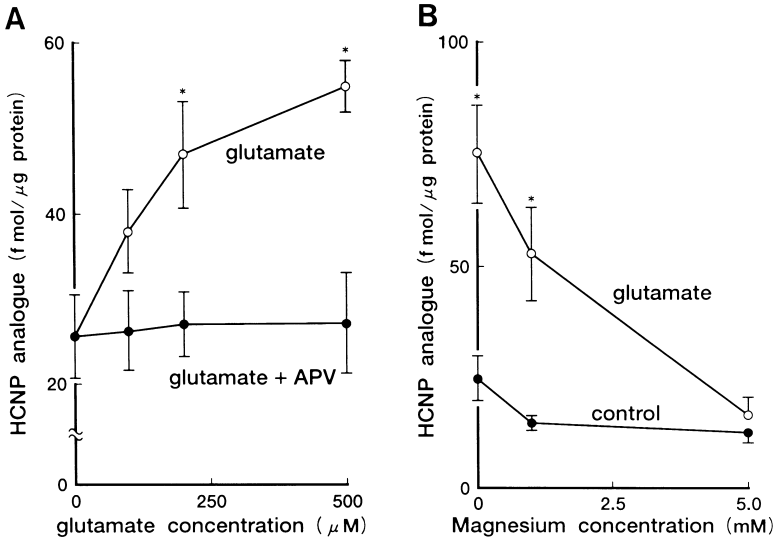

Fig. 5. Characterization of NMDA receptor-mediated release of HCNP analogue in hippocampal tissue culture. (A) HCNP analogue release was dependent on glutamate concentration (open circle), and 200 μM APV abolished the release (solid circle). (B) The glutamate-mediated (500 μM) release of HCNP analogue was inversely related to the magnesium concentration in the incubation solution (open circle), while the baseline release without glutamate (control: solid circle) was not significantly affected. Each point represents the mean of the number of fmol of HCNP analogue per μg protein in each of three dishes. Vertical bars represent SD. $* p < 0.01$ vs. glutamate + APV (A) or control (B).

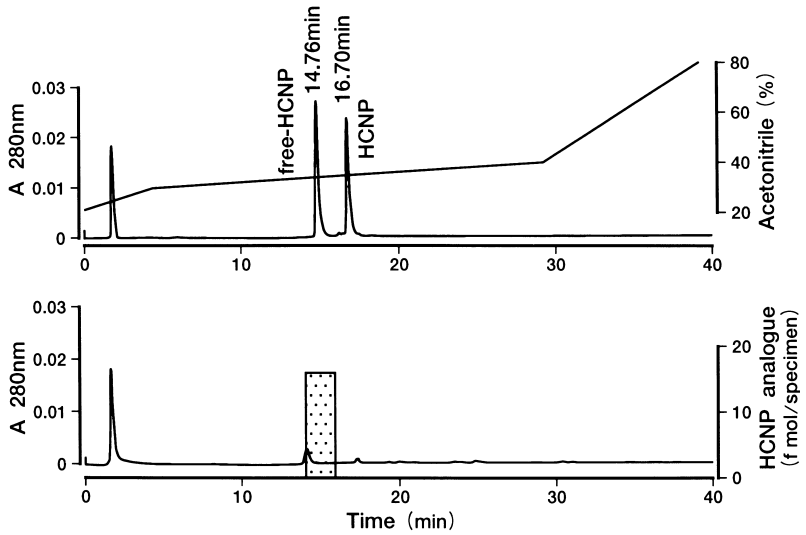

Fig. 6. Identification of HCNP analogue in the incubation solution after chemical stimulation. The supernatant of a culture incubated with 4 μM calcium ionophore A23187 in the incubation solution was collected and analyzed by a combination of HPLC and RIA [30]. The standard free HCNP and HCNP were eluted in different fractions in HPLC (upper). The anti-free HCNP antibody-reactive component in the supernatant was eluted only in the fraction with a retention time identical to that of free HCNP (below).

2,3-dione (CNQX) (Fig. 4BFig. 5A). In addition, the release was dependent on the concentration of glutamate and magnesium in the incubation buffer. A significant increase in antigen release was observed with a glutamate concentration above 200 μM (Fig. 5A), and a magnesium concentration of 5 mM resulted in an almost complete inhibition of the glutamate-mediated release of the antigen, even though the baseline release without glutamate was not greatly affected (Fig. 5B). Furthermore, the calcium ionophore A23187 (calcimycin) mimicked the effect of glutamate on the culture (Fig. 3), suggesting that the glutamate-induced antigen release is specifically mediated by the NMDA receptor, and that it is regulated by calcium influx through its receptor. This NMDA receptor-mediated release of the antigen was dependent on the age of the culture; the release can be identified after as little as 6 days in vitro, but a much more substantial release was observed after 9 days in culture (Fig. 2A).

Our previous results have indicated that both HCNP and free HCNP are present in rat hippocampal tissue [30], and that free HCNP can be considered to be the active form in vivo because it is more potent than HCNP-proper [31]. Thus, to identify which molecule is released from the tissue on NMDA-receptor activation, a combination method involving HPLC and RIA was employed. Only a peptide with a retention time identical to that of free HCNP was detected by HPLC fractionation in the supernatant whether the culture was incubated with calcium ionophore A23187 (Fig. 6) or with glutamate (data not shown).

4. Discussion

We have previously demonstrated that in neurons of the medial septal nuclei, HCNP-related components are not detectable by immunohistochemical methods [26], and that the expression of their precursor mRNA is relatively low [40]. The expression of these components (and that of their mRNA) is highest in pyramidal neurons of the hippocampus, particularly in the CA3 region and the hilus of the hippocampal dentate gyrus [26,40] where there is a dense cholinergic innervation from the medial septal nuclei [8,10]. We have also shown that the concentration in the hippocampus of HCNP analogues, as well as of their precursor protein, is age-dependent, with a peak in the first postnatal week [30].

The present data support the idea of another important proposed mechanism of action for the novel undecapeptide, in which the peptide in the neurons of the hippocampus is released by means of NMDA receptor activation during the early development of the neurons. Our results with specific agonists and a specific antagonist of HCNP release, as well as its dependence on the concentration of extracellular magnesium, suggest that the release of the peptide from hippocampal neurons is specifically mediated via the NMDA receptor. The conductance mediated by the ionotropic NMDA receptor depends on both ligand-binding (of glutamate at hippocampal synapses) and membrane depolarization to achieve peak levels of conductance [28]. In hippocampal neurons, it is thought (i) that for glutamate to generate a large conductance, removal of magnesium ion—which is blocking the calcium channels associated with the NMDA receptor at the normal resting potential—has to occur, and that this does occur when the membrane potential is depolarized through the non-NMDA type glutamate receptor [28] that is colocalized with the NMDA receptor [3], and (ii) that glutamate binding to the NMDA receptor results in ion channel opening [17] and leads to an increase in the intracellular calcium concentration [38].

NMDA itself functions both as a specific ligand and as an inducer of depolarization via the NMDA receptor [17]. In addition to glutamate and NMDA, the calcium ionophore A23187 also increased HCNP release from the hippocampal cultures used in the present experiments, supporting the notion that the ionophore increases intracellular calcium concentration in association with the NMDA receptor, but not with quisqualate-binding glutamate receptors [22]. It has been suggested that the depolarization induced by extracellular high potassium increases calcium influx through voltage-gated calcium channels in certain neurons [6]. However, in hippocampal neurons increases in intracellular calcium concentration are mediated by activation of an NMDA receptor; the voltage-gated calcium channels make little contribution to such increases [38]. This may be why application of high potassium failed to release HCNP from our hippocampal cultures.

The descending output from the hippocampus that projects toward the septal region terminates in the lateral septal nucleus, where there are no cholinergic neurons [2,36]. In fact, the HCNP released from hippocampal neurons in vivo may act in a retrograde fashion on the innervating cholinergic neurons, stimulating production of choline acetyltransferase (ChAT) by those neurons [29,32]. Studies on the development of the rat septo-hippocampal system have shown that both ChAT activity [27] and acetylcholinesterase (AchE)-positive fibers [23] in the hippocampus become evident by postnatal day 4 (P4), and that rapid growth of the hippocampal apical dendritic field that receives a septal cholinergic innervation occurs before P10 [21]. In addition, the NMDA receptor on the hippocampal neurons can be detected as early as P2 by electrophysiological methods [9]. These findings, as well as our previous data [31–33] pertaining to HCNP, suggest that we may be warranted in presuming that an NMDA-mediated calcium-dependent retrograde action of the peptide occurs in the early development of the system. It has also been suggested that NGF is involved in the early development of the septo-hippocampal cholinergic neuron by virtue of a constitutive release and/or by an activity-mediated sodium-dependent secretion predominantly at the surface of the postsynaptic hippocampal pyramidal neuron [41]. These data could be consistent with our previous results, suggesting that both HCNP and NGF, each acting in its own way, work cooperatively to regulate the differentiation of the neuron [32]. In addition to the NMDA receptor-mediated release, a spontaneous release of HCNP was observed in the present experiments. Further investigations will be required to determine whether it is specific to particular neurons, and whether, like the constitutive release of NGF, it has a role in the development of the septo-hippocampal neuron.

Long-term potentiation (LTP) is an important phenomenon in synaptic plasticity; it is partly mediated by the NMDA receptor, and it appears to be involved in memory formation as well as neuronal development [9,19]. By contrast, it has been suggested that neurotrophins [41] and other molecules—such as carbon monoxide [39], cyclic GMP [1], nitric oxide [18], and arachidonic acid [14]—all regulate LTP, and that acetylcholine released from septal neurons also affects postsynaptic hippocampal LTP [7,16]. In the septo-hippocampal system, pure NMDA receptor-based synaptic contacts become mature functional NMDA/AMPA receptor-type synapses associated with LTP during the first to second postnatal week [9]. The NMDA-receptor, but not the AMPA receptor, mediates the release of the novel undecapeptide that stimulates the differentiation of presynaptic septal cholinergic neurons, as shown in this report. These results provide strong evidence that these peptides are also involved in the processes stimulated by target-derived cues, such as the plausible reciprocal molecular interaction between the pre- and post-synaptic neurons of the septo-hippocampal system during early neuronal development and/or synaptic plasticity. In fact, similar something has been shown to occur between peripheral autonomic nerves and sweat glands though the messenger molecule has not yet been identified [11,37].

There is a precedent for the NMDA receptor mediating peptide release. It has been shown that in the sensory system of adult rats, activation of the NMDA receptor located on the terminals of primary afferent fibers causes prolonged release of substance P from the terminals of the same sensory fibers in the spinal cord, producing pain behavior [20]. We have previously demonstrated that HCNP and its more bioactive form, free HCNP [30], and expression of their precursor mRNA [40] can be found in brain sub-regions in young adult rats, predominantly in the hippocampus. Thus, these data might suggest that the HCNP analogue also has certain important functions in the hippocampus of mature animals, possibly through the NMDA receptor, as it does in the developing animal. Further, it is possible that accumulation of HCNP-related components in intracytoplasmic inclusions (HBs) in the hippocampus of elderly individuals and patients with degenerative dementia [24,25] might lead to a disturbance of the mechanism of HCNP release. However, to elucidate these points will require further studies involving in vivo experiments on appropriate adult animal models. Such studies should involve the identification (i) of the receptors and HCNP elements concerned, and (ii) of their exact locations within the nervous system.

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