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Structural and functional analogs of the novel mammalian neuropeptide, neuromedin S (NmS), in the dermal venoms of Eurasian bombinid toads

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Abstract

We report the isolation and structural characterization of two neuromedin S (NmS) analogs, (NmS-17 and NmS-33), from the dermal venoms of Eurasian bombinid toads. NmS is a novel neuromedin U (NmU)-related peptide with potent anorexigenic and circadian rhythm-modulating properties recently discovered in mammals. Cloning of NmS precursor-encoding cDNAs from skin venom-derived libraries revealed the presence of a high degree of transcript splice variation comparable to that found previously for NmU in both amphibian skin and mammalian brain. Synthetic replicates of both amphibian NmS peptides evoked robust and dose-dependent transient increases in intracellular calcium ion concentrations in CHO cells that had been stably transfected with either FM-3/GPR66 or FM-4/TGR-1 human NmU receptors. The potency and efficacy of these amphibian skin peptides at such receptors were comparable to those observed with human NmS and rat NmS. These data show that NmS and NmU genes had already diverged at the level of the Amphibia and that differential splicing of their transcribed mRNAs has been highly conserved throughout tetrapod vertebrate evolution indicative of fundamental biological function. NmS is additionally a novel neuropeptide homolog that can be added to the biologically active peptide arsenal of amphibian venom/defensive skin secretions.

Keywords: Amphibian; Venom; Peptide; Neuromedin; Cloning

Neuromedin U (NmU) was isolated by one of the present co-authors (K.K.) in 1985 from an extract of hog spinal cord due to its hypertensive and uterine smooth muscle contracting properties [1]. Two molecular forms were isolated and were designated as NmU-25 and NmU-8, the latter representing the biologically active C-terminal core of the former. NmU was found to have a classical brain-gut distribution and to be of ubiquitous occurrence in a wide range of vertebrates including human, dog, rat, guinea pig, rabbit, chicken, and frog [2–9]. In a variety of bioassays, NmU was found to possess additional peripheral biological effects including modification of intestinal ion transport, adrenocortical function, and splanchnic blood flow [10–12]. However, intracerebroventricular administration of the peptide into rats induced a significant reduction in food intake (anorexigenic effect), augmentation of stress responses, and elevations in body temperature and heart rate [13–15].

Amphibian venoms/defensive skin secretions are rich sources of biologically active peptides many of which are structural and functional homologs of endogenous vertebrate neuropeptides leading several authors to speculate that every vertebrate neuropeptide may have a frog skin secretion equivalent [16,17]. In 2000, we reported the presence of an NmU homolog in the defensive skin secretion of the Australasian tree frog, *Litoria caerulea* [18]. This

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peptide, designated NmU-23, was found to exhibit full-NmU agonist activity in preparations of rat uterine and human urinary bladder smooth muscle and to be equipotent with NmU-25 in displacing this radiolabeled ligand in a rat uterine smooth muscle membrane radioreceptor assay. Subsequently, the cDNA encoding this peptide was cloned from a library manufactured from lyophilized skin secretion of this tree frog using a novel technique developed in our laboratory [19]. In addition to the full-length preproneuromedin U transcript, deemed to be such due to its alignment with the homologous human transcript cloned from a pituitary gland library, we identified a number of splice variants largely involving splicing events within short exons immediately upstream of the NmU encoding sequence. This prompted a study of frog, rat, and human brain in which the same splice variation was observed. Thus within the tetrapod vertebrates, the NmU gene appears to possess a multiplicity of short exons within the open-reading frame that undergoes a highly conserved and discrete differential regional splicing indicative of an as yet unidentified but presumably fundamental function.

Recently, a second NmU-related peptide, designated neuromedin S (NmS), has been identified in and isolated from rat, mouse, and human brain tissues [20]. This was achieved by reverse deorphanization of the NmU receptors, FM-3/GPR66, and FM-4/TGR-1, stably transfected into CHO cells, and interrogated with brain peptide extracts using a calcium mobilization assay. Using radioimmunoassay systems that employ phylogenetically conserved, site-specific antisera to neuropeptides, we have screened reverse phase HPLC-fractionated venoms/defensive skin secretions from some 140 different species of amphibians for at least 20 neuropeptides. In this screen, we confirmed the presence of NmU immunoreactivity in *L. caerulea* skin secretion [18], although this was somewhat heterogenous indicating the presence of molecular forms in addition to the major NmU-23. In addition, we detected NmU immunoreactivity in a closely related species, the white-lipped tree frog, *Litoria infrafrenata*. What was most surprising was the presence of NmU immunoreactivity in skin secretion fractions of the three species of bombinid toad subjected to analysis. All other species were consistently negative. Structural and bioinformatic analyses of the bombinid toad immunoreactive peptides indicated that they were in fact NmS homologs. Molecular cloning of these toad skin NmS cDNAs from venom-derived libraries revealed a spectrum of splice variants that reflected our previous findings for NmU transcripts in *L. caerulea* skin secretion [19]. Synthetic replicates of frog NmS-17 and frog NmS-33 interacted with stably transfected GPR66 and TGR-1 NmU receptors in a manner similar to endogenous human and rat NmS homologs. These data demonstrate, for the first time, that NmU and NmS genes had already diverged within the amphibians and that the systematic study of amphibian defensive skin secretions can provide many valuable insights into the molecular evolution of vertebrate regulatory peptides.

Materials and methods

Acquisition of skin secretions. Young adult specimens of the Oriental fire-bellied toad (*Bombina orientalis*, $n = 5$), the Chinese giant fire-bellied toad (*Bombina maxima*, $n = 3$), and the European yellow-bellied toad (*Bombina variegata*, $n = 10$) were captive-bred and were maintained in separate species terraria at a temperature of 22 °C under a 12 h/12 h light/dark cycle with three feeds of multi-vitamin loaded crickets per week. Following a 12-week period of acclimatization, venom was obtained by gentle transdermal electrical stimulation (4-ms pulse width, 50 Hz, 5–7 V) of deionized water-moistened skin for three periods of 15 s duration [21]. The viscous white granular gland secretions were washed from the skins of the toads using a stream of deionized water into pre-chilled (2 °C) glass beakers, snap-frozen in liquid nitrogen, and lyophilized. The secretions from each of the three species of toads were separately pooled. Approximately 10–15 mg dried weight of skin secretion could be obtained from each specimen on a monthly basis.

Reverse phase HPLC/MS. Ten milligrams of lyophilized venom from each species of toad were separately subjected to reverse phase HPLC/MS analysis using a gradient formed from 0.05/99.95 (v/v) trifluoroacetic acid (TFA)/water to 0.05/39.95/60.0 (v/v/v) TFA/water/acetonitrile in 60 min at a flow rate of 1 ml/min. A Thermoquest gradient reversed phase HPLC system, fitted with an analytical column (Phenomenex, C-18, 25 × 0.45 cm), and interfaced with a Thermoquest LCQ™ Deca electrospray ion-trap mass spectrometer was employed. The effluent from the chromatographic column was flow-split with approximately 10% entering the mass spectrometer source and 90% directed towards a fraction collector. Dead volume between column and fraction collector was minimal (20 µl). The molecular masses of polypeptides in each chromatographic fraction were further analyzed using matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry (MALDI-TOF MS) on a linear time-of-flight Voyager DE PRO mass spectrometer (Perseptive Biosystems, MA, USA) in positive detection mode using α-cyano-4-hydroxycinnamic acid as the matrix. Internal mass calibration of the instrument with known standards established the accuracy of mass determination as ±0.1%. The peptides possessing masses coincident with those of NmS analogs deduced from cloned cDNAs were each subjected to primary structural analyses by automated Edman degradation using an Applied Biosystems 491 Procise sequencer in pulsed-liquid mode or by MS/MS fragmentation sequencing using the LCQ™ Deca electrospray ion-trap instrument.

Analysis of reverse phase HPLC fractions by NmU/NmS radioimmunoassay. Fifty microliter samples from each chromatographic fraction were removed and lyophilized prior to radioimmunometric analysis using a system described in detail previously [18]. Briefly, antiserum GP 9320 was raised in a guinea pig immunized with a (Lys^0)-NmU-8/glutaraldehyde/ovalbumin conjugate. The assay buffer, in which all reactants were diluted, consisted of 0.04 M sodium phosphate, pH 7.2, containing 0.14 M sodium chloride and 2% (v/v) horse serum. The assay volume was 400 µl consisting of 100 µl of diluted NmU-8 antiserum (1:38,000), 100 µl of monoradioiodinated NmU-8 tracer (100 Bq; 2 fmol), and 100 µl of NmU-8 standard (0–250 fmol/ml) or unknown sample. Addition of tracer was delayed for 24 h and bound/free tracer was separated after a further 24 h by addition of 1 ml of 0.05% dextran-coated microfine charcoal and centrifugation at 1100*g* for 30 min. All procedures were carried out at 4 °C and under the conditions described, the sensitivity of the assay, defined as the least amount that could be detected above zero with 95% confidence, was 1.2 fmol NmU-8/assay tube. The antiserum cross-reacted fully on a molar basis with NmU-8, NmU-25, frog NmU-23, human NmS, and rat NmS but exhibited no cross-reactivity with a wide range of vertebrate neuropeptides.

Cloning of NmS precursor cDNAs from venom-derived libraries. Five milligrams of lyophilized venom from each species were dissolved separately in 1 ml of cell lysis/mRNA stabilization buffer (Dynal Biotec, UK). Polyadenylated mRNA was isolated from the stabilization buffer/skin secretion mixtures using magnetic oligo(dT) beads as described by the

manufacturer (Dynal Biotec, UK) and reverse-transcribed. The cDNA was subjected to 3′- and 5′-RACE procedures to obtain full-length prepro-NmS nucleic acid sequence data using a SMART-RACE kit (Clontech UK) essentially as described by the manufacturer. Briefly, the 3′-RACE reactions employed an NUP primer (supplied with the kit) and a degenerate sense primer (NmS-S1: 5′-GGIATHGTIGGIMGICCITT-3′) that was complementary to the internal amphibian NmS amino acid sequence, –GIVGRPF–. 3′-RACE products were gel-purified and cloned using a pGEM-T vector system (Promega Corporation) and sequenced using an ABI 3100 automated DNA sequencer. The sequence data obtained from these 3′-RACE products were used to design a gene-specific primer, (NmS-AS1: 5′-TGTGTCCATAAGATCAGGCAGAAT-3′) to a sequence within the 3′-non-translated region. 5′-RACE reactions were performed using this primer in conjunction with the NUP and the resultant products were gel-purified, cloned, and sequenced as described above. Following acquisition of these data, a second gene-specific sense primer (NmS-S2: 5′-CCTCTACCACTGCTGCTTGCGATC-3′) was designed to a site within the putative signal peptide domain and was employed in 3′-RACE reactions. Products were likewise gel-purified, cloned, and sequenced as described previously. All identified sequences and splice variants were represented at least ten times in the several hundred clones that were sequenced.

Chemical synthesis of toad NmS-17 and NmS-33. Subsequent to unequivocal primary structural characterization of the toad defensive secretion peptides NmS-17 and NmS-33, each was separately synthesized using solid-phase Fmoc chemistry and an Applied Biosystems 433 peptide synthesizer. Following cleavage from the resin and deprotection, each respective peptide was purified by reverse phase HPLC, a process that was monitored and quality-controlled by means of electrospray mass spectrometry using an LCQ DECA instrument (Thermo-Electron Corporation, San Jose, CA, USA).

Production of human NmU-receptor transfected cell lines. CHO cells were stably transfected with either human FM-3/GPR66 (GenBank Accession No. BC036543) or with FM-4/TGR-1 (AF242874) NmU/NmS receptors by cloning into pcDNA3.1 vectors. The cell lines (CHO/FM-3-14 and CHO/FM-4-16), that exhibited the greatest elevations in free cytosolic Ca^{2+} ion concentrations when challenged with human NmU, were used in this study. The intracellular calcium mobilization assay was performed using a FLIPR system (Molecular Devices) that has been described in detail previously [20]. All peptide solutions contained 1% (w/v) bovine serum albumin and each synthetic peptide was subjected to amino acid analysis prior to construction of solutions for dose–response studies.

Results

Identification and structural characterization of toad venom NmS peptides

NmS/NmU immunoreactive peptides were identified in reverse phase HPLC fractions of venom from each of the three species of bombinid toad investigated. The profile obtained with *B. orientalis* is shown in Fig. 1. Two immunoreactive peptides were resolved in fractions of *B. maxima* and *B. orientalis* venoms in contrast to the single peptide in *B. variegata* that was coincident in retention time with the more abundant and hydrophilic peptide resolved in the other congeneric species. This more hydrophilic peptide was found to be of identical molecular mass (1964 Da—non-protonated) in all three species. A combination of automated Edman degradation and MS/MS fragmentation sequencing established the primary structure as: DSSGIVGRPFFLFR PRNamide. The minor, and more hydrophobic peptides from *B. maxima* and *B. orientalis* were found to be of similar (3800 and 3818 Da—non-protonated, respectively) but not identical molecular masses. The primary structure of the *B. maxima* peptide was established by automated Edman degradation as: FLFQFSRAKDPSLKIGDSSGIVGRPF FLFRP(RNamide). The last C-terminal amino acid residues were not detectable in the final cycles of Edman degradation but their inclusion matched the previously observed molecular mass of this peptide. The *B. orientalis* peptide was also subjected to automated Edman degradation and the two C-terminal residues were likewise not detected. However, as in the first instance, their inclusion provided an exact match to observed molecular mass. The *B. orientalis* peptide differed in primary structure from the *B. maxima* homolog at two sites—a Thr for Ala substitution at position 8 and a Thr for Ile substitution at position 15. This finding explained the discrepancy in molecular mass observed between the two

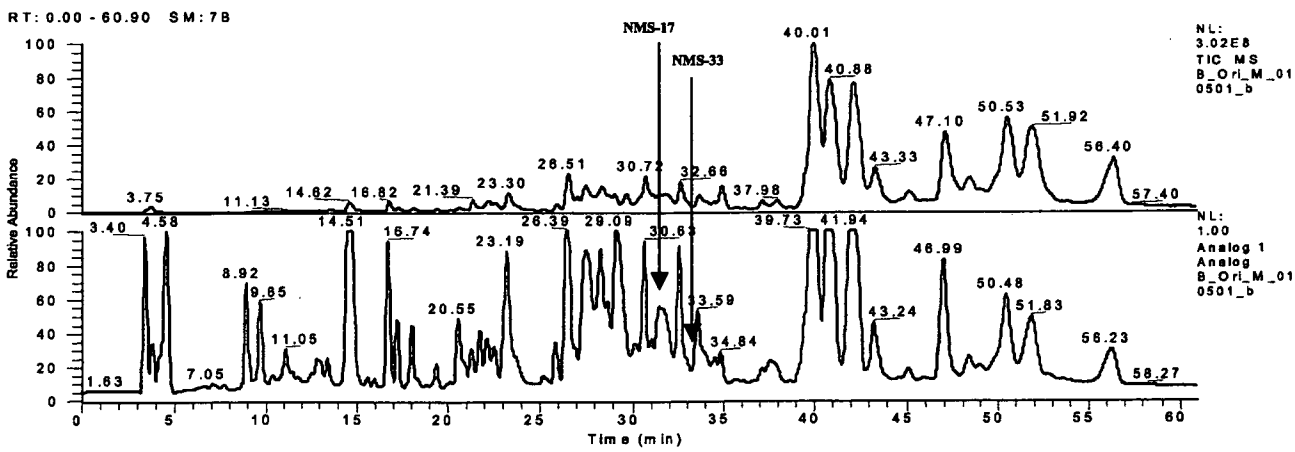

Fig. 1. A typical LC/MS spectrogram of skin venom from the Oriental fire-bellied toad, *B. orientalis*. The upper panel illustrates total ion count entering the mass spectrometer and the lower panel illustrates UV absorbance profile at λ214 nm. Both *y* axes are in arbitrary units where the highest peak is default expressed as 100% of signal to illustrate component relative abundance. The retention times of NmS-17 and NmS-33 are indicated by arrows.

```
Toad  NmS-17                          DSSGIVGRPFFLFRPRNa
Toad  NmS-33              FLFQFSRAKDPSLKIGDSSGIVGRPFFLFRPRNa
Human NmS-33              ILQRGSGTAAVDFTKKDHTATWGRPFFLFRPRNa
Mouse NmS-36          LPRLLRLDSRMATVDFPKKDPTTSLGRPFFLFRPRNa
Rat   NmS-36          LPRLLHTDSRMATIDFPKKDPTTSLGRPFFLFRPRNa
                                      *      ***********

Frog  NmU-25          LKPDEELQGPGGVLSRGYFVFRPRNa
Frog  NmU-23            SDEEVQVPGGVISNGYFLFRPRNa
Human NmU-25          FRVDEEFQSPFGSRSRGYFLFRPRNa
Rat   NmU-23          YKVNE-YQGP-GAPSGGFFLFRPRNa
Pig   NmU-25          FKVDEEFQGPIVSQNRRYFLFRPRNa
Dog   NmU-25          FRLDEEFQGPIASQVRRQFLFRPRNa
                            * *       * *****
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Fig. 2. Comparative alignments of homologous amphibian and mammalian NmS and NmU sequences. * Denotes amino acid residue identities; *a* denotes C-terminal amide. Defining NmS motif is clearly a completely conserved C-terminal undecapeptide amide marked in bold italic contrasting with NmU whose defining motif is a completely conserved C-terminal pentapeptide amide marked in bold italic. Gaps have been inserted in the sequence of rat NmU-23 to maximize alignment.

peptides. The primary structures of toad NmS-17 and toad NmS-33 are compared with NmS peptides from human, mouse, and rat in Fig. 2. In addition, NmU peptides from amphibian and mammalian source tissues are included for comparison. The defining motif of NmS is evidently a fully conserved C-terminal undecapeptide amide that contrasts with the defining motif of NmU peptides, that is, a C-terminal pentapeptide amide. The C-terminal region of NmU has been established as the bioactive core of the molecule [1] and it is likely that this will also hold true for NmS peptides. However, NmS appears to have been more rigorously conserved in this region than its NmU relation during tetrapod evolution.

Cloning of NmS precursor cDNAs from venom-derived libraries

Using the strategy described in the Methods section, a single diffuse band was obtained following electrophoresis of 3′-RACE PCR products generated from each venom-derived library. Cloning of these PCR products revealed their heterogeneity as, following sequencing of over 200 clones, it was evident that six different transcript isoforms were present in *B. maxima* and in *B. orientalis* venom-derived libraries, but only a single transcript, corresponding to one of the *B. maxima/orientalis* isoforms, was present in the library from *B. variegata* venom. This may be of evolutionary significance as the first two species are of Asiatic origin whereas the latter is found in Central Europe. The longest open-reading frames encoded by the cloned cDNAs from each species are aligned in Fig. 3 and those of human, mouse, and rat are included as a sub-set for direct comparison. Figs. 4 and 5 show the aligned splice variant isoforms 1 through 6 obtained from *B. maxima* and *B. orientalis* venom-derived cDNA libraries, respectively. In common with the transcripts arising from the NmU gene in amphibians and mammals, the major region of splice variation occurs immediately upstream of the NmS-encoding domain. All

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Human MKHLRPQFPLILAIYCFCMLQIPSSGFPQPLADPSDGLDI
Mouse MKHPLPHYSPILFIYCFCMLQIPSSGASPPLADSPDGLDI
Rat   MKHPFPQFPPILVIYCFCMLQIPSSGASPPLAGPPDGLDA
      *** *    ** *************   ***   ****
BM    MRSEKHLLPLPLLLAICCLGTLHLSSGFPQSVPSYLEGLD
BO    MRSEKHLPPLPLLLAICCLGTLHPSSGFPQSVPSYMEALD
BV    MRSEKHLLPLPPLLAICCLGTLHLSSGFPQSVPSYLEGLD
      ******* *** *********** *********** * **

Human VQLEQLAYCLSQWAPLSRQPKDNQDIYKRFLFHYSRTQEA
Mouse VDPERLAYFLKQREIHSNQPKENQDVYKRFLFHYSRTRKP
Rat   VDPERLAHFLNQRETCSNQPKESRDVYKRFLFHYSRAWKS
      *  * **  * *    * ***   * **********
BM    IPESERHAFCFSQWTALQDQEQIPSFVMDLCSSIYNRMKV
BO    IPESEKLAFCFSQWTALPDQEQIPSFVMDLCSSIYNRMKV
BV    IPESE----------------IPSFVMDLCSSITNRMKV
      *****                ************ *****

Human THPVKTGFPPVHPLMHLAAKLANRRMKRILQRGSGTAAVD
Mouse THPVSAEFAPVHPLMRLAAKLASRRMKRLPRLLRLDSRMA
Rat   THPVNSEFAPVHPLMRLAAKLPSRRMKRLPRLLHTDSRMA
      ****   * ****** *****  *******
BM    NEENNHEIYKRFLFQFSRAKDPSLKIGESQIATAEYTKRD
BO    NEENNHEIYKRFLFQFSRTKDPSLKTGESQIATAEYTKRD
BV    NEENNHEIYKRFLFOFSRAKDPSLKIGESQIATAEYTKRD
      ****************** ****** ***************

Human ---FTKKDHTATWGRPFFLFRPRNGRNIEDEAQIQW
Mouse TVDFPKKDPTTSLGRPFFLFRPRNGRYTDNNFQ---
Rat   TIDFPKKDPTTSLGRPFFLFRPRNGRYTDKV-Q---
        * *** *   ************       *
BM    SSGIVGRPFFLFRPRNGRKVSINEH
BO    SSGIVGRPFFLFRPRNGRKVSINEH
BV    SSGIVGRPFFLFRPRNGRKVSINEH
      *************************
```

Fig. 3. Alignment of full-length translated open-reading frames of NmS precursors from human, mouse, rat, and the toads, *B. maxima* (BM), *B. orientalis* (BO), and *B. variegata* (BV). * Denotes conserved residues in mammals; ⋆denotes conserved residues in toads.

NmS cDNA isoforms from the bombinid toads studied here have been deposited in the EMBL Nucleotide Sequence Database under the Accession Nos. AM115659 through AM115671.

Pharmacological investigation of stable NmU receptor-transfected CHO cell lines

Synthetic replicates of toad NmS-17 and toad NmS-33 were investigated for activity using CHO cell lines (CHO/FM-3-14 and CHO/FM-4-16) that had been stably transfected with either human FM-3/GPR66 or FM-4/TGR-1 NmU/NmS receptors. Human and rat NmS were tested at the same time in all experiments as internal controls for comparative purposes. The dose–response curves for each peptide ligand and at each NmU receptor-transfected CHO cell line are shown in Fig. 6. All NmS peptides tested generated classical sigmoidal dose–response curves, as assessed by quantitative changes in fluorescence intensity within the concentration range (10^{-13} M through 10^{-7} M) employed, and were of a high and comparable order of potency effecting receptor activation in the sub-nanomolar range. The calculated EC_{50} values for each NmS peptide at each receptor are summarized in Table 1 for clarification of

```
BM1   MRSEKHLLPLPLLLAICCLGTLHLSSGFPQSVPSYLEGLD
BM2   MRSEKHLLPLPLLLAICCLGTLHLSSGFPQSVPSYLEGLD
BM3   MRSEKHLLPLPLLLAICCLGTLHLSSGFPQSVPSYLEGLD
BM4   MRSEKHLLPLPLLLAICCLGTLHLSSGFPQSVPSYLEGLD
BM5   MRSEKHLLPLPLLLAICCLGTLHLSSGFPQSVPSYLEGLD
BM6   MRSEKHLLPLPLLLAICCLGTLHLSSGFPQSVPSYLEGLD

BM1   IPESERHAFCFSQWTALQDQEQIPSFVMDLCSSIYNRMKV
BM2   IPESE----------------IPSFVMDLCSSIYNRMKV
BM3   IPESERHAFCFSQWTALQDQEQIPSFVMDLCSSIYNRMKV
BM4   IPESERHAFCFSQWTALQDQEQIPSFVMDLCSSIYNRMKV
BM5   IPESERHAFCFSQWTALQDQEQIPSFVMDLCSSIYNRMKV
BM6   IPESERHAFCFSQWTALQDQEQIPSFVMDLCSSIYNRMKV

BM1   NEENNHEIYKRFLFQFSRAKDPSLKIGESQIATAEYTKRD
BM2   NEENNHEIYKRFLFQFSRAKDPSLKIGESQIATAEYTKRD
BM3   NEENNHEIYKRFLFQFSRAKDPSLKIG----------D
BM4   NEENNHEIYKR----------ESQIATAEYTKRD
BM5   NE----------ESQIATAEYTKRD
BM6   NE----------FSRAKDPSLKIGESQIATAEYTKRD

BM1   SSGIVGRPFFLFRPRNGRKVSINEH
BM2   SSGIVGRPFFLFRPRNGRKVSINEH
BM3   SSGIVGRPFFLFRPRNGRKVSINEH
BM4   SSGIVGRPFFLFRPRNGRKVSINEH
BM5   SSGIVGRPFFLFRPRNGRKVSINEH
BM6   SSGIVGRPFFLFRPRNGRKVSINEH
```

Fig. 4. Alignment of full-length translated open-reading frames of NmS precursors generated by splice variation (BM1 through 6) and cloned from a skin secretion library of the toad, *B. maxima*. KR (-Lys-Arg-) indicates propeptide convertase processing sites generating the N-terminals of NmS peptides and GR (-Gly-Arg-) indicates propeptide convertase/amidation enzyme site that generates the C-terminal asparaginamide. Sequences encoded by "spliced out" exons are indicated by hatched lines.

```
BO1   MRSEKHLPPLPLLLAICCLGTLHPSSGFPQSVPSYMEALD
BO2   MRSEKHLPPLPLLLAICCLGTLHPSSGFPQSVPSYMEALD
BO3   MRSEKHLPPLPLLLAICCLGTLHPSSGFPQSVPSYMEALD
BO4   MRSEKHLPPLPLLLAICCLGTLHPSSGFPQSVPSYMEALD
BO5   MRSEKHLPPLPLLLAICCLGTLHPSSGFPQSVPSYMEALD
BO6   MRSEKHLPPLPLLLAICCLGTLHPSSGFPQSVPSYMEALD

BO1   IPESEKLAFCFSQWTALPDQEQIPSFVMDLCSSIYNRMKV
BO2   IPESEKLAFCFSQWTALPDQEQIPSFVMDLCSSIYNRMKV
BO3   IPESEKLAFCFSQWTALPDQEQIPSFVMDLCSSIYNRMKV
BO4   IPESEKLAFCFSQWTALPDQEQIPSFVMDLCSSIYNRMKV
BO5   IPESE----------------IPSFVMDLCSSIYNRMKV
BO6   IPESEKLAFCFSQWTALPDQEQIPSFVMDLCSSIYNRMKV

BO1   NEENNHEIYKRFLFQFSRTKDPSLKTGESQIATAEYTKRD
BO2   NE---------FLFQFSRTKDPSLKTGESQIATAEYTKRD
BO3   NE------------------------ESQIATAEYTKRD
BO4   NEENNHEIYKR--------------------------D
BO5   NE-----------------------------------D
BO6   NEENNHEIYKRFLFQFSRTKDPSLKTG----------D

BO1   SSGIVGRPFFLFRPRNGRKVSINEH
BO2   SSGIVGRPFFLFRPRNGRKVSINEH
BO3   SSGIVGRPFFLFRPRNGRKVSINEH
BO4   SSGIVGRPFFLFRPRNGRKVSINEH
BO5   SSGIVGRPFFLFRPRNGRKVSINEH
BO6   SSGIVGRPFFLFRPRNGRKVSINEH
```

Fig. 5. Alignment of full-length translated open-reading frames of NmS precursors generated by splice variation (BO1 through 6) and cloned from a skin secretion library of the toad, *B. orientalis* KR (-Lys-Arg-) indicates propeptide convertase processing sites generating the N-terminals of NmS peptides and GR (-Gly-Arg-) indicates propeptide convertase/amidation enzyme site that generates the C-terminal asparaginamide. Sequences encoded by "spliced out" exons are indicated by hatched lines.

this point. Toad NmS-17 and NmS-33 were obvious cognate ligands for both human receptors with similar orders of potency compared to human NmS, and rat NmS that in turn have been found previously to be comparable to human NmU [20]. Toad NmS-17 was virtually indistinguishable from human NmS in terms of potency at the expressed human FM-4/TGR-1 receptor (0.231 ± 0.015 nM vs. 0.237 ± 0.027 nM; $n = 5$, mean ± SEM) whereas it was more potent than human NmS as a ligand for the FM-3/GPR66 receptor (0.085 ± 0.005 nM vs. 0.139 ± 0.013 nM).

Discussion

Neuromedin S has been identified in this study as a new amphibian venom/defensive skin secretion peptide. Reverse phase HPLC fractions of venom from all three species of discoglossid toad from the genus, *Bombina*, which were included in a radioimmunometric screen for bioactive peptides in a sample of 140 species of amphibian, were found to contain peptides that were reactive with an antiserum raised to NmU. *B. maxima* and *B. orientalis* venom fractions contained two immunoreactive peptides, in contrast to that of *B. variegata*, in which a single immunoreactive peptide was detected. Isolation and primary structural analysis of all five NmU-immunoreactive peptides indicated that they exhibited greater structural similarity to the novel neuropeptide, neuromedin S (NmS) [20], than to NmU peptides from the same spectrum of tetrapod vertebrates (Fig. 2).

Neuromedin S (NmS) is a recently discovered neuropeptide in man and rodents which is an obvious structural homolog of NmU that cross-reacts fully with C-terminally directed NmU antisera, interacts with both nominate NmU receptors, and shares biological effects, such as hypertension induction, smooth muscle contraction, and induction of anorexia [20]. However, detailed RT-PCR analysis of NmS distribution revealed that it was mainly expressed in the central nervous system, spleen, and testis [20]. Specifically within the brain, NmS expression was localized predominantly to the core region of the suprachiasmatic nucleus within the hypothalamus—a regulatory center for circadian rhythm that intracerebroventricular administration of NmS was found to shift. This discrete nucleus also expresses mRNA encoding the TGR-1 NmU receptor that was found to be NmS-preferring and thus endogenous NmS may interact with in an autocrine or paracrine manner.

In amphibians, NmU was discovered in the skin secretion of the Australasian White's tree frog (*L. caerulea*) in our laboratory using an approach similar to that adopted in the present study [18]. This peptide, of 23 amino acid residues, was found to be of similar molar potency to porcine NmU-25 in contraction of rat uterine smooth muscle and

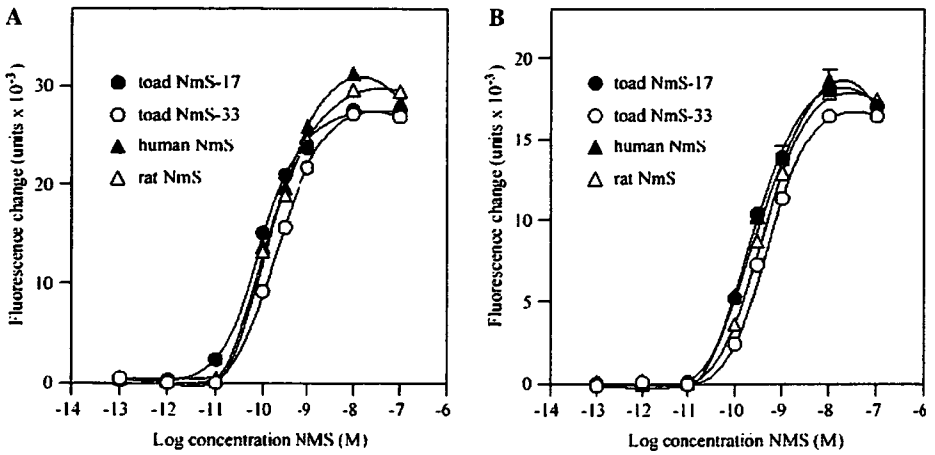

Fig. 6. Response curves for toad NmS-17 (filled circle), toad NmS-33 (open circle), human NmS (filled triangle), and rat NmS (open triangle) in the calcium mobilization assay using CHO cells stably expressing FM-3/GPR66 (A) or FM-4/TGR-1 (B) human NmU receptors. The calcium mobilization assay using the FLIPR system has previously been described in detail [20]. Data points represent the means ± SEM of six replicates.

Table 1
Half-maximal response concentrations (EC_{50}, in nM) of amphibian and mammalian NmS peptides for effecting calcium mobilization in FM-3/GPR66 or FM-4/TGR-1 stably transfected CHO cell lines

Peptide	FM-3/GPR66	FM-4/TGR-1
Toad NmS-17	0.085 ± 0.005	0.231 ± 0.015
Toad NmS-33	0.222 ± 0.018	0.439 ± 0.020
Human NmS	0.139 ± 0.013	0.237 ± 0.027
Rat NmS	0.148 ± 0.012	0.358 ± 0.021

EC_{50} was determined by the FLIPR assay [20], and data were analysed using GraphPad Prism ($n = 6$, mean ± SEM).

of similar affinity to rat NmU in displacing monoradioiodinated rat NmU ligand from rat uterine smooth muscle membranes. Molecular cloning of the frog NmU precursor cDNA from a frog skin library indicated that a series of splice variants were present in addition to the "full-length" open-reading frame transcript judged to be so by alignment with and structural similarities to the homologous human NmU precursor transcript [2]. Interrogation of a fetal human brain cDNA library indicated the same splice variants as discovered in the skin of the amphibian were also generated as a consequence of NmU gene expression in man and that the sites of splice variation occurred precisely at exon/intron boundaries within the human gene that consists of 10 exons [20]. Differential splicing of NmU mRNA is thus a highly conserved phenomenon within the tetrapod vertebrates—a finding that is certainly of biological relevance.

Molecular cloning of NmS cDNAs from libraries constructed using lyophilized venoms of *B. maxima* and *B. orientalis*, using a novel technique developed in our laboratory [19], revealed a series of six transcripts in each species that represented splice variants displaying a striking similarity to those obtained for NmU in amphibian skin and mammalian brain (Figs. 4 and 5). The venom library of *B. variegata* in contrast, apparently contained a single NmS-encoding transcript that represented one of the splice variants present in the other two congeneric species. The reason for this difference is unclear but of note is that *B. variegata* has a geographical distribution (Central Europe) that is different from the other Oriental species. The high degree of phylogenetic conservation in the splice variation observed following expression of the NmU gene in mammals and amphibians can be explained mechanistically by the structure of the human NmU gene that contains 10 exons. The coincident nature of amphibian and human transcripts would imply a very similar if not identical organisation of the amphibian NmU gene. The NmS gene in humans, although exhibiting relatively low nucleotide similarity (53%) with the NmU gene, is likewise composed of 10 exons with comparably conserved exon/intron boundaries. Though no evidence so far exists of differential splicing events occurring in the expression of the human NmS gene, the data presented in this study unequivocally demonstrate that such events occur following expression of the amphibian homolog. As in the case of the NmU gene, these differential splicing events occur predominantly within relatively short exons encoding peptide domains upstream of the NmS-encoding sequence within the precursor protein. Of particular note is the fact that several of these discrete exon-encoded peptide domains terminate in a typical -KR- propeptide convertase processing site such that differential splicing events within this region have the potential to alter the sites of cleavage and hence the nature of the peptide products so generated. This effect explains the presence of the two molecular variants of NmS detected in the venom of two species examined in the present study. NmS-17 was the predominant molecular form of NmS in two species of toad and the only molecular form in the third. This is considerably shorter in chain length than the 33-mers from human and the 36-mers from rat and mouse. Although a 33-mers NmS was isolated from the venom of two of the three species of toads studied, it arises in a different manner in the toads when compared with the human. Fig. 3 shows the alignments of mammalian and amphibian "full-length"

open-reading frames and the typical propeptide convertase processing sites (-KR-) that are indicated. As can be clearly seen, these differ in location between mammalian and amphibian precursors. The -KR- processing site in the amphibian precursors that resides immediately upstream of the NmS-17 encoding domain exhibits a site substitution in the mammalian homologs to -KK-. The specificity of the endogenous propeptide convertases in mammals obviously does not permit cleavage of this motif as no attenuated forms of mammalian NmS were reported in the original publication [20]. The generation of toad NmS-33 occurs as a result of a different process to the 33-mers human homolog. Alternative splicing of exons immediately upstream of the NmS encoding sequence in the amphibian gene (*B. maxima* and *B. orientalis*) results in the generation of a series of putative isomeric precursors. One of these isoforms within each of the two species (BM3 and BO6) encodes NmS-33 as a result of splicing out of an exon that encodes a peptide terminating in the -KR- processing site which generates NmS-17. The N-terminal domain of NmS-33 constitutes the N-terminal domain of the novel 33-mers peptide speculated to exist in the NmU precursor [2] and found to exist as a 34-mers in the human NmS precursor [20]. This novel NmU/NmS gene associated peptide was found to be a potent prolactin-releasing factor when administered intracerebroventricularly in rats [20]. However, intracerebroventricular administration of NmU in rats potently suppresses prolactin release [22]. Thus it would be intriguing to examine the effect of toad NmS-33, a peptide containing both contra-active domains, on prolactin release in this bioassay. While this was beyond the scope of the present study to assess, the ability of both toad NmS-17 and NmS-33 to activate stably transfected NmU receptors in CHO cell lines, as monitored by the generation of transient intracellular calcium fluxes, was studied. Both peptides were found to be equipotent with human NmS and rat NmS in activating both subtypes of NmU receptors, FM-3/GPR66 and FM-4/TGR-1, confirming the previous assertion that NmS peptides are cognate ligands for both of these NmU receptors [20]. Whether NmS or NmU peptides interact with these receptors appears to be determined by the differential spatial distribution of both components of this regulatory system. The fact that both NmU and NmS genes are obviously very closely related in many ways begs the question of their evolutionary origins. In other similar situations in peptide evolutionary biology, it is a general assumption that the degree of phylogenetic conservation of active site residues reflects two things: fundamental physiological importance and perhaps derivation. In Fig. 2, the primary structures of amphibian and mammalian NmS and NmU peptides are compared. Within this comparison that spans the tetrapod vertebrates, NmS displays a fully conserved C-terminal undecapeptide amide whereas a similar set of NmU peptides display a fully conserved pentapeptide amide. The C-terminal amidated region of NmU has been established as the active core with N-terminal extensions having subtle effects on bioactivity in a species-specific manner. This appears to be likewise true for NmS when comparing potencies of rat/mouse NmS-36 with toad NmS-17. In view of this evidence, we would contend that the original gene encoded NmS and that the gene duplication event that gave rise to NmU must have preceded tetrapod vertebrate evolution as both are separate entities in amphibians. Thus the results of this study have provided important insights into regulatory peptide biology and have posed several fundamental questions that will form the basis of further in-depth investigations to dissect the relative roles of NmS and NmU peptides in regulation of important physiological events such as control of feeding behavior and circadian rhythm modulation.

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Des-Acyl Ghrelin Induces Food Intake by a Mechanism Independent of the Growth Hormone Secretagogue Receptor

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Ghrelin, an acylated peptide produced predominantly in the stomach, stimulates feeding and GH secretion via interactions with the GH secretagogue type 1a receptor (GHS-R1a), the functionally active form of the GHS-R. Ghrelin molecules exist in the stomach and hypothalamus as two major endogenous forms, a form acylated at serine 3 (ghrelin) and a des-acylated form (des-acyl ghrelin). Acylation is indispensable for the binding of ghrelin to the GHS-R1a. Ghrelin enhances feeding via the neuronal pathways of neuropeptide Y and orexin, which act as orexigenic peptides in the hypothalamus. We here studied the effect of des-acyl ghrelin on feeding behavior. Intracerebroventricular (icv) administration of rat des-acyl ghrelin to rats or mice fed *ad libitum* stimulated feeding during the light phase; neither ip nor icv administration of des-acyl ghrelin to fasting mice suppressed feeding. The icv administration of des-acyl ghrelin induced the expression of Fos, a marker of neuronal activation, in orexin-expressing neurons of the lateral hypothalamic area, but not neuropeptide Y-expressing neurons of the arcuate nucleus. Peripheral administration of des-acyl ghrelin to rats or mice did not affect feeding. Although icv administration of ghrelin did not induce food intake in GHS-R-deficient mice, it did in orexin-deficient mice. In contrast, icv administration of des-acyl ghrelin stimulated feeding in GHS-R-deficient mice, but not orexin-deficient mice. Des-acyl ghrelin increased the intracellular calcium concentrations in isolated orexin neurons. Central des-acyl ghrelin may activate orexin-expressing neurons, perhaps functioning in feeding regulation through interactions with a target protein distinct from the GHS-R. (*Endocrinology* 147: 2306–2314, 2006)

GHRELIN IS A 28-amino-acid peptide isolated from human and rat stomach as an endogenous ligand for the GH secretagogue receptor (GHS-R) (1). The GHS-R, a G protein-coupled seven-transmembrane domain receptor, was initially identified as a receptor for small synthetic molecules termed GH secretagogues (GHSs), such as L-692,429, GHRP-6, and MK-0677, all of which act on the pituitary to stimulate GH secretion (2, 3). Two GHS-R subtypes are generated by alternative splicing of a single gene: the full-length type 1a receptor (GHS-R1a) and a carboxyl-terminally truncated form, the GHS-R type 1b (GHS-R1b), that encodes a protein containing transmembrane domain one to five (2, 3). The GHS-R1a is the functionally active, signal transducing form of the GHS-R, whereas the GHS-R1b is devoid of high-affinity ligand binding and signal transduction activity. Ghrelin molecules, predominantly produced by endocrine cells of the gastric oxyntic glands (4, 5), exist in two major molecular forms, ghrelin and des-*n*-octanoyl ghrelin (des-acyl ghrelin) (6). These two ghrelin molecules are also produced in the rat hypothalamus, as demonstrated by the combination of reverse-phase HPLC (RP-HPLC) with two separate RIAs recognizing ghrelin and des-acyl ghrelin (7, 8). All ghrelin species identified in fish, amphibians, birds, and many mammals possess a unique structural modification of the hydroxyl group of their third residue, which is either serine or threonine, by *n*-octanoic acid (9). This acylation is essential for the binding of ghrelin to the GHS-R1a (1, 10, 11); thus, the acylated form has been designated as ghrelin in our original description (1). Administration of ghrelin stimulates food intake in humans and rats (12–16) but does not change feeding behavior in GHS-R-deficient mice (17), suggesting that ghrelin enhances food intake via GHS-R-mediated signaling.

Several recent *in vitro* studies have demonstrated that radiolabeled ghrelin and des-acyl ghrelin bound to the membranes of PC-3 prostate tumor cells, H9c2 cadiomyocytes and isolated adipocytes, none of which expressed the GHS-R (18–20). This binding could be displaced by ghrelin, des-acyl ghrelin, and synthetic GHSs. Ghrelin and des-acyl ghrelin exhibit similar GHS-R-independent biological activities, including a cytoprotective effect on cultured cardiomyocytes

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Abbreviations: AgRP, Agouti gene-related protein; CRF, corticotropin-releasing factor; 2-DG, 2-deoxy-D-glucose; GHS, GH secretagogue; GHS-R, GH secretagogue receptor; HKRB, Krebs-Ringer bicarbonate buffer; icv, intracerebroventricular(ly); LHA, lateral hypothalamic area; MCH, melanin-concentrating hormone; NPY, neuropeptide Y; PVN, paraventricular nucleus; RP-HPLC, reverse-phase HPLC.

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(18), the inhibition of cell proliferation of breast carcinoma cell lines (19), the reduction of glycerol release from rat epididymal adipocytes (20), an ionotropic effect on guinea pig papillary muscle (21), and the promotion of bone marrow adipogenesis (22). Although the signaling molecules downstream of des-acyl ghrelin remain undefined, des-acyl ghrelin appears to share a subset of biological activities with ghrelin in peripheral tissues through an unidentified receptor or a target protein unique from the GHS-R.

The coordination of the regulation of food intake and energy expenditure occurs in the hypothalamus. Glucoprivic states induced by fasting or treatment with 2-deoxy-D-glucose (2-DG), a selective inhibitor of carbohydrate metabolism, increase feeding through the activation of orexigenic peptides, neuropeptide Y (NPY) and agouti gene-related protein (AgRP) in the arcuate nucleus (23). Secretion of des-acyl ghrelin from the rat hypothalamus increased in glucoprivic states induced by fasting or treatment with 2-DG (7). The axonal terminals of neurons that produce ghrelin and des-acyl ghrelin make direct synaptic contacts with NPY- and orexin-expressing neurons, which participate in hypothalamic feeding regulation (16, 24). Both ghrelin and des-acyl ghrelin may have a direct central action on the control of feeding.

We here investigated the effect of central or peripheral administration of des-acyl ghrelin on food intake in rats and mice and Fos expression, a marker of neuronal activation (25), in neurons that produce the orexigenic hypothalamic peptides, NPY/AgRP, orexin, or melanin-concentrating hormone (MCH). We studied the functional signaling downstream of des-acyl ghrelin using orexin-deficient mice and rats pretreated with antiorexin-A and -B IgGs. We confirmed that des-acyl ghrelin increased the intracellular calcium concentrations in orexin neurons dispersed from the lateral hypothalamic area (LHA) by the calcium-imaging analysis. We demonstrated that des-acyl ghrelin increased feeding by activation of orexin neurons in the LHA. We examined whether des-acyl ghrelin-induced food intake was mediated by the GHS-R pathway using GHS-R-deficient mice. Des-acyl ghrelin appears to regulate feeding via a receptor or target protein independent of the GHS-R.

Materials and Methods

Animals

We used male Wistar rats (Charles River Japan, Inc., Shiga, Japan), weighing 300–350 g, male C57BL/6 (Charles River Japan, Inc.), weighing 24–28 g, and male ddy mice (Kiwa Laboratory Animals Co., Ltd., Wakayama, Japan), weighing 35–39 g. Orexin-deficient mice (12-wk-old, male) and GHS-R-deficient mice (12-wk-old, male) were generated by targeted mutation of embryonic stem cells as reported (17, 26). All animals were individually housed in plastic cages at a constant room temperature in a 12-h light (0800–2000 h)/12-h dark cycle and given standard laboratory chow and water *ad libitum*. All procedures were approved by University of Miyazaki Animal Care and Use Committee and were in accordance with the Japanese Physiological Society's guidelines for animal care. Anesthesia was given as an ip injection of sodium pentobarbital (Abbot Labs., Chicago, IL). Intracerebroventricular (icv) cannulae were implanted into the lateral cerebral ventricles of rats and mice. Proper placement of the cannulae was verified at the end of the experiment by dye administration. Intravenous cannulae were implanted into the rat right jugular vein. Only animals that exhibited progressive weight gain after surgery were used.

Peptide synthesis

Rat ghrelin and des-acyl ghrelin were purchased from Peptide Institute, Inc. (Osaka, Japan). Adequate purification of synthesized peptides was ascertained by RP-HPLC, ion-exchange-HPLC, capillary zone electrophoresis, amino acid sequencing, and mass spectrometry (MALDI-MS). Ghrelin and des-acyl ghrelin were separately eluted, each as single peaks by RP-HPLC performed using a TSK ODS SIL 120A column (4.6 × 150 mm) (Tosho Co., Tokyo, Japan) with a linear gradient of 10–60% acetonitrile (CH_3CN) containing 0.1% trifluoroacetic acid (Fig. 1).

Fos expression

Ghrelin (200 pmol/10 μl saline), des-acyl ghrelin (200 pmol/10 μl saline), or saline was injected icv into Wistar rats or GHS-R-deficient mice 90 min before transcardial perfusion with fixative containing 4% paraformaldehyde. The brain was sectioned into 20- or 40-μm-thick samples. Fos-specific immunohistochemistry was performed as described (27). Hypothalamic sections from rats and GHS-R-deficient mice were incubated for 2 d with goat anti-Fos antiserum (Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1:1500), then stained using the avidin-biotin complex method (Vectastain Elite ABC kit; Vector Laboratories, Inc., Burlingame, CA). These sections were also stained with either rabbit antiorexin-A (dilution 1:3000) (16, 27) or rabbit anti-MCH (Phoenix Pharmaceuticals, Inc., Belmont, CA; dilution 1:200) antisera. We observed orexin- and MCH-expressing neurons by light microscopy. For immunofluorescence microscopy, we incubated hypothalamic sections of GHS-R-deficient mice with goat anti-Fos antiserum (dilution 1:1500) for 2 d at 4 C, then performed an additional 2 h incubation with

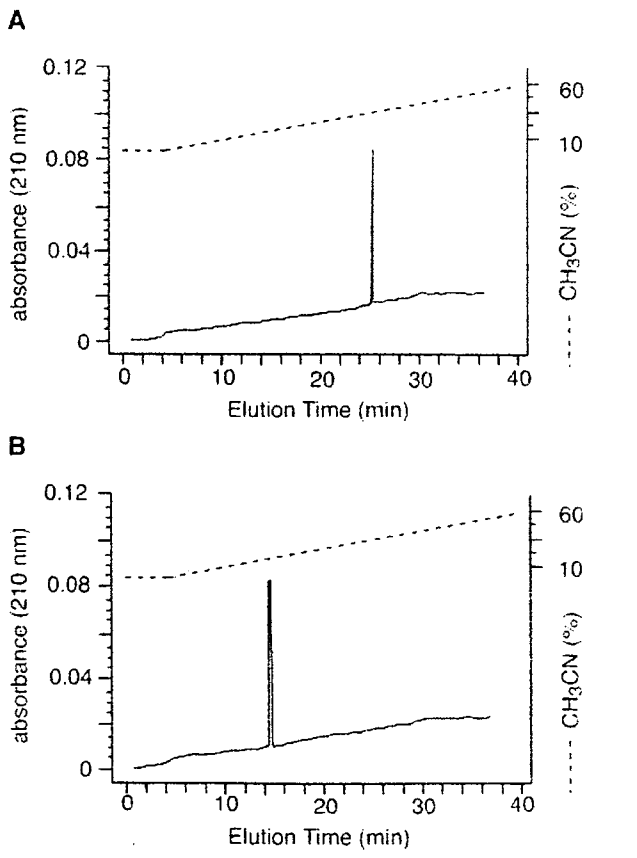

FIG. 1. RP-HPLC analysis of synthetic rat ghrelin (A) and des-acyl ghrelin (B) used for experimentation. Each peptide (0.3 nmol) was loaded onto a TSK ODS SIL 120A column using a linear gradient of 10–60% CH_3CN containing 0.1% trifluoroacetic acid at a rate of 1.0 ml/min for 40 min. Each peptide is eluted as a single peak whose elution position was identical with that of the corresponding synthetic peptide.

Alexa 488-conjugated donkey antigoat IgG antibody (Molecular Probes, Inc., Eugene, OR; dilution 1:400). After washing with PBS (pH 7.4), samples were incubated with a rabbit antiorexin-A antiserum for 2 d at 4 C and Alexa 546-labeled goat antirabbit IgG antibody (Molecular Probes; dilution 1:400) for a final 2 h. Slides were observed on a fluorescence microscope (BH2-RFC; Olympus, Tokyo, Japan).

Food intake

Experiments were performed 1 wk after the implantation of icv or iv cannulae. First, ghrelin or des-acyl ghrelin (each at 200 pmol/10 μl saline) was administered icv at 1000 h to rats fed *ad libitum* (n = 10 per group). The 1-, 2-, and 4-h food intake amounts were then measured. Second, des-acyl ghrelin (1 nmol/10 μl saline) was administered icv to rats (n = 6 per group) 10 min before the beginning of the dark phase, after which the 30-min food intake was measured. Third, ghrelin (1.5 nmol) or des-acyl ghrelin (1.5 or 5 nmol/100 μl saline) was administered iv to rats at 1000 h through an iv cannula. Fourth, des-acyl ghrelin (1 or 5 nmol/2 μl saline) was administered ip at 1000 h to C57BL/6 mice fed *ad libitum* (n = 8 per group). Fifth, 3 h after an icv administration of antiorexin-A and -B (each at 0.25 μg/2.5 μl saline), anti-NPY (0.5 μg/5 μl saline), or normal rabbit serum (0.5 μg/5 μl saline) IgGs, ghrelin or des-acyl ghrelin (each at 200 pmol/5 μl saline) was administered at 1200 h to rats (n = 10–12 per group). Sixth, ghrelin or des-acyl ghrelin (each at 200 pmol/2 μl saline) was administered icv at 1000 h to orexin-deficient mice or their wild-type littermates (n = 6–8 per group). Seventh, ghrelin (200 pmol/2 μl saline), des-acyl ghrelin (200 pmol/2 μl saline) or NPY (1 nmol/2 μl saline; Peptide Institute, Inc.) was administered icv at 1000 h to GHS-R-deficient mice or their wild-type littermates (n = 6–8 per group). With the exception of the first and second experiments, 2-h food intake was measured in all tests. Eighth, ghrelin or des-acyl ghrelin (each at 1 nmol/2 μl saline) was administered icv at 1000 h to ddy mice fed *ad libitum*. Ninth, des-acyl ghrelin (1 nmol/2 μl saline) was administered icv at 1000 h to ddy mice that had fasted for the previous 16 h (n = 8 per group). Tenth, des-acyl ghrelin (1 nmol/50 μl saline) was administered ip at 1000 h to ddy mice that had fasted for the previous 16 h (n = 8 per group). After the injections of ddy mice in the eighth, ninth, and tenth experiments outlined above, we measured 20-min, 1-h, and 2-h food intake. These feeding tests were performed using a cross-over design experiments in which animals were randomized to receive either test substance with a washout period of 3 d between each administration.

Measurement of cytosolic Ca^{2+} concentration ($[Ca^{2+}]i$)

The LHA of rat brain was punched out according to the *Atlas of the Rat Brain* (28). The tissue was washed twice with HEPES and Krebs-Ringer bicarbonate buffer (HKRB) [129 mM NaCl, 5.0 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 10 mM HEPES (pH 7.4)] containing 10 mM glucose. The LHA was incubated in HKRB supplemented with 1 mg/ml papain (Sigma-Aldrich, St. Louis, MO), 5 mg/ml deoxyribonuclease, and 0.025% BSA for 20 min at 36 C in a shaking water bath, then LHA cells were dispersed by mechanical desegregation for 4 min. The cell suspension was diluted with HKRB and centrifuged at 100 × *g* for 5 min. The pellet was resuspended in HKRB and distributed onto the glass well (Nunc 96 Microwell Optical Bottom Plate; Nalge Nunc International, Rochester, NY). Measurement of $[Ca^{2+}]i$ was carried out 2–4 h after the preparation of cells. The cells were loaded with Fluo-3 for 20 min in HEPES buffer solution [10 mM HEPES, 140 mM NaCl, 5.0 mM KCl, 1.2 mM $MgCl_2$, 2.0 mM $CaCl_2$, 10 mM glucose, and 2 μm Fluo-3/acetoxymethylester (Dojindo Labs, Kumamoto, Japan) (pH 7.2)]. They were washed twice with HEPES buffer, then filled with 100 μl HEPES buffer. One min after, 50 μl of 3 μM rat des-acyl ghrelin were added into the well. $[Ca^{2+}]i$ was determined by measuring fluorescence signal from the Ca^{2+} indicator Fluo-3/acetoxymethylester, with 480 nm excitation and 530 nm emission using a cooled charge-coupled device camera, and the ratio image was produced in Functional Imaging Cell-Sorting System (IMACS; Hamamatsu Photonics, Hamamatsu, Japan). The level of $[Ca^{2+}]i$ in a single neuron was recorded for 6 min after the administration of des-acyl ghrelin. After $[Ca^{2+}]i$ measurement, the neurons were fixed with 4% paraformaldehyde overnight. They were incubated with rabbit antiorexin-A antiserum (dilution 1:1500) for 2 d at 4 C, then Alexa 350-conjugated goat antirabbit IgG antibody (dilution 1:400) for 2 h (16, 27). The picture of calcium imaging was collated with the immunohistochemical picture. Fluorescence signals from Fluo-3 were converted automatically to pseudo colors in IMACS. The levels of $[Ca^{2+}]i$ were assigned pseudo colors ranging from blue of the lowest value through yellow to red of the highest value. Fluorescence signals from Alexa-350 were shown in white.

Measurement of locomotor activity

Locomotor activity of rats was measured using a rat locomotor activity recording system (Muromachi Co. Ltd., Tokyo, Japan) comprising infrared sensors, an interface and a computer. The infrared sensors were placed above the cages and measured all locomotor activity. A cage with the infrared sensor was placed in an isolated chamber with a controlled light/dark cycle. Rats were given icv des-acyl ghrelin (1 nmol/10 μl saline), human orexin-A (Peptide Institute, Inc.; 1 nmol/10 μl saline) or saline administration icv at 0900 h (n = 5 per group), then these rats were immediately returned to their individual cages. Locomotor activity counts were made 30 min and analyzed by Compact ACTAM Software (Muromachi Co. Ltd.).

GH response

Ghrelin or des-acyl ghrelin (each at 1.5 nmol/100 μl) was administered iv to rats (n = 6 per group) at 1000 h. Blood samples (80 μl) were obtained from the tail veins at 0, 15, 30, and 60 min after administration. After removal, six anterior pituitary glands of rats were immersed in Hanks' balanced salt solution, then incubated at 37 C for 30 min. Each one pituitary grand was placed in a polystyrene well (16 mm in diameter; Iwaki Glassware Co., Tokyo, Japan) filled with oxygenated medium (DMEM containing 2.5% fetal calf serum and 2.5% bovine serum). After rinsing twice in 500 μl medium for 1 min each, 750 μl medium was added into each well for 5 min. The medium was then collected into plastic microtubes to evaluate basal GH secretion. The pituitary glands were then stimulated for 5 min with medium containing either ghrelin or des-acyl ghrelin (1 μM). The medium was collected into plastic microtubes to quantify GH concentration with a Biotrak Rat GH RIA kit

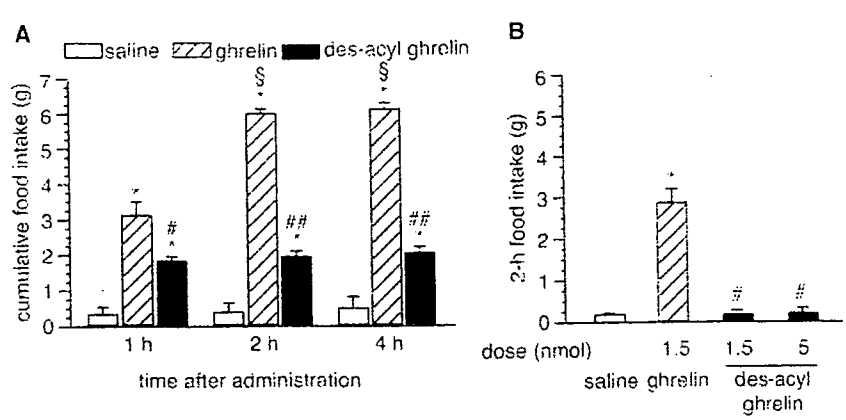

FIG. 2. Effect of ghrelin or des-acyl ghrelin on food intake in rats. A, Intracerebroventricular administration of des-acyl ghrelin or ghrelin (each at 200 pmol) at 1000 h. *, $P < 0.001$ *vs.* saline; #, $P < 0.05$; ##, $P < 0.001$ *vs.* ghrelin; §, $P < 0.001$ *vs.* 1-h food intake. B, Intravenous administration of ghrelin (1.5 nmol) or des-acyl ghrelin (1.5 or 5 nmol) at 1000 h. *, $P < 0.001$ *vs.* saline; #, $P < 0.001$ *vs.* ghrelin.

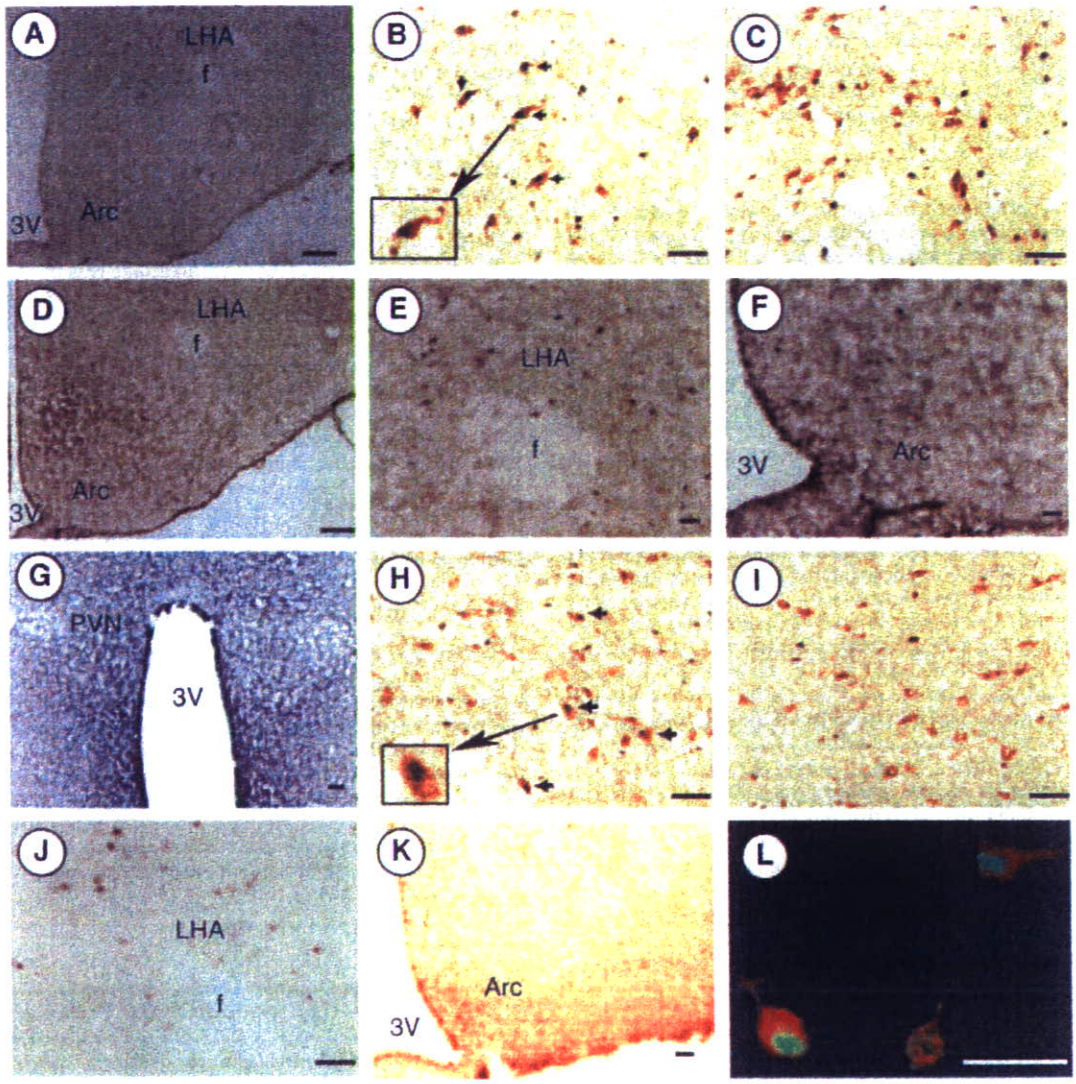

FIG. 3. Fos expression in the hypothalamus after icv administration of ghrelin or des-acyl ghrelin (each at 200 pmol). A, Fos expression (*dark blue-black*) in the LHA and arcuate nucleus of rats given ghrelin. B, Costaining (*arrows*) of Fos (*dark blue-black*) and orexin (*brown*) in rats given ghrelin. The *inset* is a higher magnification of Fos expression in an orexin-expressing neuron. C, No Fos (*dark blue-black*) expression in MCH-expressing neurons (*brown*) after ghrelin administration. D, Fos expression (*dark blue-black*) in the hypothalamus of rats given des-acyl ghrelin. E, Fos expression (*dark blue-black*) in the LHA of rats given des-acyl ghrelin. No Fos (*dark blue-black*) expression in the arcuate nucleus (F) or the PVN (G) after des-acyl ghrelin administration. H, Costaining (*arrows*) of Fos (*dark blue-black*) and orexin (*brown*) in rats given des-acyl ghrelin. The *inset* is a higher magnification of Fos expression in an orexin-expressing neuron. I, No expression of Fos (*dark blue-black*) is observed in MCH-expressing neurons (*brown*) after des-acyl ghrelin administration. J, Fos expression (*dark blue-black*) in the LHA of GHS-R-deficient mice given des-acyl ghrelin. K, No Fos expression (*brown*) in the arcuate nucleus of GHS-R-deficient mice given des-acyl ghrelin. L, Costaining of Fos (*green*) and orexin (*red*) in the LHA neurons of GHS-R-deficient mice given des-acyl ghrelin. 3V, Third ventricle; Arc, arcuate nucleus; f, fornix. *Scale bars*, A, D, 200 μm; B, C, E, F, H, G, H, I, J, K, L, 50 μm.

(Amersham, Buckinghamshire, UK). The experiment was concluded by treatment with 60 mM KCl to induce depolarization.

Statistic analysis

Data (mean ± SEM) were analyzed by ANOVA and the *post hoc* Scheffé's F test. Differences were considered to be significant when the P values were less than 0.05.

Results

Des-acyl ghrelin-induced food intake

The icv administration of either ghrelin or des-acyl ghrelin to rats stimulated food intake (Fig. 2A). Although ghrelin increased food intake for at least 2 h after administration, the effect of des-acyl ghrelin lasted for only 1 h. Des-acyl ghrelin also significantly increased the early dark-phase food intake in rats in comparison to saline administration (des-acyl ghrelin, 2.04 ± 0.37 g/30 min; saline, 0.96 ± 0.23 g/30 min, $P < 0.05$). Next, we studied the effect of peripherally administered des-acyl ghrelin on feeding. A single iv administration of ghrelin significantly increased feeding, whereas des-acyl ghrelin did not (Fig. 2B). An ip administration of des-acyl ghrelin to C57BL/6 mice fed *ad libitum* did not increase food intake (des-acyl ghrelin 1 nmol, 0.05 ± 0.03 g/2 h; 5 nmol, 0.04 ± 0.02 g/2 h; saline, 0.05 ± 0.02 g/2 h).

Fos expression

Intracerebroventricular administration of ghrelin induced Fos expression in the LHA and arcuate nucleus (Fig. 3A). In contrast, icv administration of des-acyl ghrelin induced Fos in the LHA, but not the arcuate nucleus or the paraventricular nucleus (PVN) (Fig. 3, D–G). By double immunohistochemistry, ghrelin induced Fos in 32 ± 7% of orexin-immunoreactive neurons (Fig. 3B). Des-acyl ghrelin induced Fos in 22 ± 5% of orexin-immunoreactive neurons (Fig. 3H). In the LHA, neither ghrelin nor des-acyl ghrelin induced Fos in MCH-immunoreactive neurons (Fig. 3, C and I). Intracerebroventricular administration of des-acyl ghrelin to GHS-R-deficient mice induced Fos in the LHA, but not the arcuate nucleus (Fig. 3, J and K). In GHS-R-deficient mice, des-acyl ghrelin induced Fos expression in 28 ± 2% of orexin-immunoreactive neurons, whereas ghrelin did not induce the expression of Fos in any hypothalamic neurons (Fig. 3L).

Functional relationship between des-acyl ghrelin and orexin in feeding

We examined the effects of orexin and NPY blockades on des-acyl ghrelin-induced food intake. Both ghrelin and des-acyl ghrelin increased food intake in rats given an icv administration of control IgG (Fig. 4). Pretreatment with anti-orexin-A and -B IgGs, however, reduced ghrelin-induced food intake by 29% from the amounts seen in rats given control IgG and ghrelin, whereas pretreatment with anti-orexin-A and -B IgGs completely abolished des-acyl ghrelin-induced food intake. Whereas pretreatment with anti-NPY IgG reduced ghrelin-induced feeding in rats in comparison to rats given control IgG and ghrelin, anti-NPY IgG did not affect des-acyl ghrelin-induced feeding in comparison to rats given control IgG and des-acyl ghrelin (Fig. 4).

Orexin-deficient mice were used to verify the functional relationship between des-acyl ghrelin and orexin in feeding regulation. Although ghrelin induced food intake in orexin-deficient mice, the potency of this induction in these mice was significantly reduced from that seen in wild-type littermates (Fig. 5A). Des-acyl ghrelin stimulated feeding in wild-type mice, but not in orexin-deficient mice (Fig. 5A). To investigate whether des-acyl ghrelin regulates feeding through the GHS-R, we gave an icv administration of des-acyl ghrelin to GHS-R-deficient mice. Des-acyl ghrelin, but

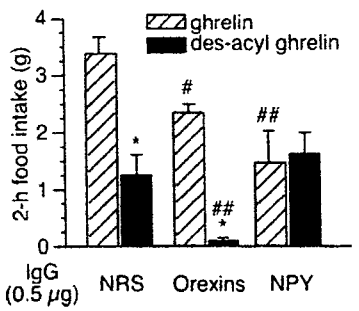

FIG. 4. The effect of antiorexin-A and -B (each at 0.25 μg) and anti-NPY (0.5 μg) IgGs on ghrelin- or des-acyl ghrelin-induced feeding. Ghrelin or des-acyl ghrelin (each at 200 pmol) was given to rats 3 h after icv administration of IgG. The 2-h food intake was then measured. NRS, Normal rabbit serum. *, $P < 0.01$ *vs.* ghrelin; #, $P < 0.05$; ##, $P < 0.01$ *vs.* NRS IgG.

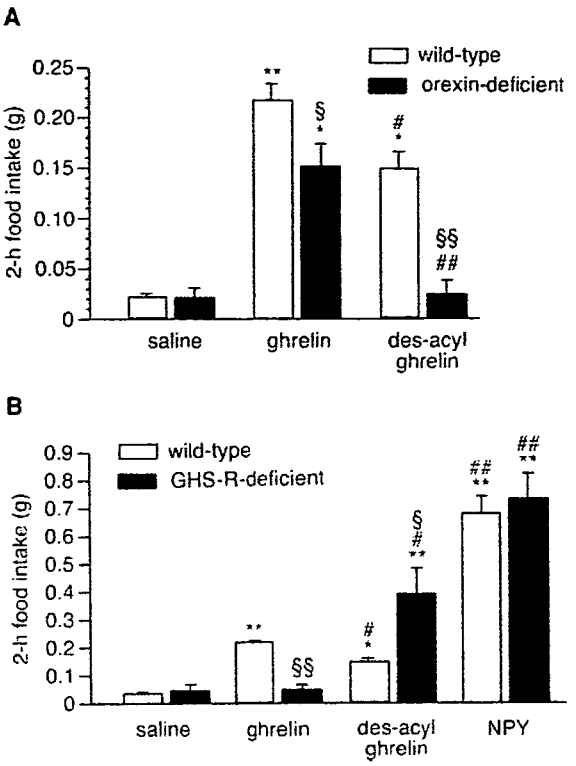

FIG. 5. A, Effect of icv administration of ghrelin or des-acyl ghrelin (each at 200 pmol) on 2-h food intake in orexin-deficient mice. *, $P < 0.05$, **; $P < 0.01$ *vs.* saline; #, $P < 0.05$; ##, $P < 0.01$ *vs.* ghrelin, §, $P < 0.05$, §§, $P < 0.01$ *vs.* wild-type mice. B, Effect of icv administration of ghrelin (200 pmol), des-acyl ghrelin (200 pmol), or NPY (1 nmol) on 2-h food intake in GHS-R-deficient mice. *, $P < 0.05$; **, $P < 0.001$ *vs.* saline; #, $P < 0.001$ *vs.* ghrelin; §, $P < 0.05$; §§, $P < 0.01$ *vs.* wild-type mice.

not ghrelin, stimulated feeding in GHS-R-deficient mice (Fig. 5B). Des-acyl ghrelin-induced feeding in GHS-R-deficient mice was more potent than that induced in wild-type littermates. NPY was used as a positive control to evaluate the orexigenic effects on GHS-R-deficient mice. NPY-induced food intake was similar in both GHS-R-deficient mice and their wild-type littermates (Fig. 5B).

Intracerebroventricular administration of des-acyl ghrelin increased food intake in ddy mice fed *ad libitum* (Fig. 6A). Neither icv nor ip administration of des-acyl ghrelin suppressed food intake in ddy mice that had fasted for 16 h (Fig. 6, B and C).

Cytosolic [Ca^{2+}]i response in orexin neurons

We studied the cytosolic [Ca^{2+}]i response of orexin-expressing neurons to des-acyl ghrelin. Some cells dispersed from the rat LHA showed increased cytosolic [Ca^{2+}]i in response to des-acyl ghrelin administration (Fig. 7, A and B). These cells showed orexin immunoreactivity by immunohistochemistry (Fig. 7C).

Locomotor activity

We examined the effect of des-acyl ghrelin on locomotor activity. Intracerebroventricular administration of des-acyl

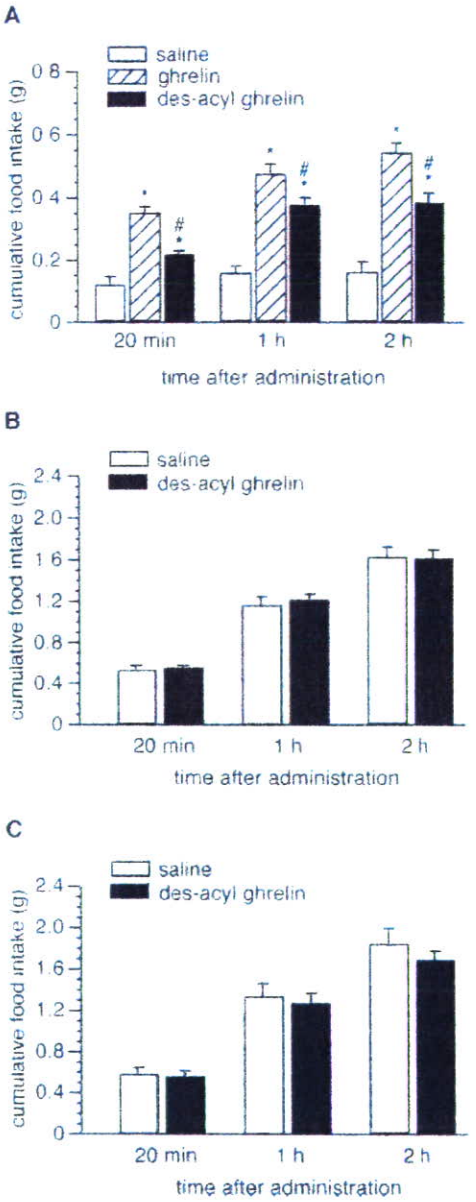

FIG. 6. Effect of des-acyl ghrelin on food intake in ddy mice. A, Intracerebroventricular administration of ghrelin or des-acyl ghrelin (each at 1 nmol) at 1000 h to mice *ad libitum*. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* saline; #, $P < 0.01$ *vs.* ghrelin. Neither icv (B) nor ip (C) administration of des-acyl ghrelin (1 nmol) suppressed feeding in mice that had fasted for 16 h.

ghrelin to rats significantly increased locomotor activity compared with saline administration (Fig. 8). Orexin-A also significantly increased locomotor activity in these rats.

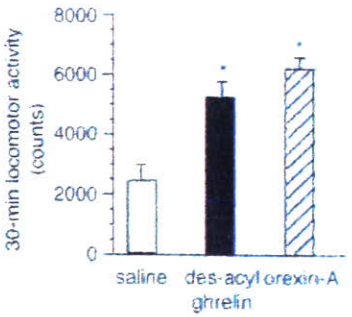

FIG. 8. Effects of icv administration of des-acyl ghrelin or orexin-A (each at 1 nmol) on locomotor activity in rats. *, $P < 0.05$ *vs.* saline.

GH response

We studied the release of GH in response to peripheral des-acyl ghrelin administration. Intravenous administration of ghrelin elicited a marked increase in plasma GH levels, with the peak occurring 15 min after administration (Fig. 9A). Intravenous administration of des-acyl ghrelin did not stimulate GH release. We examined the effect of des-acyl ghrelin on GH release from isolated samples of the rat anterior pituitary. GH concentrations in the culture medium of the anterior pituitary cultures increased in response to ghrelin administration, but not to des-acyl ghrelin administration (Fig. 9B).

Discussion

At present, the amino acid sequences of ghrelin peptides in 21 species of fish, amphibians, birds, and mammals have been determined (9). All of the ghrelin molecules identified possess a serine or threonine as the third amino acid residue. A hydroxyl group of this amino acid forms an ester with a monocarboxylic acid of medium-chain fatty acid (6). Ghrelin peptide is present in the stomach of humans, rats, and mice as two major molecular forms: ghrelin and des-acyl ghrelin (6). In the plasma, ghrelin accounts for only 2–20% of total ghrelin immunoreactivity (6, 7, 28–30). This is likely due to the shorter half-life of ghrelin than that of des-acyl ghrelin because plasma ghrelin rapidly disappears from the circulation because of binding to the GHS-R in the systemic tissues (31). Deacylation of ghrelin to des-acyl ghrelin, which rapidly occurs in the plasma, is also responsible for the reduced half-life of ghrelin. Two enzymes involved in the deacylation of ghrelin have been identified: high-density lipoprotein-associated paraoxonase functions in the plasma, whereas lysophospholipase I, a thioesterase active against palmitoyl-Gsα and plamitoyl-coenzyme A, functions in the stomach (32–34). In contrast, the enzyme that catalyzes the acyl modification of ghrelin has not been identified.

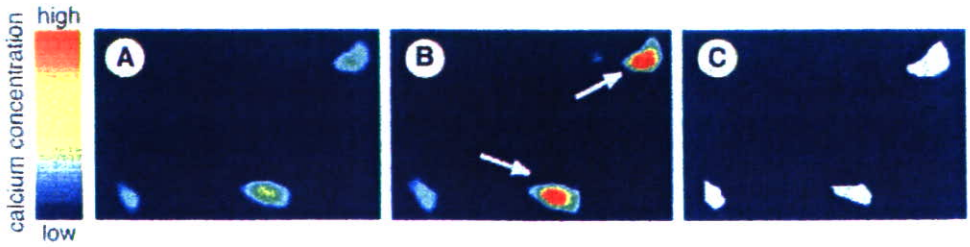

FIG. 7. Effect of des-acyl ghrelin on $[Ca^{2+}]i$ response in isolated orexin-expressing neurons. A, A picture shows the basal level of $[Ca^{2+}]i$ in neurons (*blue*) before administration of des-acyl ghrelin. B, Des-acyl ghrelin increased cytosolic $[Ca^{2+}]i$ in two neurons (*arrows*). C, Immunostaining of orexin-expressing neurons (*white*) after the measurement of $[Ca^{2+}]i$ response.

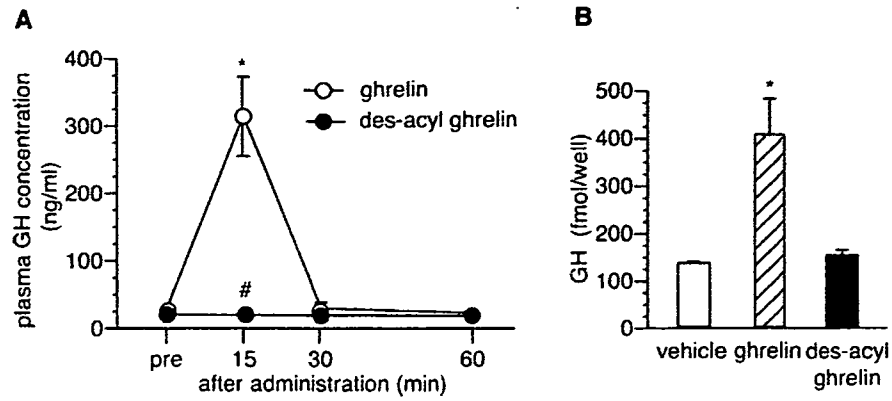

FIG. 9. A, Effect of iv administration of ghrelin or des-acyl ghrelin (each at 1.5 nmol) on the plasma GH concentration in rats. *, $P < 0.001$ *vs.* preadministration; #, $P < 0.001$ *vs.* ghrelin at the same time point. B, Effect of ghrelin or des-acyl ghrelin administration (each at 200 pmol) on GH release from rat pituitary gland (n = 6 per group). *, $P < 0.001$ *vs.* control vehicle.

Acylation of ghrelin is essential for ghrelin's GH-releasing activity (1, 9, 10); several recent *in vitro* studies have shown that des-acyl ghrelin exhibits biological activities on the cell proliferation and metabolism of cardiomyocytes, adipocytes, myocytes, and myelocytes (18–22). Although many of these cells did not express the GHS-R, des-acyl ghrelin bound to their cell membranes (18–20). We here examined the orexigenic activity of des-acyl ghrelin. We confirmed the purity of ghrelin and des-acyl ghrelin by several biochemical methods before using these substances in feeding experiments. Both ghrelin and des-acyl ghrelin were completely pure by RP-HPLC, ion-exchange-HPLC, capillary zone electrophoresis, and mass spectrometry. Des-acyl ghrelin did not stimulate GH release when either peripherally administered to rats or applied directly to the rat pituitary *in vitro*. Intracerebroventricular administration of des-acyl ghrelin significantly induced feeding during both the light and dark phases in rats. Intracerebroventricular administration of des-acyl ghrelin also increased food intake in GHS-R-deficient mice and their wild-type littermates. Two recent studies reported the anorexic activity of des-acyl ghrelin in rats and mice (35, 36). In these studies, ip administration of des-acyl ghrelin suppressed feeding in rats had fasted for 16 h (35). Both ip and icv administrations of des-acyl ghrelin suppressed feeding in ddy mice that had been fasting for 16 h; icv administration of des-acyl ghrelin did not significantly change the light phase food intake in ddy mice fed *ad libitum* (36). These studies described that icv and ip administrations of des-acyl ghrelin expressed Fos in the PVN neurons, presumably corticotropin-releasing factor (CRF) neurons (35, 36). We also examined the effect of des-acyl ghrelin on feeding in ddy mice because the anorexic effect noted above contrasted the orexigenic effect observed in C57BL/6 mice. An icv administration of des-acyl ghrelin significantly increased the light phase food intake of ddy mice fed *ad libitum*. The icv administration of des-acyl ghrelin did not suppress food intake in ddy mice that had fasted for 16 h. In addition, an icv administration of des-acyl ghrelin did not express Fos in any neurons of the PVN where CRF-producing neurons are present. Because the effects of peptides in feeding experiments are hampered by unsatisfactory habituation (37, 38), all of the rats and mice used in these experiments were satisfactorily acclimated to handling before ip and icv injections. We do not know why our findings conflicted with previous results; des-acyl ghrelin, however, reproducibly stimulated feeding in rats, C57BL/6 mice and ddy mice. Des-acyl ghrelin, which was synthesized in the rat hypothalamus, was released in response to fasting (7). The ratio of des-acyl ghrelin to ghrelin in the rat hypothalamus was 2:1 under *ad libitum* conditions, and this ratio did not change in upon fasting. Des-acyl ghrelin, as well as ghrelin, may serve as orexigenic peptides in the hypothalamus.

Ghrelin-producing neurons localize to the hypothalamic arcuate nucleus and adjacent to the third ventricle between the dorsal, ventral, paraventricular, and arcuate hypothalamic nuclei of rats and mice (1, 24). Ghrelin fibers synapse on NPY/AgRP neurons in the arcuate nucleus and orexin neurons in the LHA (16, 24, 39). We investigated the signaling events downstream of des-acyl ghrelin that stimulates feeding. Fos expression, induced by icv administration of des-acyl ghrelin, was restricted to orexin-expressing neurons in the LHA. Des-acyl ghrelin-induced food intake was completely abolished in rats by pretreatment with antiorexin IgG, but not anti-NPY IgG or control serum IgG. Des-acyl ghrelin did not stimulate feeding in orexin-deficient mice. These results indicate that des-acyl ghrelin-induced feeding is mediated by the activation of the orexin pathway. Orexin-A and -B are hypothalamic peptides functioning in the regulation of feeding, energy homeostasis, and arousal (40). Approximately 3000 orexin-expressing neurons are present in the LHA of rats and mice. Orexin-positive nerve fibers have wide projections onto a variety of brain regions, such as the arousal centers in the forebrain and brain stem and the feeding center within the hypothalamus (40). Orexin-expressing neurons are heterogeneous in their anatomical projections and physiological functions, playing multifaceted roles in the brain. Ghrelin fibers project to orexin-positive neurons (16) and ghrelin stimulated elecrophysiological activity of isolated orexin neurons in the whole-cell patch-clamp study (41); we demonstrate here that ghrelin and des-acyl ghrelin acted on orexin-expressing neurons and that des-acyl ghrelin increased intracellular calcium concentration in isolated orexin neurons. There are three possible subtypes of orexin neurons: those that express the GHS-R as a receptor for ghrelin, those expressing an as-yet unknown target protein of des-acyl ghrelin, neurons possessing both proteins. Orexin also functions to maintain wakefulness (40). We examined the effect of des-acyl ghrelin on locomotor activity. As expected, icv administration of des-acyl ghrelin increased locomotor activity, suggesting that des-acyl ghrelin may increase wake-

fulness and locomotor activity for food seeking by stimulating orexin neurons.

We next investigated the functional relationship between des-acyl ghrelin and the GHS-R using GHS-R-deficient mice. The icv administration of des-acyl ghrelin to GHS-R-deficient mice induced food intake and Fos expression in orexin-expressing neurons. The icv administration of ghrelin to GHS-R-deficient mice did not stimulate food intake. Des-acyl ghrelin did not bind to GHS-R-expressing Chinese hamster ovary cells and did not inhibit the binding of ghrelin to rat pituitary culture cells expressing the GHS-R (1, 10, 42), implying that des-acyl ghrelin does not compete with ghrelin for the binding to the GHS-R. Thus, des-acyl ghrelin is thought to stimulate feeding via a mechanism independent of the GHS-R.

A number of gastrointestinal peptides transmit satiety or starvation signals to the nucleus of the solitary tract via the vagal afferents and/or to the hypothalamus via the bloodstream (43). Although iv administration of ghrelin stimulated both vagal afferents and feeding, iv administration of des-acyl ghrelin affected neither (44). Peripheral administration of des-acyl ghrelin to rats and mice did not affect feeding. Receptors on vagal afferents are generated by nodose ganglion neurons, transported to the nerve terminals through axonal transport (45). These results indicate that a receptor or a target protein binding to des-acyl ghrelin is not expressed in nodose ganglion neurons. The plasma concentration of des-acyl ghrelin increased upon fasting (7). The peripheral des-acyl ghrelin does not act to suppress feeding.

In summary, centrally administered des-acyl ghrelin increased feeding through activation of the orexin pathway. In addition to its peripheral actions, which include cell proliferation, inhibition of apoptosis, and fat metabolism (18–22), des-acyl ghrelin may function in hypothalamic feeding regulation. Central administration of des-acyl ghrelin to GHS-R-deficient mice stimulated feeding, suggesting that des-acyl ghrelin acts on a target protein that is specific for des-acyl ghrelin and independent of the GHS-R. Ghrelin and des-acyl ghrelin act in the regulations of peripheral cell functions through a common putative target protein (18–22). Ghrelin and des-acyl ghrelin function as orexigenic peptides in the hypothalamus. Des-acyl ghrelin may have basal effects of ghrelin-related peptides. Further studies examining the physiological and neuroanatomical interactions between des-acyl ghrelin and its target will establish roles of ghrelin peptides in the regulation of feeding and energy homeostasis.

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Maternal Ghrelin Plays an Important Role in Rat Fetal Development during Pregnancy

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Ghrelin, an acylated peptide serving as an endogenous ligand for GH secretagogue receptor (GHS-R), was originally isolated from rat and human stomach. In this study, we report the critical role of maternal ghrelin in fetal development. High levels of ghrelin receptor (GHS-R) mRNA were detected in various peripheral fetal tissues beginning at embryonic d 14 and lasting until birth. Fetal GHS-R expression was also confirmed in fetal tissues by immunohistochemistry. Autoradiography revealed that both des-acyl ghrelin and acyl ghrelin bind to fetal tissues. Chronic treatment of mothers with ghrelin resulted in a significant increase in birth weight in comparison to newborns from saline-treated mothers. Even when maternal food intake after ghrelin treatment was restricted through paired feeding, significant stimulation of fetal development still occurred. Conversely, active immunization of mothers against ghrelin decreased fetal body weight during pregnancy. A single ghrelin injection into the mother increased circulating ghrelin levels in the fetus within 5 min of injection, suggesting that maternal ghrelin transits easily to the fetal circulation. High levels of des-acyl ghrelin were detected in fetal blood and amniotic fluid. Both acylated and des-acyl ghrelin increased [^{3}H]thymidine and 5-bromo-2′-deoxyuridine incorporation of cultured fetal skin cells in a dose-dependent manner, and calcium-imaging analysis revealed that acyl and des-acyl ghrelin increased the Ca^{2+} influx in discrete cultured fetal skin cells, respectively. These results indicate that maternal ghrelin regulates fetal development during the late stages of pregnancy. (*Endocrinology* 147: 1333–1342, 2006)

GHRELIN, RECENTLY purified from rat and human stomachs as an endogenous ligand for the GH secretagogue receptor (GHS-R), is a 28-amino acid peptide with an *n*-octanoylation modification at Ser^3 (1). This octanoylation is important for the stimulation of GH secretion from the pituitary gland (1). Although cells immunostained for ghrelin are distributed widely in the stomach, hypothalamus, pituitary gland, liver, kidney, pancreas, and placenta, the main source of circulating ghrelin is considered to be the gastrointestinal tract (2–6). Both acylated and des-acyl ghrelin are observed in the peripheral circulation, the levels of des-acyl ghrelin being higher (7, 8). Several studies on the physiological function of ghrelin have demonstrated that, in addition to stimulating GH secretion, ghrelin also stimulates food intake and body weight gain independent of GH secretion (8–13). It is likely that the appetite-stimulating effect of peripheral ghrelin is due to action via the afferent vagal nerve (14). In contrast, the central effect is thought to be via neuropeptide Y and agouti-related peptide secretion from the arcuate nucleus in the hypothalamus (11, 15). Administration of ghrelin continuously to rodents resulted in fat deposition and obesity (9). These effects of ghrelin on appetite and fat deposition are counteracted by leptin (11). These results imply that ghrelin may play an important role in the regulation of food intake and energy expenditure.

The ghrelin receptor GHS-R, highly conserved from teleost fish to humans, is expressed widely in both central and peripheral organs, including the brain, pituitary gland, and pancreas (16–19). The broad distribution of GHS-R suggests that ghrelin may play important roles in addition to those mentioned above. It has been also demonstrated that ghrelin might be involved in stomach motility (10), gastric acid secretion (20), insulin and gastrin release (21), the cardiovascular system, and stress reactions (12). In addition, we demonstrated previously that neonatal rats treated daily with ghrelin for 2 or 3 wk from birth showed faster eye and vaginal opening than those of saline-treated group (22), suggesting that ghrelin may be involved in neonatal development. Therefore, it has been assumed that ghrelin from the maternal stomach or placenta during pregnancy may play a role in fetal development. In the present study, we examined the possible involvement of maternal ghrelin in rat fetal development.

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Abbreviations: BrdU, 5-Bromo-2′-deoxyuridine; E, embryonic day; GHS-R, GH secretagogue receptor; mcKLH, mariculture keyhole limpet hemocyanin.

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Materials and Methods

Animals

Wistar rats were housed under controlled temperature (23 ± 1 C) and regulated 12-h light 12-h dark conditions (lights on at 0700 h). Female rats were mated on the day of proestrus at approximately 3 months old.