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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

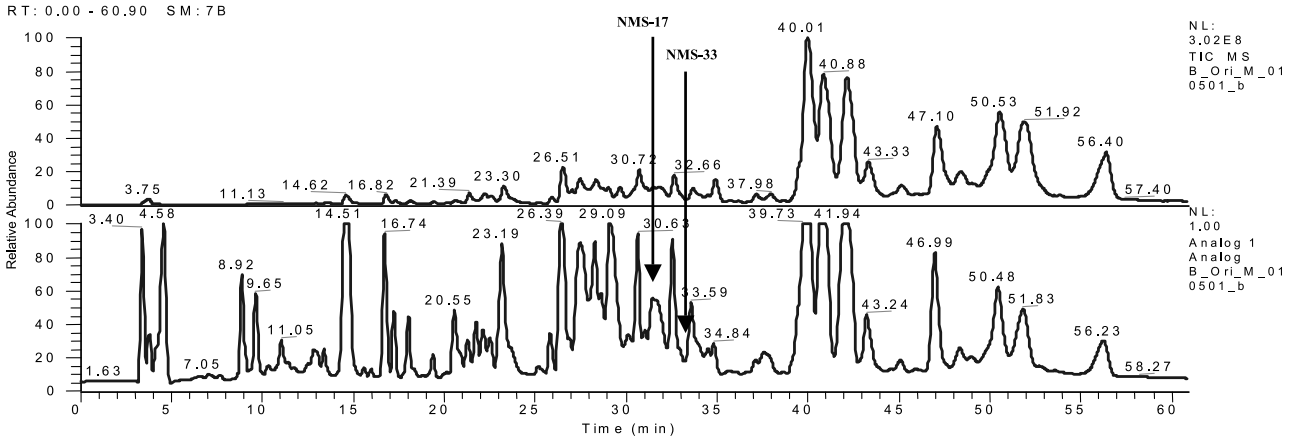

Fig. 1. A typical LC/MS spectrogram of skin venom from the Oriental fire-bellied toad, *B. orientalis*. The upper panel illustrates total ion count entering the mass spectrometer and the lower panel illustrates UV absorbance profile at λ214 nm. Both *y* axes are in arbitrary units where the highest peak 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

```
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
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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
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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

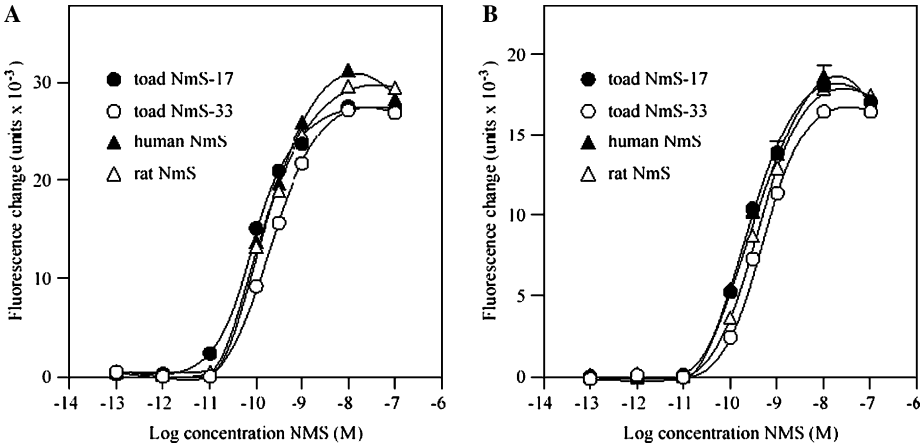

Fig. 6. Response curves for toad NmS-17 (filled circle), toad NmS-33 (open circle), human NmS (filled triangle), and rat NmS (open triangle) in the calcium mobilization assay using CHO cells stably expressing FM-3/GPR66 (A) or FM-4/TGR-1 (B) human NmU receptors. The calcium mobilization assay using the 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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